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The puzzle of lichen symbiosis

Pieces from Thamnolia

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Abstract
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Symbiosis brought important evolutionary novelties to life on Earth. Lichens, the symbiotic entities formed by fungi, photosynthetic organisms and bacteria, represent an example of a successful adaptation in surviving hostile environments. Yet many aspects of the lichen symbiosis remain unexplored. This thesis aims at bringing insights into lichen biology and the importance of symbiosis in adaptation. I am using as model system a successful colonizer of tundra and alpine environments, the worm lichens *Thamnolia,* which seem to only reproduce vegetatively through symbiotic propagules. When the genetic architecture of the mating locus of the symbiotic fungal partner was analyzed with genomic and transcriptomic data, a sexual self-incompatible life style was revealed. However, a screen of the mating types ratios across natural populations detected only one of the mating types, suggesting that *Thamnolia* has no potential for sexual reproduction because of lack of mating partners. Genetic data based on molecular markers revealed the existence of three morphologically cryptic *Thamnolia* lineages. One lineage had a clear recombination structure and was found in the tundra region of Siberia, shorelines of Scandinavia, and Aleutian Islands. The other lineage was allopatric with the previous, and was highly clonal; only two haplotypes were found across the alpine region of central and southeastern Europe. However, the third lineage was sympatric with the other two, had a worldwide distribution, and although highly clonal, showed a recombinant population structure. Our data could not reveal whether the signs of recombination resulted from rare recombination events due to the extreme low frequency of the other mating type or ancestral variation before the loss of sexual reproduction. However, investigation of *Thamnolia's* green algal population showed that in different localities, different algal genotypes were associated with the same fungal genotype. Furthermore, data suggest that *Thamnolia* carried several algal genotypes within its thalli and shared them with other distantly related but ecologically similar fungal species.

Keywords: Thamnolia, lichen, symbiosis, photobiont, mycobiont, phylogeography, MAT-loci, barcoding, NGS, genome, transcriptome, Ice Age

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“Shoot for the moon. Even if you miss, you'll land among the stars.”
Norman Vincent Peale

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Onuţ-Brännström, I., Tibell, L., Johannesson, H..(in press) A world-wide phylogeography of the whiteworm lichens Thamnolia reveals three lineages with distinct habitats and evolutionary histories. Ecology and Evolution

II Onuţ-Brännström, I., Johannesson, H., Tibell, L.. *Thamnolia tundrae* n. sp., a cryptic species and putative glacial relict. *Manuscript*

III Onuţ-Brännström, I., Benjamin, M., Scofield, D., Heiðmarsson, S., Andersson, M.G.I., Lindström E.S., Johannesson, H.. Differential sharing of photobionts in sympatric populations of *Thamnolia* and *Cetraria* lichens: evidence from next generation sequencing. *Manuscript*

IV Onuţ-Brännström*, I., Ament-Velásquez*, S.L., Hiltunen, M., Resl, P., Vanderpool, D., Yamamoto, Y., Spribille, T., Scofield, D., Johannesson, H.. Constraint to sex by a single mating type? Genomic and population analyses reveal insight into the reproductive biology of *Thamnoli*a. *Manuscript*

*These authors contributed equally to this study.
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Contents

Abbreviations

bp	base pairs
DNA	Deoxyribonucleic acid
F	fertilization
ITS	internal transcribed spacer
kb	kilo base
MAT	mating-type genes in fungi
Me	meiosis
Mt	mitosis
Myr	million years
NGS	Next Generation Sequencing technology
PCR	Polymerase chain reaction
pl.	plural
RNA	Ribonucleic acid
rpm	rotations per minute
sing.	singular

Preface

I started this amazing adventure on the 1st of July 2012, when I began my years as doctoral student of Uppsala University. I was fortunate to be able to continue my undergraduate work on lichens and I had big plans!

Imagine lichens symbiosis as a two-sided coin: with the main symbiotic fungus on one side and the photosynthetic alga on the other side. If you just look at one side you will entirely miss the other one. How about instead of flipping the coin, you spin it? Then your mind can register both sides in the same time. So, I thought back then, if I could study lichens as a 'spinning coin', then I would truly be able to comprehend their biology. How? Easy! We are in the genomics era! Have you never heard of genomes, metagenomes, transcriptomes, and metatranscriptomes? Have you never heard of Illumina HiSeq, MiSeq, and IonTorrent? The whole universe was mine; my supervisors were all in and *Thamnolia* samples were arriving in troves from friends and lichenologists from all over world! My grant applications were successful and the field trips were great! And then I started the lab work. What can I say? *Thamnolia* did not easily let go of its DNA and RNA. Handling genomic data was not a walk in the park either. It felt as if I was moving one step forward, only to go three backwards. Slowly but surely, I managed both the lab work and data analysis. I am not sure I managed to see lichens as 'spinning coins'. For sure I have the biggest *Thamnolia* collection in the world. I followed Mr. Peale's advice and I shot for the moon. I know I landed among stars, and made some interesting discoveries about this lichen species. I dare to say that I with this thesis contribute valuable information needed to complete the great puzzle of lichen symbiosis.

Introduction

What are lichens? Simply said, they are the symbiotic phenotype of a heterotrophic fungus associated with photosynthetic organisms such as green microscopic algae or cyanobacteria (Honegger, 1993; Muggia et al., 2011). In practice, the answer is much more complicated and attempts to capture lichens symbiotic nature can be illustrated by a whole range of definitions. There are the intricate ones like: 'intimate and long-term symbiosis...[of organisms] joined to form a new biological entity different from its individual components' (Sacristán et al., 2007). Then, there are the more evocative ones such as 'self-contained miniature ecosystem' (Dal Grande et al., 2014a) or the very metaphorical ones: 'fungi using solar panels' (Toby Spribille, personal communications) and 'spinning coins' (my own invention).

Symbiosis brought important evolutionary novelties to life on Earth: from the formation of Eukaryotic cell (Cooper, 2000), to the land plant colonization (Heckman et al., 2001). Lichens represent an example of a successful adaptation strategy, which enables habitat-dependent organisms, to survive the hostile environments of the harsh Antarctic (Perez-Ortega et al., 2012) and the Namibian dessert (Lalley & Viles, 2005). They can even be exposed to outer space radiation and come back alive (Meessen et al., 2015)! While many different assumptions and hypothesis are made about the lichens life cycle, reproduction, dispersal, ecology and genetics the knowledge about their adaption mechanisms to a wide range of environment is still largely unknown. Despite the numerous efforts to puzzle them out, lichens remain mostly a black box and whenever an "answer" is given a whole fountain of new questions explode. However, in the last two decades, when molecular methods and advanced microscopy technologies became available much progress was made and the pieces are finally coming together. There are many good examples of lichen studies that start to reveal the evolutionary processes and patterns that govern their symbiosis. Throughout this introduction, I will stick to the ones that inspired my work and I believe, represent important stepping-stones to understand lichens.

Naturally, the work of this thesis does not claim to entirely solve the puzzle of lichen biology. Nonetheless, using the worm lichens *Thamnolia* as a model system, it brings new insights in lichen reproduction, dispersal and algal recruitment.

1.1 A crash course in lichen biology

For better understanding of the thesis content, in this crash course I recap some basic knowledge on lichen biology and draw attention to some interesting features of lichen symbiosis.

1.1.1 Two of the players: the mycobiont and the photobiont

Lichens come in all forms and colors, ranging from minute dots or undefined crusts ("crustose lichens") to distinct plant-like entities ("macrolichens") that can have shrubby ("fructicose") or leafy ("foliose") morphologies. They can be found on all sorts of substrates: from natural ones such as rocks, soil, wood and trees, to artificial ones: plastic, glass, and iron surfaces (Sanders, 2005).

In recent years, research has provided new insights into the partners involved in lichens symbiosis and the view is changing from a two-partner system to a more dynamic system, linking multiple participants. Entire bacterial communities, which live in (Grube *et al.*, 2015) and on (Mushegian *et al.*, 2011) lichens appear to influence the symbiosis (Aschenbrenner *et al.*, 2014; Wedin *et al.*, 2015). Basidiomycete yeasts that were once thought to be lichen parasites seem to be part of the symbiosis by forming the upper layer (cortex) of many lichen species (Spribille *et al.*, 2016). In this thesis, however, I focused my efforts to the study of two of the potentially many symbiotic partners of a macrolichen: the ascomycetous fungus (*the mycobiont*), which forms most of the lichen thallus and is inclosing within its plant-like body (Sanders & Lücking, 2002), the unicellular green algae (*the photobionts*).

The symbiotic relationship between the mycobiont and the photobionts are not yet entirely understood. Some consider the relationship mutualistic (Smith, 1980): the fungus obtains carbohydrates from the green algae (Honegger, 1998) and in return, the algae appear—*nota bene*: little empirically tested (Sadowsky & Ott, 2015)—to be protected from unfavorable conditions (*e.g.*, UV light, cold, aridity) (Huneck, 1999; Lücking *et al.*, 2014; Molnár & Farkas, 2014). Others view lichens more as a 'controlled parasitism,' similar to a plant-fungal pathogen interaction, in which the photobiont has to defend itself against a fungus that searches for food resources (Athukorala & Piercey-Normore, 2015). Support of the 'controlled parasitism hypothesis' comes from experimental work, which showed that not all photobionts are able to survive recruitment from a certain mycobiont genotype (Schaper & Ott, 2003). Regardless of the symbiotic relationship, it appears that the mycobiont – photobiont interaction is mediated through lectins that are secreted by the fungus and bind to the photobiont cell wall receptors. A failure to sequester the fungal lectins at the cell wall leads to a drastic increase of algal putrescine, followed by chloroplast disintegration and cell

death (Vivas *et al.*, 2010). The same recognition mechanism seems to be involved in the fungus' control of its internal (intrathaline) photobiont populations (Sacristán *et al.*, 2007; Diaz *et al.*, 2016).

1.1.2 Reproductive strategies in lichens

I often found it challenging to talk about lichen reproductive strategies because when we say *lichen* we often refer to the symbiotic entity. While lichens are often spreading as one unit enclosing multiple symbionts together, both the mycobiont and the photobionts can reproduce and disperse separately from each other (Honegger, 1998). Thus, one way to explain lichen reproduction is to group all different possibilities under "vertical" versus "horizontal" transmission of symbionts.

Vertical transmission is by which a lichen individual can maintain the successful symbiotic phenotype by reproducing asexually through joint symbiotic units called vegetative propagules. These propagules may be transmitted over short or long distances (Dal Grande *et al.*, 2014b) and if the conditions are suitable, they will develop into new lichens. Isidia (sing. isidium) (Fig.1a) and soredia (sing. soredium) (Fig.1c) are specialized vegetative propagules often encountered on the upper surface of macrolichens. While soredia consist of mycobiont hyphae tightly wrapped around photobionts that are released through ruptures of the lichen thallus, isidia are outgrowths of the lichen where the cortex and everything beneath the cortex is preserved (Fig.1a). Furthermore lichens can replicate by fragmentation (Fig.1b) and lichen development from vegetative propagules has been documented in several field experiments (Ott *et al.*, 1993; Sanders & Lücking, 2002; Sanders, 2005).

The situation is more complicated in the horizontal transmission of symbiotic partners. If the symbionts were dispersed separately (*e.g.* the ascospores) they would need to re-establish the symbiosis by finding all the necessary partners. Consequently, new symbiotic combinations may be formed, which can be followed by drastic phenotypic changes (Henskens et al., 2012; Wedin et al., 2015).

Ascomycetous mycobionts can reproduce both asexually through mitotic fungal spores (conidia) and sexually through meiotic spores (ascosopores). For mycobionts forming macrolichens, both ways of reproductions have always been documented in a lichenized state only, and as a consequence it is believed that these fungi are obligate symbionts (Ahmadjian, 1988; Honegger, 1998). On the surface of many lichen species, the fungal reproductive structures called apothecia (sing. apothecium) and pycnidia (sing. pycnidium) can be observed. Apothecia, in which the ascospores are formed, are relatively large and easy to recognize, they may have different shapes

and colors, and their occurrence is evidence that the mycobiont went through sexual reproduction. Pycnidia are minuscule, bottle-like structures, full of conidia. Ascopore germination and algal recruitment (Figs.1d) has been described in several beautiful experiments (Yoshimura et al., 1993; Sanders & Lücking, 2002; Sanders, 2005; Guzow-Krzeminska & Stocker-Wörgötter, 2013). However, due to their minute cell structure with very little cytoplasm, the development of lichens from conidia (Figs.1e) is still under debate (Vobis, 1977; Tibell, 1997, Honegger, 1984b). Their role is yet unknown, though indirect evidence suggests they may function like in other non-symbiotic fungi as spermatia (Honegger, 1984a; Keller & Scheidegger, 2016).

The separate transmission of photobionts is not as well documented as in the mycobionts case. Although photobionts can easily grow in cultures where they can reproduce both sexually (through meiosis) or asexually (through mitosis) (Ahmadjian, 1988; Beck, 1999), it is still debated if they can survive for long periods, as free-living cells (Ahmadjian, 1988). For example, the most frequently encountered lichenized green algae, those of *Trebouxia* species, are not found growing in big colonies as their non-symbiotic relatives, suggesting an obligate symbiotic life style. Furthermore, meiotic photobiont cells were rarely observed within lichens (Slocum *et al.*, 1980). It appears that the mycobiont is suppressing the photobiont sexual stage in order to maintain an already adapted photobiont genotype (Ahmadjian, 1988).

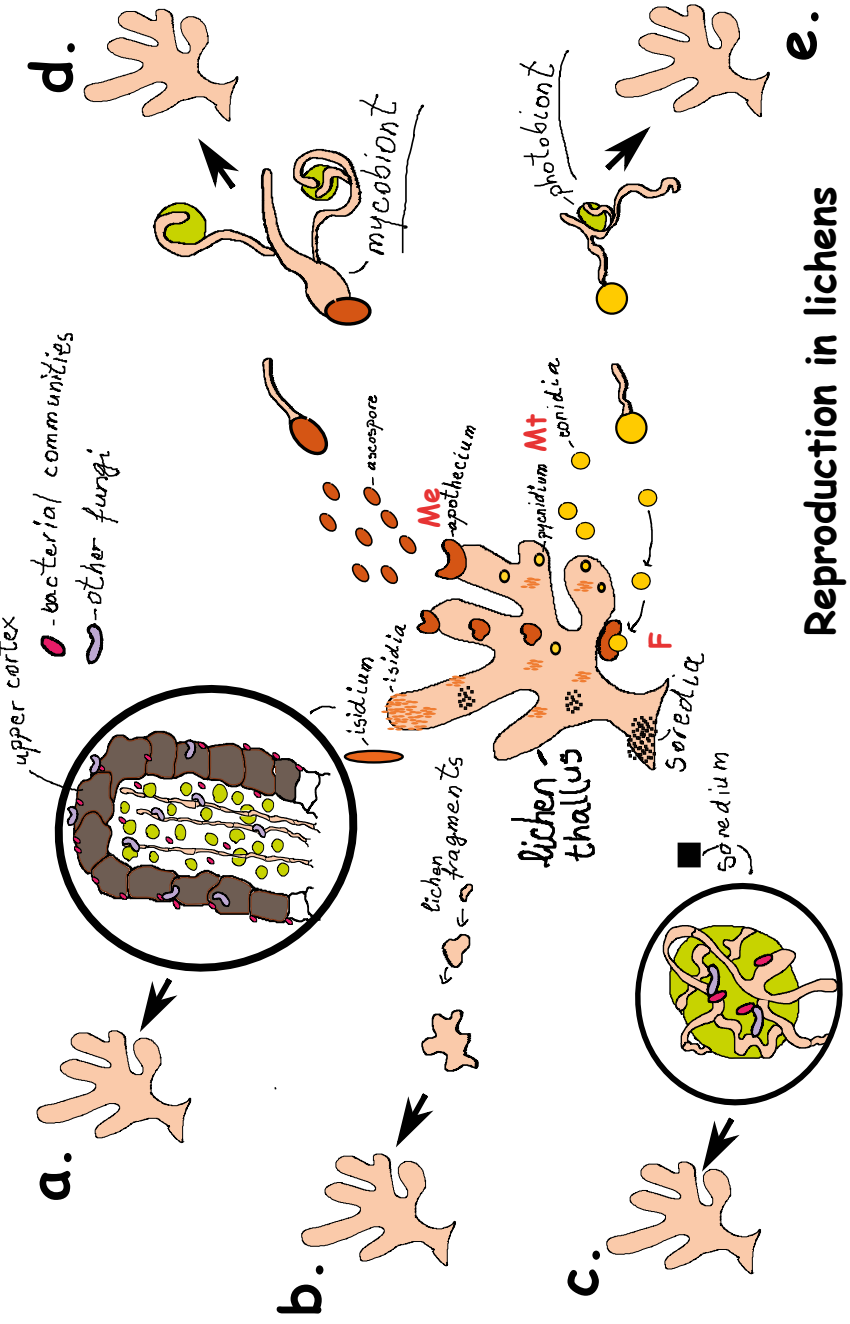

Figure 1. Schematic representation of lichen reproduction and ontogeny.

1.1.3 Possible outcomes of lichens reproductive strategies

The presence of vertical and horizontal transmission in lichens contributes to a complex and likely more flexible dispersal biology than non-symbiotic organisms. Each of the employed strategies will influence the population structure of the respective symbiotic partner and also the life history of each lichen species. Many lichen species such as *Lobaria pulmonaria,* (Widmer *et al.*, 2012) *Cetraria aculeata* (Fernandez-Mendoza et al., 2011)*,* or *Letharia vulpina* (Högberg *et al.*, 2002) largely reproduce through symbiotic vegetative propagules. Furthermore, there are some examples of sterile lichens such as species from *Lepraria* (Nelsen & Gargas, 2008)*, Siphula ceratites* (McVean, 1956)*,* or *Thamnolia vermicularis* (Culberson, 1963) in which apothecia have never been described. Some of these highly asexual lichens have narrow distribution ranges and they are threatened by extinction (Högberg *et al.*, 2002; Widmer *et al.*, 2012), while others are abundant and have wide distributions in several continents (Sheard, 1977; Fernandez-Mendoza *et al.*, 2011). Genetic based studies showed that in all the above examples, both the mycobiont and the photobiont present low genetic variation and high clonality. Often, identical mycobiont and photobiont genotypes are found over very long distances in widely distributed lichen species (Printzen & Ekman, 2002; Nelsen & Gargas, 2009a; Fernandez-Mendoza *et al.*, 2011; Printzen *et al.*, 2013). How have these lichen species adopt an asexual reproduction strategy? Is it advantageous (*e.g*. a highly-adapted genotype) or is it the only strategy left to disperse (*e.g*. there are no mating partners)? In the case of lichens the answers must be given at several levels: separate for symbionts and as a whole for the symbiotic entity.

1.1.4 Mycobiont mating system

For all fungi, the sexual reproduction is regulated by specialized parts of the genome called mating-type (MAT) loci (Fraser & Heitman, 2003). All ascomyceteous fungi, including the lichen forming ones (e.g. Scherrer *et al.*, 2005; Singh *et al.*, 2012; Tuovinen, 2017) have a bipolar system with two allelic forms of one MAT locus that gives the mating type, *i.e.*, sexual identity, of a fungal strain (Butler, 2007). Because of their lack of reciprocal homology, the allelic forms of the MAT loci are also called idiomorphs. In different fungal systems, different nomenclatures are used and in this thesis, I will refer to the MAT idiomorphs as *MAT1-1* and *MAT1-2,* following the suggestion of (Turgeon & Yoder, 2000). In heterothallic (sexual self-incompatible) fungi, each individual harbors one or the other idiomorph in their haploid genome, and mating occurs between two individuals of opposite mating types. In the case of homothallic fungi, the need of finding a mating partner is usually overcome by the occurrence of both MAT idiomorphs in the same haploid genome. However, there are exceptions, such as the fungal human pathogen *Cryptococcus neoformans* (Lin et al., 2005) and

Neurospora africana, (Gioti *et al.*, 2012) in which selfing may occur even when only one idiomorph is present.

At the molecular level, the idiomorphs can be recognized by the presence of genes with two highly divergent protein domains: the gene *MAT1-1-1* is characterized by the α-domain, while *MAT1-2-1* by the HMG-box domain (Martin *et al.*, 2010). The general genetic architecture of a *Pezizomycotina* MAT locus is visualized in Figure 2. The MAT loci are in all ascomycetes flanked by two protein-coding genes: *APN2*, encoding a DNA-lyase, and *SLA2*, encoding a gene important for the membrane cytoskeleton formation (Butler, 2007).

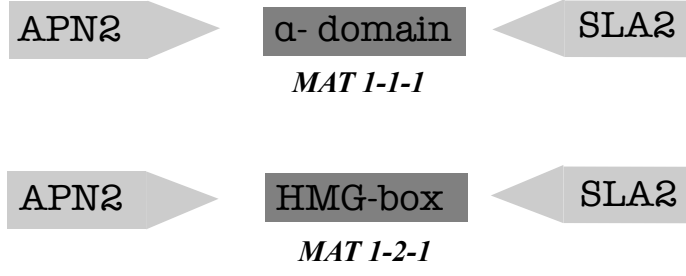

Figure 2. A schematic representation of the molecular architecture of the MAT idiomorphs and the two flanking genes in Pezizomycotina.

1.2 Chemical based identification of lichen species

Although lichens are symbiotic association, their species name is attached to the main mycobiont that typically forms the lichen thalus. Phenotypic features such as thallus morphology, anatomy, reproductive structures (*e.g.*, isidia, soredia, apothecia, the fungal spore shape and size), together with chemistry, are playing an important role in identifying lichen species.

Lichens contain numerous secondary metabolites. Some of these have antioxidant properties or may act as sun blockers and thus reduce harmful solar irradiation (Huneck, 1999; Lücking et al., 2014; Molnár & Farkas, 2014). Studying lichen secondary metabolites in the 1980s has been likened to the importance of the use of molecular methods today. It was a must as Brodo expressed in 1986: '..no serious lichen systematist can ignore chemical characteristics of the taxa being considered and, indeed, a revision not including chemical data is very likely to be rejected as incomplete' (Brodo, 1986). In many cases, morphologically indistinguishable individuals can contain different chemical compounds, and sometime the chemotypes distribution seems to follow a geographical pattern (Sheard, 1977). Consequently, the

use of lichen chemical variation in taxonomy seemed like a rational decision (Culberson, 1968; Culberson, 1986). A turning point came when molecular methods utilizing DNA markers were readily available to taxonomists, and phylogenetic relationships could be inferred to delimit lichen species. Many species recognized by chemical characters failed to show monophyly (Lumbsch & Leavitt, 2011). Although it is still not known if lichen chemistry has a genetic or environmental component, it is often assumed that the secondary metabolites are produced by the mycobiont (Lumbsch, 1998). However, in light of the Spribille et al. (2016) study, in which a strong correlation between the production of vulpinic acid and the presence of the basideomyceteous yeast in the lichen cortex of two *Bryoria* morphospecies was demonstrated, this widely useful lichen taxonomy tool needs to be at least treated with caution.

1.3 Lichen phylogeography

I see phylogeography studies as detective work in which population genetic tools are used to understand the historical processes behind the present geographic distributions of populations. These studies may reveal how important geological processes such as the Pangaea split (Westrand & Korall, 2017) or drastic climatic changes such as the Last Ice Age period molded the present day biodiversity. Furthermore, these type of studies may also be used to predict how future climatic changes will affect the present day distribution of species (Kozma *et al.*, 2016).

1.3.1 Lichens, suitable candidates for testing the glacial survival hypothesis

The last Pleistocene glaciation that ended approximately 10,000 years ago appears to have had a quite dramatic effect, especially in Northern Europe and North America. Here it is believed that the massive ice-sheets that covered those regions eliminated all former biodiversity (the 'tabula rasa' hypothesis), and the pattern of the present-day species distribution can only be explained by the subsequent colonization of organisms from the non-glaciated areas (Nordal, 1987). However, an alternative explanation is offered by the glacial survival' hypotheses, argues for the existence of populations that survived *in situ*, within the ice shield. Examples of such isolated ice-free places are coastal mountains (Nordhagen, 1936), nunataks (Knaben, 1959), or tundra areas that were not permanently covered by ice (Dahl, 1946). Although there is no direct way to distinguish post Pleistocene immigrants from *in situ* survivals, genetic patterns such as genotype uniqueness in the former glaciated areas (Westergaard *et al.*, 2011) and/or reduced popula-

tion size (Stewart *et al.,* 2010) have been used as arguments for the 'glacial survival' hypotheses.

Among the greatest challenges for organisms living in cold climates is the ability to survive freezing, to endure lack of liquid water and sustain high UV radiation (Kappen 1993, Perez-Ortega et al. 2012). Many vascular plants do cope with some of these challenges by temporarily inactivating their metabolism. However, in the most extreme conditions, only poikilohydric organisms, which can tolerate cell and tissue desiccation and at the same time recover without physical damage, can remain metabolically active (Kappen 1993, Perez-Ortega et al. 2012). In cold environments, lichens are frequently able to use snow as a water source, continue their photosynthesis while frozen, and immediately recover after long freezing periods (Kappen 1993). Therefore, lichens with their high tolerance of extreme environments are suitable candidates to test the glacial survival hypothesis.

1.3.2 Population genetics on lichens: challenges and outcomes

Population genetics methods available today offer wonderful tools not only to explore the impact of the last Ice Age period (Pleistocene) on different lichens species (Geml *et al.*, 2010; Fernandez-Mendoza & Printzen, 2013; Bendiksby *et al.*, 2014) but also to understand lichen biology and life histories (*e.g.*, dispersal, mating behavior) (Werth, 2010). However, phylogeography studies on lichens are not as straight forward as in other organisms. Firstly, from a genetics point of view, lichens should be treated as hologenomes (Bordenstein & Theis, 2015). Therefore symbiont-specific molecular markers or bioinformatic methods must be used to separate each individual genome. Secondly, all population genetic methods for their application require some genetic variability in the investigated population, and furthermore, most of them were designed for sexual reproducing organisms. Yet many lichen species are highly clonal, thus requiring a large amount of genetic data to overcome the low genetic variability. Furthermore, the basic assumption of random mating is often violated.

Most of the studies of lichen population genetics so far have been focused on the mycobiont (Högberg *et al.*, 2002; Buschbom, 2007; Geml *et al.*, 2010; Werth, 2010; Fernandez-Mendoza & Printzen, 2013; Leavitt *et al.*, 2013; Sork & Werth, 2014), but though very few on the photobionts (Werth & Sork, 2014). Some studies, however, have considered both the mycobiont and the associated photobionts (Fernandez-Mendoza *et al.*, 2011; Widmer *et al.*, 2012; Chen *et al.*, 2016). Although each of these studies has its own system with their specific characteristics, common patterns that support the prediction made for plants and animals (Schonswetter *et al.*, 2005; Parducci *et al.*, 2012; Nägele *et al.*, 2015) can be observed. For example, in the context of Last Ice Age Period, previously identified refugial areas such as south

central Alaska or southern Europe also appear to likewise have been sanctuaries for lichen species such as *Cavernularia hultenii* (Printzen *et al.*, 2003) or the rare *Lobaria pulmonaria* (Widmer *et al.,* 2012).

1.4 Lichen ecology and evolution

The recent years research in lichenology showed an increased interest towards molecular ecology based studies, which led to some exciting and sometimes unexpected discoveries about mycobionts and photobionts ecology and evolution.

1.4.1 Mycobionts and photobionts are not co-evolving lineages

In many species of lichens, the mycobiont-photobiont association is not maintained over evolutionary time, and in different environments, the same fungal genotype can be found to be associated with different photobiont genotypes (Beck *et al.,* 2002; Nelsen & Gargas, 2008; Nelsen & Gargas, 2009b; Fernandez-Mendoza *et al*., 2011). This phenomenon has been called *algal-switching* (Piercey-Normore & DePriest, 2001) and it appears to play a key role in the adaptation of lichens to new environmental conditions (Fernandez-Mendoza *et al.,* 2011; Peksa & Skaloud, 2011; Dal Grande *et al*., 2014b; Werth & Sork, 2014). However, the mechanism of *algal-switching* is poorly understood. Is it happening by complete replacement of one photobiont genotype by another? Or is it a gradual process where diverse populations of photobionts are maintained within the lichen thallus but their proportion depends on the environmental conditions? It is easier to envision it in lichens that disperse solely with fungal spores (*e.g.* Geiser & McCune, 1997). To be able to form the lichen, the germinating spores (Fig. 2d, 2e) would need to associate with locally available photobionts, especially during long distance dispersal events and these may not be of the original genotypes. Personally, I found it especially difficult to imagine the *algal-switching* mechanism in the big fructicose and foliose lichens that grow extremely slowly, and in many cases rarely produce fungal fruiting bodies. May it then be as Grube & Spribille (2012) suggested, that in the beginning (or early stages), the macrolichens disperse by vegetative propagules and subsequently produce fungal spores, which will restart the lichen cycle? Interestingly, algal-switching occurs even in lichen species where fungal fruiting bodies have never been described and where co-evolution of the mycobiont and symbiont due to co-dispersal was to be expected (Nelsen & Gargas, 2008; Nelsen & Gargas, 2009b). In this case photobiont switching as a gradual process may seem more plausible.

1.4.2 Mycobionts share photobionts with similar ecological requirements

Rikkinen (2002) showed that genetically different mycobionts under similar ecological conditions share the same photobiont populations (the lichen guild hypothesis). He further suggested that lichen species that are mainly or always spreading through vegetative propagules (asexual lichens) are the source of photobionts for the spore-dispersed lichens (sexual lichens). Subsequent studies showed the dependency between the sexual and asexual reproducing lichens (*e.g.* Belinchón *et al.*, 2015) and the existence of interconnected ecologically similar fungal communities (*e.g.* Peksa & Skaloud, 2011).

1.4.3 Mycobionts associate with locally adapted photobionts

Once the fact that photobionts are shared in different lichen communities was revealed, the next step was to understand what creates the background photobiont community. Do mycobionts associate with compatible photobionts that happen to be there only because they migrated from other places? Or are those photobionts living in a particular environment because they are adapted to it? The answer is not yet clear. Several studies suggest that there is a strong correlation between photobiont genetic identity and environment at both the microhabitat level (Werth & Sork, 2014) and ecoregions level (Fernandez-Mendoza *et al.*, 2011; Werth & Sork, 2014). An experiment-based study showed that coexiting *Trebouxia* lineages (living in the same lichen), which were grown separately under different temperature and light conditions, have different physiological performances regarding growth and photosynthetic rates (Casano *et al.*, 2011). When the same two photobiont lineages were investigated in lichens specimens across a wide geographic range, it was shown that the abundance ratio of two photobiont lineages within the lichen was correlated with the climate that represented the best preferred experimental growing conditions (Catala *et al.*, 2016).

1.4.4 Multiple photobiont genotypes in one lichen thallus

Molecular studies using nuclear markers seem to indicate that an individual lichen thallus contains only one algal genotype (*e.g.* Nelsen & Gargas, 2008; Fernandez-Mendoza *et al.*, 2011; Cao *et al.*, 2015). However, these types of studies are based on Sanger sequencing technology and best suited for homogeneous samples, of which lichens are not. When other technologies such as: cloning of PCR (Catala *et al.*, 2016), microsatellites-markers (Dal Grande *et al.*, 2014a), fingerprinting (Muggia *et al.*, 2013), and independent culturing and sequencing (Voytsekhovich & Beck, 2015) were used, coexisting photobiont genotypes were always found.

1.5 The worm lichens *Thamnolia*

Many times, I have been asked why I chose to study *Thamnolia*. I might be biased in that I have been studying it now for more than 6 years. Still *Thamnolia* with its peculiar morphology, curious chemical variation, its mysterious life cycle and wide distribution range, has attracted the attention of few other lichenologists and its taxonomic status is still under debate.

1.5.1 Worldwide distribution

Thamnolia is a widely-distributed lichen genus, often encountered in Arctic and Alpine tundra environments on all continents, except Africa and Antarctica (Sheard, 1977). As the common name of this lichen suggests, it looks like big white worms caught among low grass and mosses, or sometimes just slightly attached to the soil (as depicted on the cover of this thesis).

Despite its wide distribution range, the dispersal of this lichen is not well understood. The mycobiont has never been found to carry sexual fruiting bodies and thallus fragmentation has been assumed to be important in the dispersal of worm lichens (Andrei et al., 2006-2007). Previous studies have suggested a predominantly clonal reproduction strategy with the fungus showing a very low genetic variation with no consistent signatures of recombination (Nelsen & Gargas, 2009a). The lack of sexual fungal spores, and likewise a less efficient long-distance dispersal mechanism, would lead to an assumption about a confinement to a rather small geographical area. Although it is unlikely that the heavier lichen fragments will be dispersed over very long distances only by wind, grazing animals and birds may carry them farther away. In fact small *Thamnolia* fragments have been observed to be used by the white plover as nesting material (Wright, 1992). Furthermore it has also recently been shown that *Thamnolia* specimens can produce minuscule conidia with unknown function (Lord et al., 2013). As in all other lichens, the conidia may act solely as male gametes (spermatia) and/or they play a role in dispersal. If conidia have the ability to germinate, they need to restore the symbiosis (i.e., to relichenize) by finding a new and compatible photobiont. Relichenization by conidia can be one explanation; *Thamnolia* has been found to be associated with different photobiont species of *Trebouxia* green algae in different localities.

1.5.2 Systematic placement

Thamnolia belongs to *Icmadophilaceae*, a recently recognized family of lichen forming fungi (Triebel 1993). It has a strikingly similar morphology with *Siphula ceratites* (Fig. 3), both genera have an erect thallus and lack apothecia. Therefore a common ancestry of these two genera was suggested (Poelt, 1973). In the same time the sexually reproducing species in the fami-

ly, *e.g. Dibaeis baeomyces* and *Icmadophila ericetorum* also have remarkably similar morphologies (*i.e.* pink apothecia on a green-gray crustose thallus; Fig. 3). But systematic studies based on nuclear markers showed that *Thamnolia* and *Dibaeis* Clem. are sister genera, while *S. ceratite*s is closely related to *I. ericetorum* (Platt & Spatafora 2000; Stenroos *et al.*, 2002; Fig. 3). Hence, the differences in morphologies within *Icmadophilaceae* seem to be correlated with reproductive behavior, in which a transition from a sexual to an asexual reproductive mode has drastically changed the lichen phenotype, from a horizontal thallus (crustose lichen) to an erect (macrolichen).

1.5.3 How many species of *Thamnolia* are out there ?

Another interesting aspect of *Thamnolia* species is its variation in secondary chemistry. Two distinct and mutually exclusive sets of secondary metabolites (Culberson, 1963; Santesson, 2004), which also exhibit an interesting distribution, occur in *Thamnolia* specimens. Although coexisting in some areas (Europe, North America), podetia (i.e. the worm-like thalli) containing squamatic and baeomycesic acids are encountered at higher frequency in the Northern Hemisphere, while those containing thamnolic acid are more frequently found in the Southern Hemisphere. There is also a contact zone around the equator where the two chemotypes co-occur at equal frequencies (Satô, 1963).

Even if *Thamnolia* specimens have an easily recognized morphology for the observant eye of lichenologists, some distinct phenotypes exist (Kärnefelt & Thell, 1995). The most frequently encountered morphology is shown on the cover of this thesis (also **Fig. 3**), here called ‘cylindrical’. It can be 4-5 cm in length and is hollow in the middle. The other two phenotypes are rather different. The one that I am calling the ‘flat’ morphotype looks like small white pieces of paper. The other one is very long (up to 10 cm), thin and the cylindrical branches are not hollow (Kärnefelt & Thell, 1995).

Several attempts have been made to distinguish taxa in *Thamnolia*. These have been based on distinctive phenotypes, and traits such as thallus chemistry (Culberson, 1963) or a combination of chemical and anatomical characters (Kärnefelt & Thell, 1995; Santesson, 2004). Recent molecular data, using a limited sampling, showed that the *Thamnolia* chemotypes do not form monophyletic groups. Thus the species recognition in *Thamnolia* remained unclear, and the authors suggested that the lack of chemotype monophyly could be attributed to incomplete lineage sorting, rare or historic recombination events or repeated chemotype evolution (Nelsen & Gargas, 2009a; Leavitt et al., 2016).

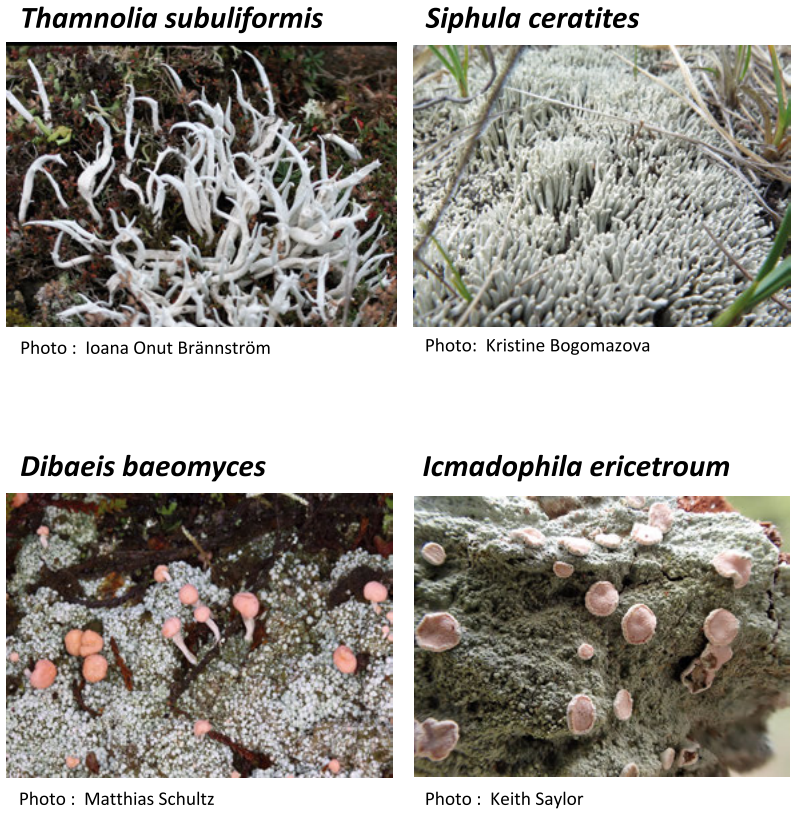

Figure 3. ***Icmadophilaceae*: morphology and mycobionts phylogenetic relationships**. *Thamnolia subuliformis* was photographed by Ioana Onut Brännström in alpine tundra (fjäll) on the peak of Njulia (Abisko, Sweden) The picture of *Siphula ceratites* was taken by Kristine Bogomazova in Scotland, West Sutherland, on a plateau above the Falls of Kirkaig. *Dibaeis baeomyces* was photographed by Matthias Schultz from sandy soils in a *Calluna* heath (Prignitz, NW Brandenburg, Germany) and *Icmadophila ericetorum* by Keith Saylor from an old-growth forest in Toketee Falls (Oregon, U.S.A). The cladogram represents the phylogenetic relationship of the four mycobiont genera (Platt & Spatafora 2000; Stenroos *et al.*, 2002)

2. Thesis aims

The overall aim of this thesis was to further the understanding of the evolutionary processes that govern lichen symbiosis and adaptation to the environment. To achieve this aim, I used the putative asexual lichenized fungal species from *Thamnolia,* as a model system.

Specifically, I was interested in understanding the reason behind *Thamnolia*'s wide distribution range and test the hypothesis that some of its Northern Hemisphere populations were Pleistocene survivors (**Paper I**).

I investigated in **Paper II** the taxonomic status of *Thamnolia* and resolved a long-lasting debate concerning the use of chemical and morphological traits in recognizing species in the genus.

The third aim of my thesis was to understand how photobiont switching occurred in *Thamnolia* and whether it shared green algae with ecologically similar but distantly related mycobionts. This aim is the focus of **Paper III**.

The fourth aim of my thesis was to explore whether *Thamnolia* is indeed a truly asexual fungal species. Furthermore, I wanted to test a hypothesis, which stipulated that *Thamnolia* sterility was due to the lack of mating partners (**Paper IV**).

3. Material and Methods

3.1 Sample collection and chemical identification

The *Thamnolia* samples were gathered from several resources: private collections, herbariums, and collections during my field trips. One important source of *Thamnolia* specimens was the *Adventure Scientists*, a non-profit organization (http://www.adventurescience.org/), which helps researchers with data and sample collection. The mycobiont axenic cultures of *Thamnolia subuliformis* (culture collection #0043) and *Dibaeis baeomyces* (culture collection #0196M) were obtained from Prof. Yoshikasu Yamamoto from the Laboratory of Advanced Bio-Production Science in the Akita Prefectural University, Japan. Philipp Resl from the Institute of Plant Sciences, University of Graz, Austria provided the culture of *Icmadophila ericetorum* The *Siphula ceratites* lichen specimen (BG-L-95765) used for hologenome sequencing was acquired from Tor Tønsberg (University Museum of Bergen The Natural History Collections).

The presence of the secondary metabolites in lichens can be determined with different methods. One such is the spot test, where a change in color occurs when a specific chemical is applied on the lichen. Another method is to use UV light, which indicates the presence of certain chemical compounds depending on whether the thallus fluoresces. Lastly, thin layer chromatography can be used to separate chemicals present in lichen based on their molecular weight. In the case of *Thamnolia*, we used UV light at 3500 A, which is a fast and reliable method to discriminate between two possible sets of chemical compounds: either thamnolic acid, or baeomycesic + squamatic acids. The specimens containing thamnolic acid will have a dull red-brownish color and will be tagged as UV-, while specimens with baeomycesic and squamatic acids will be denoted as UV+ and their upper surface will shine bright yellow, especially when the samples are freshly collected.

Information about sample identification, geographic location, chemical varieties, and collectors name can be obtained in the tables of each paper.

3.2 DNA and RNA isolation, PCR and Sequencing

Both fungi and green algae have sturdy cell walls that must be disrupted to successfully extract DNA or RNA. To make the lichen tissue brittle and in order to disrupt the cell walls, we performed a preparation step: samples were air-dried at 47°C for a half an hour and then frozen at -80°C or in liquid nitrogen. Just before DNA or RNA isolation, the frozen samples were mechanically pulverized with a tissue-lyzer machine at high speed for 1 to 2 minutes at 25 rpm. For all our samples, including the old herbarium material, we used commercial kits for the isolation of DNA and RNA and we followed the manufacturers' protocol. We made sure to prolong the first incubation step to at least one hour and invert the samples every 10 minutes to maximize extraction. *Thamnolia* is a highly clonal species and to avoid nucleotide misincorporation, we used high-fidelity polymerases during amplification. Further details pertaining to DNA, and RNA isolation and PCR protocols can be obtained in the Material and Methods of each paper.

Several sequencing technologies were used to generate the genetic data for each of the four studies: the traditional Sanger sequencing and three next generation sequencing (NGS) methods: Illumina MiSeq, Illumina HiSeq and Ion Torrent. The NGS was performed at the National Genomic Infrastructure, Science for Life (SciLifeLab, Uppsala, Sweden), while Sanger sequencing was conducted both at SciLifeLab and the Evolutionary Biology Centre (Uppsala, Sweden). The Sanger technology offers high accuracy and an ability to amplify fragments up to 1000 basepairs (bp). Therefore, Sanger sequencing was suitable in the phylogeography (**Paper I)** and systematic **(Paper II**) studies, where we used symbiont specific molecular markers. It was further used to identify the predominant photobiont genotype in the lichen samples **(Paper III**), and to verify and complete the bioinformatically inferred genomic architecture of the MAT loci (**Paper IV**). However, Sanger sequencing is most appropriate when targeting only the highly predominant amplicon from a PCR reaction. In complex environmental samples, such as that of a lichen thallus, other methods would need to be applied. In **Paper III**, we were interested to capture all the *Trebouxia* genotypes from a lichen sample. Thus, we used the Ion Torrent NGS technology that can sequence DNA amplicons from mixed samples, of median sizes of cca 250 bp. This method is often used in studies aimed at describing the hidden diversity of a community or a heterogeneous sample, with the use of DNA barcodes (short genetic markers) (Menkis *et al.*, 2016; Nguyen *et al.*, 2016; Veldman *et al.*, 2017).

In ascomyceteous fungi, the mating-type loci together with their flanking genes, *APN2* and *SLA2*, can stretch over several kilobases (kb) and may sometimes be situated in different parts of the genome. Furthermore, the two idiomorphs MAT 1-1 and MAT 1-2, have high sequence dissimilarity, even

between closely related species. When we started this project, only one available study had characterized sections of the MAT loci of two mycobiont species, by using PCR amplification (Scherrer *et al.*, 2005). However regarding the low sequence similarities of Ascomyceteous MAT loci (Butler, 2007), the NGS technologies seemed the most efficient method to describe their architecture in *Icmadophilaceae* species, and to ensure a correct assignment of their reproductive behavior (heterothallic versus homothallic). The transcriptomes obtained from *Thamnolia* Lineage C. and *D. baeomyce*s were used for gene annotation. To ensure a high genome coverage we used a combination of Illumina MiSeq, which gives longer read length and Hiseq, which gives shorter reads but a better coverage.

3.3 Phylogenetic analyses

Several phylogenetic analyses methods were used for the four studies. Haplotype networks are suitable to understand the evolutionary relationships between recently diverged lineages. They can offer information on recombination (Huson & Bryant, 2006), and can be used to infer migration patterns (Schaal *et al.*, 2003). Therefore, I used two programs: TCS (Clement *et al.*, 2000) and Splits Tree (Huson & Bryant, 2006) to investigate *Thamnolia*'s life history (**Paper I**). However, both programs utilize genetic distances algorithms based on parsimony with no model of sequence evolution. Maximum likelihood and Bayesian methods address this issue, taking into account models of nucleotide and/or amino-acid substitution rates in order to infer the most probable tree. I inferred the Maximum-Likelihood phylogenies for **Papers I, II** and **III** with Garli (Bazinet *et al.*, 2014), and in the case of problematic alignments with many ambiguous aligned sites, I used BaliPhy (Redelings, 2014) that simultaneous estimates alignment and phylogeny in a Bayesian framework (**Paper I** and **II**).

3.4 Population genetics methods

To investigate the divergence times among *Thamnolia* lineages (**Paper I**), I used previously inferred substitution rates of the ITS region (Leavitt *et al.*, 2012) and the mean genetic distance between lineages to calculate approximate divergence time.

Population genetic parameters such as Nei's nucleotide diversity (Nei & Kumar, 2000) and genotypic diversity (Caron *et al.*, 2014) were calculated to estimate the level of polymorphism, while Tajima's D (Tajima, 1989) and Fu's F statistics (Fu & Li, 1993) were used to understand demography and selection in *Thamnolia* lineages (**Paper I)**.

To investigate whether there are signs of recombination at the genomic level in *Thamnolia*, I used two recombination detection methods. The first method is called 'The four gamete test', and it searches for combinations of all four alleles at two given polymorphic loci in the DNA data (Hudson & Kaplan, 1985). As long as the mutation rate is lower than the recombination rate, then the existence of the four gametes can only be explained by recombination (Hudson & Kaplan, 1985). Alternatively, the Index of Association method considers variable sites in an alignment and quantifies the levels of linkage disequilibrium (Smith *et al.*, 1993). Values close to 0 show linkage equilibrium (LE), which suggests molecular recombination, while values close to 1 suggest a highly clonal population structure.

3.5 Identifying and describing the MAT loci

The MAT idiomorphs and their flanking genes were identified from genomes of *Thamnolia* Lineage C*, D. baeomyces* and *I. ericetorum* obtained from the axenic fungal cultures and the lichen thallus of *S. ceratites*. After the assembly of the mycobiont genomes using ABySS (Simpson *et al.*, 2009) and MaSuRCA (Zimin *et al.*, 2013), a BLAST strategy was used to search for the scaffolds containing the MAT loci. As queries, we used *SLA2, APN2,* and the conserved domains of *HMG-box* and *α-domain* (see Fig.3) previously identified in the mycobiont of *Xanthoria parietina* (Scherrer et al., 2005) and other Pezizomycotina fungi. *De novo* gene predictions, alignment with already described protein sequences, and the predicted transcripts based on RNAseq information were used for the annotation of the scaffolds containing the MAT idiomorphs either (**Paper IV**).

3.6 Determining the mating-type ratio in *Thamnolia* populations

To determine the mating-type ratio in *Thamnolia* populations, I designed specific primers that amplifying only one of the MAT idiomorphs. The presence/absence of each idiomorph was screened by PCR (**Paper IV**).

3.7 Investigating photobiont switching and sharing

To investigate if *Thamnolia* mycobionts associate with different photobiont lineages, specific *Trebouxia* primers were used to amplify the photobiont genotypes from the genome. Photobiont sharing between ecologically similar mycobionts was investigated through two sequencing methods: Sanger

sequencing on individual lichen samples and Ion Torrent high throughput sequencing on pooled lichen tissues (**Paper III**).

4. Results and Discussions

4.1 Three mycobiont lineages in *Thamnolia* influenced by the last glacial period

In the worldwide phylogeography study (**Paper I**), we revealed the existence of three mycobiont lineages in *Thamnolia* with different geographic distribution. Lineage A was confined to the Northern Hemisphere and adapted to the tundra region of both the North American and Eurasian continents. Notably some of the localities where Lineage A was found (*e.g.* the Aleutian Islands or the shorelines of Norway) were previously presumed glacial refugia (Brubaker *et al.* 2005, Parducci *et al.* 2012, Schafer *et al.* 2010). Therefore, based on the distribution and the population genetic structure of Lineage A, with its highly diverse and unique haplotypes, we believe that this lineage survived the last Ice Age *in situ*.

Lineage B had an even more restricted distribution, given that in all our worldwide sampling, we found it in the alpine region of the Alps (Austria and Switzerland) and Western Carpathians mountains (Poland and Romania). Several phylogeography studies support the hypothesis that at least southern Europe was partially or completely non-glaciated during the Pleistocene, and therefore represented a sanctuary for many species (*e.g.* Schonswetter *et al.* 2005, Nägele *et al.,* 2015). High diversity or presence of unique haplotypes was shown in several organisms collected from Central and South Europe (Provan & Bennett, 2008), including the rare lung-lichen *Lobaria pulmonaria* (Widmer *et al.* 2012). Although Lineage B had an extremely low genetic diversity (only two genotypes), the restricted European distribution of this lineage suggests a Pleistocene influence where it could have retreated to the non-glaciated region of Europe and possibly adapted to the alpine region.

Lineage C seems to be adapted to tundra and alpine environments; it was found worldwide on all continents except Africa and Antarctica. This lineage was highly clonal and it seems capable of long distance dispersal: The same haplotype was found in North America, Sweden and Nepal. Furthermore, the haplotype network analyses suggested a Northern Hemisphere, even a Scandinavian origin of Lineage C. Due to the insufficient variability of the nuclear markers used in this study, I was not able to confidently infer migration

patterns. However, both the haplotype networks analyses and the negative Tajima's D indicate that Lineage C went through a bottle neck, followed by recent population expansion and/or a selective sweep. Based on these data, I speculate that Lineage C originated somewhere in Europe or Asia where it might already have been adapted to tundra and alpine environments. When the Pleistocene glaciers started to retreat, Lineage C expanded with the tundra left behind, crossed the Beringian bridge as the megafauna (Fox-Dobbs *et al.*, 2008), plants (Eidesen *et al.*, 2013) or humans (Perego *et al.*, 2009), and spread to the Americas. Similar migration patterns were revealed with microsatellites data in the highly asexual and cosmopolitan lichen species *Cetraria aculeata* (Schreb.) Fr. (Fernandez-Mendoza & Printzen, 2013), with which *Thamnolia* is often found growing side by side. But these are hypotheses needing to be tested, and due to the high clonality of this lineage, I believe that it is only possible under a population genomics framework using NGS data.

4.2 Can we use morphology and chemistry in *Thamnolia* taxonomy?

The simplest answer to this question is: no, neither morphology nor chemistry can reliably be used as taxonomic characters to recognize species in *Thamnolia* (**Papers I** and **II)**. All morphotypes described (Kärnefelt & Thell, 1995), including the most extreme ones such as *T. papelillo* (with very flat podetia) and *T. juncea* (with very the long podetia) (Santesson, 2004) genetically are grouped together with the cylindrical thalli from Lineage C (**Paper II)**. However, when it comes to chemistry the situation is a bit more complicated. All samples from Lineage A are UV+ and have cylindrical podetia, while all samples from Lineage B likewise are cylindrical but are UV-. Yet Lineage C, which partly is sympatric with both A and B, contains individuals of both chemistries and all the morphologies described here (**Papers I** and **II)**. Nevertheless, chemistry and morphology, in combination with geographical location, can be indicators of the lineage: Lineage B, which is UV-, was always sympatric with UV+ specimens of Lineage C. In other words, if *Thamnolia* specimens were collected from Austria, Switzerland, Romania or Poland, we can be fairly confident that the UV- specimens belonged to Lineage B, while the UV+ specimens are from Lineage C. Likewise, the situation is similar in the case of Lineage A (always UV+); Lineage A was much more often sympatric with UV- specimens from Lineage C (**Papers I** and **II)**. Still the three *Thamnolia* lineages can only be confidently distinguished by DNA data, and both the ITS1 and ITS2 regions can be used as for barcoding.

The studies that tried to tackle chemotype monophyly (Nelsen & Gargas, 2009a; Leavitt *et al.*, 2016) considered *Thamnolia* samples from Lineages A and C as one homogenous population. These studies showed partially solved phylogenies with specimens of both chemistries, together with one supported clade of just UV+ and one low supported clade of UV- individuals. However, the well-supported UV+ clade was composed of individuals of *Thamnolia* Lineage A only. The authors attributed their results to incomplete lineage sorting or rare recombination but in fact, they were drawing conclusions based on a heterogeneous sample of Lineage A and C.

In **Paper II**, a taxonomic review of the genus *Thamnolia* was made in which we considered the most-used morphologically described species and chemical varieties: *T. vermicularis* (Sw.) Schaer., *T. subuliformis* (Ehrh.) W.L. Culb., *T. papelillo* R. Sant. (both chemical varieties), *T. juncea* R. Sant. (both chemical varieties), *T. subvermicularis* Asah., and *T. taurica* Wulfen ex Jacquin. Except for the last two mentioned species, we could amplify DNA from the ITS region from their holotypes or neotypes. One big achievement was obtaining DNA data from the neotype of *T. subuliformis,* which belonged to the Olof Swartz herbarium and was probably collected at the end of 18th or early 19th century. Obtaining DNA from herbarium types, and incorporating the geographic location and podetium chemistry information, enabled us to follow The International Code of Nomenclature for algae, fungi, and plants (ICN) and taxonomically name the three *Thamnolia* lineages: Lineage B, which is always UV-, should be called *T. vermicularis,* a name previously used for the UV- specimens. Although *T. subuliformis* was used by Culbersson (1963) to describe the UV+ specimens, this name is much older, and therefore we propose to assign it to the samples from Lineage C. None of the previous names could be used to identify Lineage A. Thus, we choose a new species name that connects with the habitat where it is found: *T. tundrae* Brännström & Tibell. **Paper II** is currently under review; therefore, until the proposed scientific names are accepted, throughout this thesis, I will use the provisional names from **Paper I**: Lineages A, B and C.

4.3 *Thamnolia* switches photobionts

We confirmed the previous findings that *Thamnolia* can associate with different photobiont lineages from the genus *Trebouxia* (Nelsen & Gargas, 2009b). Species recognition in *Trebouxia* is controversial, but we could assign the *Trebouxia* found in *Thamnolia* to five distinct *Trebouxia* clades (**Paper I** and **II**). Yet, our data showed that in fact, only the mycobiont belonging to Lineage C was found associated with photobionts belonging to all five *Trebouxia* lineages. Lineage A was only found associated with the photobionts of the two related clades of *Trebouxia simplex* (**Paper I**), while

Lineage B was found associated with one of this closely related clades (**Paper I).**

An interesting geographic pattern was revealed in the distribution of *Thamnolia* photobionts (**Paper I,** Fig. 5). One clade was frequently found in samples from North America, another was more frequently found in samples from northwestern Asia, while Central Asia was dominated by *Thamnolia* Lineage C associated with a third *Trebouxia* clade. The region of Europe and Alaska seem to be a contact zone, where the three *Trebouxia* clades are more equally distributed. Do these results suggest an adaptive potential of Lineage C to varying environments that explain its successful dispersal beyond the boundaries that confine Lineages A and B? Can the geographic pattern seen in the photobiont distribution be explained by algal adaptation to a specific environment? If these hypothesis are true then Lineage C wide distribution could be explained by its ability to associate with many photobiont genotypes and therefore access different habitats then the ones where Lineages A and B are found. Although suggestive, our present data (**Paper I)** cannot confirm the hypothesis of adaptation. A similar geographic pattern was described for the photobionts associated with *Cetraria aculeata,* where it was revealed that photobiont distribution was highly correlated with geography and environment (Fernandez-Mendoza & Printzen, 2013). A correlation between environment and photobiont distribution has also been shown in other lichens (Perez-Ortega *et al.*, 2012; Dal Grande *et al.*, 2014b; Sork & Werth, 2014; Werth & Sork, 2014; Catala *et al.*, 2016). In the same time the photobiont geographic pattern we see in **Paper I (**Fig.5) can also be explained by asexual propagation through lichen propagules and switching to a new photobiont lineage when reproduction by fungal spores occurs.

4.4 *Thamnolia* and *Cetraria* share photobionts

Thamnolia and *Cetraria* are two cosmopolitan, but distantly related mycobiont genera (Miadlikowska *et al.*, 2014) that are often found growing together and can associate with several photobiont lineages. Consequently, we hypothesized that these two genera belong to the same ecological lichen guild (Rikkinen *et al.*, 2002) and will share photobionts. Therefore the first aim of the **Paper III** was to investigate if sympatric populations of *Thamnolia* and *Cetraria* are sharing their photobionts. Furthermore, in **Paper I**, we observed the occurrence of two *Trebouxia* genotypes in one *Thamnolia* sample. Thus, the second aim of **Paper III** was to investigate if *Thamnolia* might carry within its thalli several photobiont genotypes. The results partially confirmed our first hypothesis of photobiont sharing: in Iceland, the two mycobiont genera share photobionts from one of the previously described *Trebouxia* clades (**Paper I**). This photobiont clade was predominantly found in North America (**Paper I**). On Öland, *Thamnolia and Cetraria* mycobionts were

each associated with different photobionts. *Thamnolia* was found to associate with photobiont populations from the previously described *Trebouxia* clade of northwestern Asia (**Paper I**) whereas *Cetraria* mycobionts were found associated with the same photobionts as their Icelandic relatives but also with photobionts from other *Trebouxia* lineages.

Ion Torrent sequencing revealed the existence of multiple photobiont genotypes found in different proportions within both *Thamnolia.* Interestingly Ion Torrent amplicons obtained from *Cetraria* thalli were closely related with Sanger photobiont sequences obtained in a separate study (Nelsen & Gargas, 2009b) from *Thamnolia* samples from China (**Paper III**). These results are contradictory with the results based on Sanger sequencing that were obtained by us (**Paper III**) and by the previous study in *Thamnolia* (Nelsen & Gargas, 2009b). In both studies the Sanger data showed just one photobiont genotype in each *Thamnolia* podetium, supporting the idea that the results are biased by the mode Sanger sequencing works. When other technologies such as: cloning of PCR (Catala *et al.*, 2016), microsatellites-markers (Dal Grande *et al.*, 2014a), fingerprinting (Muggia *et al.*, 2013), and independent culturing and sequencing (Voytsekhovich & Beck, 2015) were used, coexisting photobiont genotypes were always found.

In conclusion, although we observed sharing of photobionts between two distantly related mycobiont species, we remain uncertain if the factors driving sharing are local adaptation of photobionts. The photobiont sharing between *Thamnolia* and *Cetraria* on Iceland and the lack of sharing on Öland could also be also explained by demography or photobiont background diversity. Yet, the existence of multiple genotypes within a lichen thallus (**Paper III**) suggested to me a new hypothesis about the switching mechanism in asexually reproducing macrolichens such as *Thamnolia* and *Cetraria.* I hypothesized that if macrolichens spread by fragments the photobionts would not be replaced but accumulated within their thalli and carried around as in a 'suitcase'. Depending on the climatic conditions the most suitable photobionts genotypes would be used and farmed by the mycobiont within the lichen thallus. I speculate that the more often the lichen is formed from symbiotic propagules (Figs. 1a, 1b, 1c), the more photobiont genotypes would be accumulated. In these circumstances, spreading by vegetative propagules should be beneficial and fungal reproduction should be detrimental.

4.5 Has *Thamnolia* any mating partner?

Icmadophilaceae is a lichen family where mating system seems to be associated with morphology features. The sexually reproducing species have strik-

ingly similar morphologies, in having a crustose thallus and pink fruiting bodies. The asexual species usually have an erect, often white thallus (Fig.3). In **Paper IV** of this thesis, we investigated the genomic architecture at the mating-type locus of four species from four genera of *Icmadophilaceae*. Genomic and transcriptomic data revealed that *Thamnolia* (Lineage C) and the sexual reproducing species from the sister genus *Dibaeis baeomyces* (Fig.3) have a heterothallic genomic architecture. This implies that both are sexually self-incompatible species, and in order to mate, they need a partner of a different mating type (MAT 1-1 or MAT 1-2). Both sequenced genomes of *Thamnolia* (Lineage C) and *Dibaeis baeomyces* contained the MAT 1-2 idiomorph and had the HMG-box domain, together with the flanking genes: *APN2* and *SLA2*, located on the same scaffold. In contrast, two species from the sister genera, *S. ceratites* and *I. ericetorum* presented a homothallic genomic architecture (MAT 1-1 and MAT 1-2), which suggests a self-compatible mating system. The sexually reproducing *I. ericetorum* had both MAT loci on different scaffolds, while the asexual *S. ceratites* had both MAT loci on the same scaffold.

To test the hypothesis that *Thamnolia* is asexual because of losing its mating partner, we screened a worldwide sample of *Thamnolia* (all three lineages) with specific primers designed to amplify the HMG-box domain defining the MAT 1-2 idiomorph (Fig.2). Except for one negative PCR reaction, all our tested samples were positive for the MAT 1-2 idiomorph, and Sanger sequences confirmed the correct amplification. This result suggests that the other idiomorph, MAT 1-1, is completely lost from *Thamnolia* populations or is found in extremely low frequencies. The sample showing a negative result might be in fact a MAT1-1 individual. However, this sample was collected in 1968: therefore the negative result might be due to low DNA quality. Another possibility could be that some of the *Thamnolia* samples are in fact homothallic and therefore have both MAT loci within one genome. Because the sequence of *Thamnolia*'s MAT 1-1 idiomorph remained unknown, we designed degenerate primers based on the α-domain sequences belonging to *S. ceratites* and *I. ericetorum.* After a re-screening of a subset of the previously investigated *Thamnolia* specimens (all three lineages), with the exception of the two positive controls of *S. ceratites* and *I. ericetorum*, we were not able to amplify α-domain in either of the samples.

We interpreted our results as a strong support for our hypothesis that *Thamnolia* is a sexual self-incompatible fungus with a complete or extreme skewed ratio toward one mating type. The lack or the extreme low frequencies of mating partners of the opposite type could have led to a complete asexual life style in *Thamnolia*. The existence of a fungal population that is originating from one adapted clone of one mating type was previously shown in *Cryptococcus neoformans* (Lin *et al.*, 2005). Two studies investigating the decline of two European red listed species *Lobaria pulmonaria*

(Widmer *et al*., 2012) and *Letharia vulpina* (Högberg *et al.,* 2002) also showed a bias in the mating-type ratio in obligate outcrossing mycobiont species. Yet such a drastic biased mating-type ratio was never shown in a mycobiont lineage with a global distribution. None of the three *Thamnolia* lineages contained specimens of MAT 1-1 idiomorphs, result suggesting that the lost of mating partners seems to precede the genus diversification. However this hypothesis might be contradicted by the recombination tests applied on *Thamnolia* lineages.

4.6 Genetic signatures of recombination in *Thamnolia*

The results of **Paper IV** strongly indicated that one of the mating types (MAT 1-1) was lost in *Thamnolia* before the split of the three highly clonal lineages. Yet when sensitive recombination tests were applied, both Lineages A and C showed genetic signatures of recombination (**Paper I**). These results could be explained by historical sexual reproduction precluding the split of the three lineages of *Thamnolia*. They can also be explained by very low frequencies of MAT 1-1, which would lead to very low frequencies of sex. Although just 8 individuals were investigated, Lineage A had a clear recombining population structure. Therefore it can be speculated that the opposite mating type might be found in this lineage. Although the screening tests showed that all investigated specimens of Lineage A were of the same mating type as Lineage C, there is a possibility that individuals of the opposite mating type were not sampled.

Other explanations for the genetic signatures of recombination can be attributed to single mating type sexual reproduction as shown in *Cryptococcus neoformans* (Lin *et al.,* 2005) or other processes such as mitotic recombination and parasexuality (Pontecorvo & Sermonti, 1954).

A last possible explanation could be that the missing mating type is not lichenized and was therefore never collected because individuals of this mating type do not look like *Thamnolia.* However, free-living mycobionts forming macrolichens have not yet been described.

5. Assembling puzzle pieces together

The work within this thesis endeavored to clarify the evolutionary processes that govern lichen symbiosis and adaptation by collecting and assembling the puzzling pieces of *Thamnolia*. Here, I lay out the pieces that were found.

Based on the genomic architecture of the mating-type locus, I concluded that *Thamnolia* is a fungus with a sexual self-sterile mating system. However, when I screened my global sample of *Thamnolia*, I found only one mating type. These findings support the hypothesis that asexuality in *Thamnolia* can be attributed to the lack of mating partners or an extremely skewed ratio of mating types.

In spite of a predominant asexual reproduction, still diversification seems to occur in *Thamnolia.* The phylogeography study of my worldwide sample using a multilocus dataset showed the existence of three cryptic mycobiont lineages with different geographic distributions, habitat preferences, population structures, and chemical variation. Although neither morphology nor chemistry can confidently be used to recognize species in *Thamnolia,* a combination of these phenotypic traits, together with geographic location, can be a good indicator that a given sample belongs to a specific lineage. However, only genetic markers can confidently be used to delimit them, and there is sufficient variation in both ITS1 and ITS2 that these markers can be used as fungal barcodes. Due to the low genetic diversity revealed by the molecular markers used, the divergence of the three lineages could be only roughly estimated to ages between 0.6 and 6.6 Myr, based on ITS mutation rates and nucleotide diversity. A more confident estimation can be obtained with much more genetic data, and I believe that genomic data based on NGS can be very useful in this regard.

Lineage A was only found in the Northern Hemisphere in the tundra region of Siberia, Aleutian Islands, the shore lines and mountains of Scandinavia. It contained only individuals with baeomycesic and squamatic acids (UV+ chemical variation) and with cylindrical morphology. Based on its narrow distribution range in tundra environments, we propose Lineage A as a new species: *Thamnolia tundrae.* Population genetic analyses revealed a recombinant population structure and relatively lower clonality than the other two lineages. In my collection, Siberia was the least sampled site. I suspect that an extensive collection in this region and the use of more genetic

data will reveal many more haplotypes. I also suspect that Lineage A might in fact harbor individuals from the other mating type, and therefore the presence of a few clones is due to the occurrence of sexual recombination. Based on its geographic distribution and haplotype uniqueness, I concluded that Lineage A is an *in situ* glacial survivor of the Pleistocene.

Lineage B had an even more limited geographic distribution, being restricted to the alpine regions of central and eastern European mountains. We found it in the Alps and Western Carpathians and I suspect it to occur above the tree line also of the Apennine Mountains and the Pyrenees. This lineage had an extremely depauperate genetic diversity with only two unique haplotypes present across the sampled sites along several mountain chains. Due to the lack of genetic variability, it was not possible to test whether recombination occurs in this lineage. Taken together, after the last Ice Age, Lineage B likely went through a drastic bottleneck, but appears to have survived as other lichen species had in the non-glaciated areas (Högberg *et al.*, 2002; Widmer *et al.*, 2012). I only found individuals containing thamnolic acid (UV- chemistry) and cylindrical morphology. We propose that *Thamnolia vermicularis* (the name previously often used for the UV- cylindrical *Thamnolia* specimens) for nomenclatural reasons has to be given to Lineage B. The alpine European habitats where Lineage B is found are isolated from each other, and can easily be affected by human activities and climate change (Schmitt, 2009). The combination of habitat specialization, low genetic variability and no potential for sexual reproduction qualifies, in my opinion, Lineage B for a status as highly endangered.

The situation is very different for Lineage C. It contained individuals of both chemistries, and remarkably, of all the previously described morphotypes (i.e. 'flat', 'wide', 'cylindrical' thalli) (Kärnefelt & Thell, 1995; Santesson, 2004). This lineage had a wide distribution spanning all continents, and was partly sympatric with both Lineages A and B. It was found in both tundra and alpine habitats. Based on the nomenclatural situation we propose that Lineage C should maintain one of the frequently used older names: *Thamnolia subuliformis*. Genetic data showed that Lineage C was highly clonal, and both population structure and genetic tests of selection suggested that this lineage went through a drastic bottleneck followed by a recent clonal population expansion. Based on two sensitive recombination tests Lineage C, with a large sample size, showed genetic signatures of recombination, as was the case in Lineage A. However neither of the tests could reveal whether these signatures were a result of ancient recombination, events predating the divergence of the three lineages, contemporary and rare recombination events, or other processes such as mitotic recombination and parasexuality. The signs of recombination could also be explained if the other mating type existed in Lineage A and hybridization between the two lineages happened. One way to investigate this last hypothesis is to use a population genomics approach and an extensive sampling in the localities where Lineage C is sympatric with Lineage A. The haplotype network anal-

yses strongly suggested that Lineage C originated in Eurasia or even in Scandinavia. Although the multilocus DNA markers used were not sufficient to perform proper migration analyses, the haplotype networks and the genetic tests (e.g. Tajima's D) suggested that Lineage C was a highly successful clone that expanded throughout the world from Eurasia to the Americas and Oceania during the Pleistocene, perhaps by island hopping across the mountains or by other means of long distance dispersal.

One could argue that Lineage C may be a hybrid between Linages A and B; it has both chemical variations and is adapted to the environments conducive to both Lineages A and B. However the divergence tests, dating analyses, and phylogenetic analyses suggested a more recent divergence of Lineages A and B and ancestrality of Lineage C. The Northern Hemisphere distribution of Lineages A and B, and the Scandinavian or Eurasian assignment of the ancestral haplotype in C suggested a Northern Hemisphere origin of *Thamnolia,* similar to that of the cosmopolitan lichen *Cetraria aculeata,* with which *Thamnolia* is often found growing together. However the origin of *Thamnolia* and its migration patterns still remain open questions that may be addressed in a population genomics context. With this approach, in combination with transcriptomic data, one may also investigate the chemical variation in *Thamnolia* and determine whether it is indeed produced by the main fungal partner, after which lichen species are named.

In a paper which focused on the frequencies of chemotypes in different herbarium collections (Satô, 1963) it was observed that the UV+ *Thamnolia* specimens are more frequently found in the Northern Hemisphere, the UV- specimens are more frequently found in the Southern Hemisphere, while in the Equatorial region, they are present at equal frequencies. In the light of this paper, I think it is striking that the Ice Age refugial Lineages A and B, have individuals of one or the other chemistry, while Lineage C has them both. This peculiar distribution has as yet not been explained. One possibility may be that the secondary metabolites in *Thamnolia* are produced by a third symbiont with a life history highly influenced by the demography of the three *Thamnolia* lineages.

Another question that continues to intrigue me is: what has contributed to the success of Lineage C, in comparison with the other two, and specifically Lineage B? One explanation might come from the ability of Lineage C to associate with several green algal lineages from *Trebouxia,* while Lineages A and B were found to be associated with only two and one, respectively. Furthermore I found that Icelandic populations of Lineage C contained within their thalli several photobiont lineages that are also shared with its omnipresent neighbor *Cetraria aculeata.* By carrying several algal populations with distinctive adaptive abilities within a lichen thallus, analogous to a suitcase, an asexual lichen can have the ability to adapt to different environments. Under this scenario, vegetative propagation through symbiotic propagules, will maintain the successful symbiotic partnership but will additionally allow accumulation of new symbionts. This mode of reproduction will

likely be more advantageous than the sexual one by fungal spores, where the formation of a lichen needs to start from the reconstitution of the lichen from each symbiotic partner.

6. Concluding remarks

My study on *Thamnolia* lichen-forming fungi began as a master student when I first started to investigate the reproductive behavior and demography of this genus, and has continued to be part of my doctoral studies presented here. With the generous support from many lichenologists, friends and family, adventurers or herbariums, a vast collection of *Thamnolia* that spanned the entire distribution range of this genus has provided a valuable resource for studying *Thamnolia* biology and forms the basis for this thesis. By using of combination of taxonomic, phylogenetics, population genetics methods, and bioinformatics tools, I increased our current body of knowledge about *Thamnolia* and provided answers to questions concerning its asexual reproduction and taxonomic status. I found that the entire *Thamnolia* genus might be constrained to only vegetative dispersal due the lack of mating partners. At the same time, the existence of the three mycobiont lineages suggests that *Thamnolia* can still diversify. The ability of *Thamnolia* to adapt to varying environments may be exemplified by the adaptation of the two allopatric Lineages A and B to tundra and alpine environments, respectively, while Lineage C has more flexible habitat criteria. Our data suggest that the lineage distribution patterns can be explained by demography; it is likely that the Last Ice Age period restricted Lineage A to the Nordic refugia, caused Lineage B to retreat to the non-glaciated regions of Southern Europe, and contributed to wide distribution of Lineage C. The apparent photobiont specificity and the low phenotypic variation of Lineage A and B can result from the availability of photobionts in those locales. On the other hand, Lineage C was found to be associated with five green algal lineages and some of them were shared with a distantly related but ecologically similar mycobiont. Lineage C also possesses all the phenotypic variations noticed in *Thamnolia.* Taken together, these results are advocating an adaptive potential of Lineage C to varying environments and could explain its successful dispersal beyond the boundaries that confine Lineages A and B.

In this work, we see the effects of different reproductive strategies on the long-term future evolutionary trajectory of symbiotic organisms such as lichens. The best example is the success story of the highly clonal and maybe entirely vegetative dispersing *Thamnolia* Lineage C. Based on its worldwide distribution and its generalist life style we could argue that the asexual reproduction is not associated with any obvious loss of fitness. Therefore

asexual reproduction in lichens might not necessarily lead to an evolutionary dead end as proposed for non-symbiotic organisms. When each symbiotic partner is a generalist, symbiosis will enable formation of diverse entities that together can adapt as one organism to changing environments.

Although in an ideal world, experiments can be conducted to further understand *Thamnolia* biology and its adaptation to variable environments, the slow growth of lichens and the impossibility to manipulate them under laboratory conditions, call for the development of new methods. I propose that a population genomics approach using NGS is the best alternative solution. My study sets the foundation from which researchers can optimize their sampling strategy, particularly given the now known reproductive behavior and geographic distribution. It also provides genomic data for four taxa of Icmadophilaceae family. One by one, pieces of the *Thamnolia* symbiosis puzzle can be elucidated from such an approach. One day, I hope that further studies can finally help me to see lichen symbiosis as 'spinning coins'.

7. Svensk sammanfattning

I denna avhandling beskriver jag nya upptäckter om biologin hos lavar av släktet *Thamnolia*. Med en kombination av taxonomiska, populationsgenetiska och bioinformatiska metoder har jag besvarat frågor angående släktets asexuella reproduktion och taxonomiska status.

Baserat på den genomiska arkitekturen av det locus som bestämmer parningstyp drog jag slutsatsen att svampkomponenten av *Thamnolia* har ett obligat utkorsande parningsbeteende. Vid globala undersökningar fann jag emellertid endast en parningstyp hos *Thamnolia*. Denna upptäckt stöder hypotesen att släktets asexualitet beror på avsaknaden av en partner att para sig med.

Diversifiering verkar ändå ske hos *Thamnolia*. I min fylogeografiska analys av ett dataset bestående av ett flertal loci från *Thamnolia* världen över så upptäckte jag tre kryptiska härstamningslinjer av lavens svampkomponent, med olika geografisk utbredning, habitatpreferens, populationsstruktur och kemisk variation. Även om varken morfologi eller kemisk variation med säkerhet kan användas för att definiera arter av *Thamnolia* så kan en kombination av dessa fenotypiska egenskaper vara en god indikator. Genetiska markörer kan användas med större förtroende; ITS1 och ITS2 används ofta som ”barcode” för att identifiera svamparter, och användes även här i detta avseende.

På grund av låg genetisk diversitet i de molekylära markörerna kunde tidpunkten för uppdelningen av de tre linjerna endast uppskattas grovt till mellan 0.6 och 6.6 miljoner år sedan. För att få en mer precis uppskattning skulle mer data behöva genereras och analyseras, exempelvis genom helgenomsekvensering.

Linje A påträffades endast på norra halvklotet i tundraregioner i Sibirien, Aleuterna, samt kustlinjer och bergskedjor i Skandinavien. Endast individer med kemisk fenotyp UV+ samt cylindrisk morfologi ingår i denna härstamningslinje. Baserat på habitat och det begränsade utbredningsområdet föreslår vi i taxonomiartikeln denna linje som en ny art: *Thamnolia tundrae*. Populationsgenetiska analyser avslöjade en rekombinerande populationsstruktur och lägre nivå av klonalitet hos denna linje än de övriga två. I min *Thamnolia*-samling är Sibirien den region som har minst antal individer, och jag misstänker att ytterligare insamling i denna region samt användning av en större mängd genotypisk data skulle identifiera fler haplotyper. Möjlig-

heten finns också att även den andra parningstypen förekommer i linje A, varvid den lägre graden av klonalitet skulle kunna förklaras med förekomsten av sexuell rekombination. Med den geografiska utbredningen och skillnaderna gentemot de andra linjernas haplotyper som grund drar jag slutsatsen att linje A härstammar från en *in situ*-överlevare från den Pleistocena nedisningen.

Linje B har ett ännu mer begränsat utbredningsområde och verkar endast förekomma i den alpina regionen i Mellan- och Östeuropa. Vi hittade den i Alperna och Västra Karpaterna och jag förväntar mig att den även kan påträffas ovanför trädgränsen i Apenninerna och Pyrenéerna. Från en taxonomisk synvinkel hittade jag bara individer med kemisk fenotyp UV- och cylindrisk morfologi. Därför föreslår vi att *Thamnolia vermicularis*, vilket är den gamla nomenklaturen som beskriver cylindriska exemplar med kemotyp UV-, ska behållas och tillges linje B. Denna linje har en extremt utarmad genetisk diversitet med endast två unika haplotyper närvarande i flera bergskedjor i dess utbredningsområde. Bristen på genetisk variation gjorde att jag inte kunde testa huruvida rekombination sker eller inte i denna linje. Återigen antyder den begränsade geografiska utbredningen och linjens unika haplotyper att B genomgick en drastisk populationsflaskhals under den senaste istiden men lyckades överleva i områden utan is, likt andra lavarter (Högberg *et al.,* 2002; Widmer *et al.,* 2012). På grund av det extremt reducerade utbredningsområdet verkar linje B vara beroende av specifika habitat. De alpina europeiska habitat där den förekommer är mycket avskilda från varandra och lättpåverkade av mänskliga aktiviteter samt klimatförändringar (ref). Kombinationen av habitatberoende, låg genetisk variation och avsaknad av potential för sexuell reproduktion är min uppfattning att denna härstamningslinje kvalificeras som en starkt utrotningshotad art.

Situationen är helt annorlunda för linje C, vilken innehåller individer av båda typer av kemisk variation och samtliga tidigare beskrivna morfologier (platta, vida och cylindriska thalli) (Kärnefelt & Thell, 1995; Santesson, 2004). Denna härstamningslinje har ett enormt utbredningsområde som sträcker sig över alla kontinenter där *Thamnolia* påträffas, inklusive tundra och alpina habitat där den är sympatrisk med både linje A och B. Baserat på dessa observationer föreslår vi att linje C bör behålla det andra frekvent använda namnet: *Thamnolia subuliformis*. Genetiska data påvisar en hög grad av klonalitet, och både populationsstruktur och test för selektion visar att denna linje har genomgått en kraftig populationsflaskhals och nyligen ökat i populationsstorlek. Liksom i linje A fann jag här tecken på genetisk rekombination, baserat på en stor mängd prover och känsliga rekombinationstest. Testen kunde dock inte visa om dessa tecken härrör från gammal rekombination som skett innan uppdelningen av de tre linjerna, nutida och mycket sällsynt rekombination, eller andra typer av processer som kan ge en signal som liknar den för sexuell rekombination, så som mitotisk rekombination eller parasexualitet. Spåren av rekombination kan också förklaras om den

andra parningstypen förekommer i linje A eller om hybridisering sker mellan linje A och C. Ett sätt att undersöka detta är att genomföra en omfattande insamling av prover från de områden där linjerna är sympatriska och analysera dem med populationsgenomisk metodik.

Analyserna av haplotypnätverk tyder starkt på att linje C ursprungligen uppkom i Eurasien, och där möjligen i Skandinavien. Även om DNA-markörerna inte räcker för att göra en ingripande analys av migrationsmönster så visar haplotypnätverken och andra genetiska test (Tajimas D) att linje C sannolikt härstammar från en mycket framgångsrik klon som spridits över världen från Eurasien till Amerika och Oceanien under den Pleistocena epoken. Jag misstänker att spridningen av linje C följde tillbakadragandet av inlandsisen i de tundraområden som då uppstod, och i kombination med långdistansspridning på så vis spred sig över världen.

Är det möjligt att linje C är en hybrid mellan A och B? Den har båda typer av kemisk variation och är adapterad till båda miljöerna. Tester av divergens, datering och fylogenetiska analyser tyder emellertid inte på detta. Istället verkar linje C vara den ursprungliga och A och B ha avgrenat från denna mer nyligen. Förekomsten av A och B på norra halvklotet samt det föreslagna ursprunget av linje C i Eurasien tyder på att *Thamnolia* härstammar från norra halvklotet, likt *Cetraria aculeata*, vilken är en annan globalt förekommande lav som *Thamnolia* ofta påträffas tillsammans med. Detta är dock fortfarande en öppen fråga som skulle kunna besvaras säkrare med populationsgenomiska analyser. Tillsammans med transkriptomdata skulle även den kemiska variationen kunna undersökas, och om det verkligen är den huvudsakliga svampkomponenten som är ansvarig för denna variation. Det är slående att linjerna som överlevde i istidsrefugier, A och B, uteslutande har den ena eller den andra kemotypen, medan linje C har båda. Satô (1963) visade att exemplar av *Thamnolia* med UV+ förekommer i högre frekvens på norra halvklotet medan kemotyperna är jämnt fördelade i ekvatoriala områden, vilket aldrig fått sin förklaring. Jag tror att en möjlighet är att sekundärmetaboliter hos *Thamnolia* produceras av en tredje symbiont vars livshistoria drastiskt påverkas av demografin hos de tre härstamningslinjerna av *Thamnolia*.

En ytterligare fråga som intresserar mig är varför linje C är så framgångsrik, i synnerhet jämfört med linje B. En förklaring skulle kunna vara att linje C har förmågan att associera med flera linjer av algen *Trebouxia* medan linje A och B endast påträffas tillsammans med två respektive en. Vidare hittade jag flera linjer av algen inom samma thallus i isländska populationer av C, något som delas med den allestädes närvarande *Cetraria aculeata*. Genom att bära ett flertal populationer av algen med olika adaptiva förmågor har en asexuell lav möjligheten att anpassa sig till olika miljöer. Under detta scenario sker vegetativ reproduktion genom att både svamp- och algkomponent av laven sprids tillsammans samtidigt som förmågan att ackumulera nya fotosyntetiserande symbionter bibehålls. Denna typ av reproduktion skulle vara

fördelaktig framför sexuell reproduktion med svampsporer då den fotosyntetiserande komponenten tappas och laven behöver återbildas och starta om från början.

Resultaten av mina studier tyder på att asexuell reproduktion hos lavar inte behöver innebära en evolutionär återvändsgränd, så som föreslagits för icke symbiotiska organismer. Då parterna är generalister kan symbios möjliggöra att olika livsformer tillsammans kan anpassa sig som en unik organism till föränderliga miljöer.

8. Glossary

algal switching mycobiont-photobiont associations are not permanently maintained and the same fungal genotype was found to be associated with distinct photobiont genotypes (ref depriest)

apothecium (pl. apothecia) lichens fruiting body which harbors the ascospores in a sac-shaped structure (**ascus**, pl. **ascii**)

ascospore the spores of an Ascomyceteous fungus that are the result of recombination followed by meiosis

asexual lichens lichens that reproduce through vegetative propagules

conidium (pl. a) the mitospores of a fungus

cortex the upper layer of a stratified lichen

DNA barcode short genetic marker, which is used as a taxonomic method to identify a species.

fructicose lichen a lichen that has a bush-like morphology

foliose lichen a lichen that has a leafy-like morphology

heterothallic obligate out-crossing fungi

homothallic self-fertile fungi

isidium (pl. isidia) minute vegetative dispersal propagules of lichens formed from corticated outgrowths of the lichen surface

lichen guild a lichen community in which genetically different mycobionts are horizontally linked by sharing their photobiont population (ref Rikkinen)

macrolichens plant-like looking lichens

mycobiont the fungal partner of the lichen which forms the main structure in macrolichens

photobiont the photosynthetic partner of the lichen; it can be a microscopic green algae or cyanobacteria; there is one case in which a brown alga was described as photobiont (Sanders)

pycnidium (pl. pycndia) the bottle-like structures which harbors conidia

relichenization the resynthesis of a new lichen

soredium (pl. soredia) minute powdery vegetative propagule of a lichen composed of photobionts tightly wrapped in fungal hyphae

sexual lichens lichens that reproduce through ascospores (meiospores), which needs to encounter a new photobiont to synthetize a new lichen

9. Acknowledgments

Hanna, is this it? I really hope not but until we know, I want to thank you from the deepest of my heart. Do you remember when I met you first time? It was in your office and I came to you when I was in one of my darkest periods in my life. You didn't know me at all but you just took me in your group, gave me all your support and made me feel strong again. Then you offered to be my main supervisor for a master thesis on lichens, when no one else did or could. And you didn't stop there! Somehow I managed to convince you that lichens are very cool to study. You took me as PhD student although they were not your main scientific interest. You believed in me so much and you let me dream and go on with all my crazy plans. In the same time you channeled all these ideas I had and helped me to make them in a thesis. You are a true mentor Hanna, a great scientist and a real fighter! Never forget that! I hope that one day when I will have my own lichen research group (see, I am still dreaming), I will be able to give all these gifts you gave me to others.

Lars, you are the love of my life and the other strong pillar during all these years. When all Swedes were having a classic summer on some warm beach, in some warm country, you drove me to windy and cold places to collect my *Thamnolia.* You froze for me and you even got pneumonia while chasing worm lichens on Gotland. You patiently listen to my crazy hypothesis, my failures and my successes. You encourage me all these year and now in these last days before sending the thesis you took so much care of me. Thank you so much. I can't promise but I next time I can at least try to choose a tropical lichen (or at least some warmer climate species).

Leif, I want to thank you for giving me *Thamnolia.* Do you remember? It was your idea to study it and your instinct that some *Thamnolia*'s survived the Ice Age was good. Through you I discovered how cool lichens are, but also how to teach lichens to students in an exciting way. You guide me through the writing of my first paper, you corrected my English and strange phrases but you left the text to be mine. You even help me recognize and not define species of *Thamnolia.*

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