Lichenized fungi and the evolution of symbiotic organisation

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Summary. Lichen symbioses comprise a fascinating relationship between algae and fungi. The lichen symbiotic life style evolved early in the evolution of ascomycetes and is also known from a few basidiomycetes. These lineages of ascomycetes have diversified in the lichenized stage to give rise to a tremendous variety of morphologies. Also, their thalli are often internally complex and stratified for optimized integration of algal and fungal metabolisms. Thalli are frequently colonized by specific non-lichenized fungi and occasionally also by other lichens. Microscopy revealed various ways of these fungi to interact with their hosts. Beside the morphologically recognizable diversity of the lichen mycobionts and lichenicolous (lichen-inhabiting) fungi, many other microorganisms including other fungi and bacterial communities are now detected in lichens by culture-dependent and culture-independent approaches. The application of multi-omics approaches, refined microscopic techniques, as well as physiological studies, have added to our knowledge of lichens, not only about the taxa involved in the lichen interactions, but also about their functions.
1. Introduction And Historical Background

Lichens were generally accepted as an independent group of organisms, when Schwendener (1) discovered that lichens are the result of the association of fungi and algae. This insight was not widely accepted by contemporaries, but in fact initiated a scientific revolution as lichens later became the prime example of a mutualistic symbiosis, and indeed the phenomenon for which the term symbiosis was originally introduced in biology [(2) as “Symbiotismus”; (3)]. The lichen symbiosis has proved to be one of the most important life styles in the Ascomycota, and is also known from a few Basidiomycota. Approximately 20 000 currently known fungal species live as lichens, mostly in species-rich lineages of Ascomycota (4). The traditional view of lichens as a mutualistic symbiosis of a fungus and one or several green algae or cyanobacteria, has always been under debate (5), but it has recently been more challenged than ever by the discovery of numerous additional microorganisms that potentially occur as obligatory participants in the symbiosis.

The self-supporting associations of fungi and their partners usually form a compact and macroscopically recognizable structure, known as the lichen thallus. Different from many other fungi, which reside beneath the surface of their substrates, the lichen thallus is typically developed at the surface of the substrate or completely exposed to the atmospheric conditions, apparently without exploiting the substrates’ nutrients (Fig. 1A-C).

2. The Lichen Thallus

The lichen thallus arose early in evolutionary history with the conquest of land, and the earliest records of internally stratified cyanobacterial and green-algal lichens were from the Lower Devonian (6). These lichens are of unclear relationship to
current lichen–forming lineages in the Ascomycota, which originated at least as early
as the Carboniferous, and radiated in the Jurassic and Cretaceous to generate the
diversity of the main modern groups (7). The ability to revitalize from dry stages
helps the lichen thallus in many species to survive environmental fluctuations with
extremes of desiccation and temperatures (8, 9). Lichens are thus prominent at high
altitudes and latitudes where conditions become adverse for most other multicellular
organisms. Especially in cool habitats, lichen biomass can be substantial and lichens
can then form a substantial part of the vegetation (Fig. 2A-C). By the balance of
respiration and photosynthetic energy production in the symbiotic system, many
lichen thalli grow slowly but are long-lived. Usually, the fungi dominate biomass and
dictate the shape of lichen thalli. The scientific name of lichens also by definition
refers to the fungal partner only.

Lichen fungi shelter the algal/cyanobacterial partners beneath a protective
peripheral cortex layer (Fig. 3). These layers are developed by the conglutination of
fungal hyphae, which seems to be correlated with increased branching and
anastomoses formation. The outer walls of the fungi are thickened to form sticky
contacts with neighboring hyphae. This appears to be a similar process as found
generally with the formation of fungal fruitbodies, but in the case of lichens
conglutination and compaction occurs in the vegetative mycelium, and this could be
one of the key evolutionary innovations to make lichen thallus evolution possible.
The conglutination of vegetative hyphae forms a more or less coherent layer by
which algal cells can be covered and under which algal populations can form more or
less coherent layers within fungal plectenchyma (i.e., tissue-like structure made of
fungal hyphae). This developmental process seems to be correlated with massive
branching and anastomosing of the hyphae. While genes regulating branching,
anastomosing and conglutination are known from model fungal systems, their
presence and regulation in lichenized fungi have so far not been studied. The
morphogenetic processes seem to be initiated as soon as the fungi contact appropriate
algal cells. Once the thallus morphogenesis is initiated, the algal symbionts can
proliferate massively under the fungal protectorate. The mycobionts are triggered by
the interaction with the algae to produce diverse, more or less complex thallus
morphologies, a wide diversity of lichen-derived secondary metabolites, as well as
fruitbodies for sexual fungal reproduction and/or asexual structures for combined
propagation of symbionts. Notably, strikingly convergent thallus morphologies have
evolved in several unrelated lineages of lichenized fungi (10). The convergent shapes
include complicated morphologies, for example umbilicate thalli (leafy structures
with a central, exclusively fungal holdfast), as well as reductions of complexity, and
reversal to simple crustose lichens or even parasitic life-styles, or the formation of
thalli composed of grain-like fungal-algal propagules.

In certain lineages, there is a strong selectional trend towards vegetative
reproduction solutions that disperse the fungal and algal partners. These include
soredia, which are granules of varying size that contain algal cells and fungal hyphae,
produced in structures of varying size and morphology termed soralia (Fig. 4A).
Other types of vegetative propagules are corticate, forming easily detached
outgrowths from the lichen thallus, called isidia (Fig. 4B). In several lineages of
lichens that reproduce by vegetative means, the production of sexual spore-producing
structures is rare or unknown.

While most species of lichenized fungi produce highly organized thalli with
internal stratification, several “borderline” lichens produce poorly differentiated
thalli (11, 12, 13). A small number of fungal species also seem to be facultatively
lichenized, in the sense that they sometimes are found loosely associated with algae, and sometimes not (14, 15, 16). In the Stictidaceae, this facultative lichenization is apparently determined by the substrate they grow on, as several species can grow as saprotrophs on dead wood (Fig. 5A) and as loosely lichenized on bark (Fig. 5B). This has been termed “optional lichenization” by Wedin et al. (17). These borderline and optional lichens may be seen as present-day analogs of early thallus evolution, before more advanced forms of thallus organization arose.

3. The Lichen Fungi And Their Phylogenetic Relationships

The integration of lichenized fungi into the classification of Fungi was slow at first as very few lichenologists accepted the consequences of Schwendener’s discovery. Vainio (18) was the first to classify at least some higher ranks of the lichen fungi together with other fungi, but the very influential Zahlbruckner (19, 20) treated lichens as a separate group, Lichenes, on a level equal to the Ascomycetes and the Basidiomycetes, which prevented further progress for many years. Nannfeldt (21) argued strongly for integrating lichenized fungi into a natural fungal system, something that was acted upon by Santesson (22, 23, 24). Only in the second half of the 20th century was this integrated classification widely accepted by most mycologists, even if the study of lichen fungi nevertheless often still is considered a matter for lichenologists, and quite distinct from the study of non-lichenized fungi.

Lichenized lineages are interesting from a phylogenetic-evolutionary perspective, as they primarily have evolved and diverged in the lichenized condition with similar partners. The self-supporting, self-sufficient lichen thallus is likely to be the target of selective pressures very different from the selective forces working on
non-lichenized fungi, which creates a substantial difference from what saprobic or
parasitic fungi may undergo.

Within the lichens belonging to the Ascomycota, microscopic characters of the
sexual reproductive structures, in particular fruit-body development and organization
(25, 26, 27), and ascus structures (28, 29, 30), helped to refine the taxonomic
relationships and were largely the basis of classifications in the late pre-molecular
era. Already at that time, there was a vigorous debate about the number of times that
the lichen life style arose in the evolution of fungi. Since it was not feasible to grow
axenic fungal cultures, it was only possible to amplify mycobiont genes directly from
lichen samples after the development of fungal-specific PCR-primers, which
excluded the algal partners. With this technical advancement, Gargas et al. (31) were
able to provide a first phylogenetic analysis of 18S rRNA genes in lichenized fungi.
Their results suggested that the lichen life style arose at least twice in the evolution
of ascomycetes. Data from more sequences of lichenized lineages were analysed by
Lutzoni et al. (32) to revisit the fundamental question of the lichen origins. The idea
of two origins was not rejected, but neither was the possibility of a single origin of
lichens in Ascomycota. Both studies convincingly showed that the lichen life-style
was a rare innovation in the evolution of Ascomycota, while the latter study pointed
out that the symbiotic life style was more frequently lost in major ascomycete
groups. Later studies (33) again suggest that the lichenization originated multiple
times, which seems currently to be the generally agreed view.

During the past years our understanding of the phylogeny within the major
lineages of lichenized Ascomycota increased significantly through a number of
investigations with large numbers of species. An updated classification of
Ascomycota including the lichenized lineages can be found in Jaklitsch et al. (34),
reflecting these discoveries. Extant lichenized fungi occur in at least six of the major lineages within the Ascomycetes; the Arthoniomycetes, Coniothyriomycetes, Dothideomycetes, Eurotiomycetes, Lecanoromycetes, and Lichinomycetes (35, 36, 37, 38). One additional lichenized lineage may belong to the Leotiomycetes. Different ‘relaxed clock’ scenarios suggested that the origin and diversification of the Pezizomycotina occurred in the Cambrian (38). Prieto & Wedin (38) provided information about the timing of the main diversification events in Ascomycota, including estimates for classes, orders and several families of both lichenized and non–lichenized Ascomycota, many of which had not been previously dated. The main lineages of lichen–forming Ascomycota were shown to all have originated at least as early as the Carboniferous, with successive radiations in the Jurassic and Cretaceous generating the diversity of the main modern groups.

Comparatively few representatives of Basidiomycota are lichenized, but recently, the *Cora-Dictyonema* complex was shown to contain a massive number of earlier undistinguished species (39). An investigation focusing on the morphological character evolution in this group showed that the lichenized thallus had evolved progressively from loosely organized filamentous crusts to stereoid and corticioid basidiomata more or less incorporated in the lichenized thallus (40). This indicates that lichenized thalli may have evolved from reproductive structures in their non-lichenized ancestors.

In the overview of lichenized Ascomycota below, we select some examples of phylogenetic studies that contribute major advances in our understanding of morphological and chemical character evolution, lichen biology and interactions, or general understanding of major lineages, of each group.
Arthoniomycetes. The Arthoniomycetes contain a single order, Arthoniales, the second-largest group of lichen-forming fungi, which is a morphologically diverse group of predominantly lichenized representatives, where most form lichen symbioses together with green algae of the Trentepohliaceae. Grube (41) presented a pre-molecular era overview of taxonomic and phylogenetic concepts for the Arthoniales. This account provided discussions of selected morphological characters, and a key to the genera now placed in the order. This work served as a baseline for subsequent molecular work. Ertz & Tehler (42) presented a first comprehensive phylogeny of Arthoniales focusing on lineages previously assigned to Opegraphaceae and Roccellaceae. Their two-locus phylogenetic study showed that traditional morphological characters such as growth form, fruit body type, exciple (a fungal layer of hyphae directly surrounding and derived from the ascoma), hypothecium and ascospores colour, ascospores septation pattern, and secondary metabolism are of limited use in delimiting families and genera. They concluded that the high level of phenotypic plasticity might indicate that the Arthoniales is an old group and that phenotypically characterized genera are paraphyletic. The recent analysis of Frisch et al. (43) focused on the heteromorphic family Arthoniaceae and demonstrated that previous classifications of this family did not reflect evolutionary patterns. According to the phylogenetic hypothesis, lichen secondary metabolites, such as pulvinic acid derivates or red pigments, do not characterize monophyletic groups above the genus level. The study also revealed that lichen-parasitic life style in Arthoniaceae has evolved more than once and is found in four independent lineages of the Arthoniaceae clade and in the Bryostigma clade. Arthoniomycetes with chlorococcoid photobionts are restricted to the Bryostigma clade and Chrysotrichaceae, while the only saprophytic Arthonia species in the phylogeny are
related to *Arthonia radiata* and group with lichenized taxa. The phylogenetic data provide a coherent framework for delineating further monophyletic groups in Arthoniaceae in the future.

**Coniocybomycetes.** Coniocybomycetes is a small group (one order and family, currently two genera with ca. 30 species) of crustose lichens with prototunicate (thin-walled and evanescent) asci and mazaedia-producing (mazaedium – an accumulation of loose, maturing spores covering the ascoma surface), stalked ascomata. This is the most recently described class of Ascomycota and is apparently related to the Lichinomycetes (38), but this relationship is not supported by any obvious morphological characteristics. The Coniocybomycetes is one of the few lichen groups from which a fossil is known, which Prieto & Wedin (7) also utilized in their dating of the major groups of Ascomycota.

**Dothideomycetes.** The Dothideomycetes is a very large fungal group characterized by ascolocular ascoma development, and only some representatives are lichenized. A comprehensive phylogenetic study of representatives of 41 families using sequences from five genes was presented by Schoch et al. (44). Notably, the ancestral reconstruction of basic nutritional modes suggests numerous transitions from saprobic life histories to either plant-associated or lichenised modes. Nelsen et al. (37) integrated the primarily lichen-forming families Trypetheliaceae, Monoblastiaceae and Arthopyreniaceae in a phylogeny of Dothideomycetes. Perez-Ortega et al. (13) introduced the new order Collemopsidiales, which contains Xanthopyreniaceae, an interesting family including borderline lichens, many of which occur in rocky intertidal habitats, as well as lichenicolous fungi.
**Eurotiomycetes.** Eurotiomycetes is likewise a very large and morphologically heterogeneous group where only some representatives are lichenized, all within the subclass Chaetothyriomycetidae, which is an assemblage of ecologically diverse species, ranging from mutualistic lichenised fungi to human opportunistic pathogens. A multigene phylogenetic study of rock-dwelling fungi concentrating on the Verrucariales suggested that this lichenized order as one of the independent ascomycete groups where lichenisation has evolved on a hostile rock surface (45).

The delimitation of orders and families in Chaetothyriomycetidae was re-assessed by Gueidan et al. (35) in a broad phylogenetic study which classified four orders and ten families in Chaetothyriomycetidae.

**Lecanoromycetes.** This class comprises the largest group of lichenized fungi and one of the most species-rich classes in the kingdom of Fungi. Traditional concepts of classification included reproductive structures, in particular of ascomata and ascus apex structure (which often contain characteristic amyloid features). These traditional concepts were challenged when comprehensive sequence sampling of taxa was available (46, 47, 48). Miadlikowska et al. (49) demonstrated that the Acarosporomycetidae and Ostropomycetidae are monophyletic subclasses, whereas the delimitation of the largest subclass, the Lecanoromycetidae, remained uncertain. This and the previous phylogenies confirmed that ascus apex morphology cannot be consistently used as a cardinal character for family level classification of lichen-forming fungi (50).

One important approach in understanding the evolution of phenetic features of lichens is by studying large genera, which are pragmatically circumscribed by key
characters. In many studies these key characters proved to be misleading, and rather were symptoms of massively parallel evolution and thus produced by unrelated fungi. Examples of such misleading key characters are prototunicate asci and mazaedia, which used to characterize the “old” order Caliciales (including pin- and stubble-lichens), a group that in its “old” circumscription now has been shown to be extremely polyphyletic (51, 38) and where the families Caliciaceae and Sphaerophoraceae are now classified in the Lecanoromycetes (31, 38). Another example of this phenomenon was provided by Schmull’s et al. (52) analysis of the heterogenous Lecidea (containing 1200 species), which are usually characterized by black fruitbodies on a crust-like thalli and single-celled, hyaline ascospores. As expected, the analysed species of Lecidea sensu lato and putatively related taxa were scattered within Lecanoromycetidae, and some were even placed outside currently recognized orders in Lecanoromycetidae.

Most of the lichen fungi that form symbioses with filamentous Nostoc cyanobacteria belong to the Lecanoromycetes. Nostoc has a sheath around the filamentous thread-like colonies that swells and becomes extremely gelatinous when wet and this gives many cyanolichens a gelatinous habit when moist. Within the Lecanoromycetes, most gelatinous lichens are classified in the Collemataceae. This is yet another example of how classifications have been based on misleading key characters as a number of gelatinous, Nostoc-containing groups formerly placed in Collemataceae are nested within another Peltigerales family, the Pannariaceae (53, 54). Symbioses with Nostoc have clearly arisen several times. Depending on the reconstruction method, most or all transformations in thallus structure within Peltigerales took place from a non-gelatinous to a gelatinous, Nostoc-containing thallus (53).
Within Lecanorales, the phylogeny of the largest lichen family Parmeliaceae was studied in a number of papers, where Crespo et al. (55) showed that morphological characters discriminated the main clades well, but that the interpretation of the morphological diversity had been quite superficial. The morphological diversity was indeed found to be substantial in this family when Divakar et al. (56) showed that the lichenicolous lifestyle originated independently three times within Parmeliaceae, ca. 24 million years ago.

Gaya et al (57) studied the Teloschistales using a supermatrix approach and showed that a progressive, cumulative, addition of taxa to the matrix analysed with a resulting increasing amount of missing data, did not affect the support and resolution much, but that the monophyly of the order was inconsistent depending on the combination of loci and taxa. In the Teloschistaceae, Arup et al. (58) proposed a completely re-organized generic classification, and investigated how the apparently large morphological plasticity affected the characterization of genera and species. Secondary metabolites were frequently found to better serve as characterizing traits than morphology, at least in parts of the family.

Within Ostropomycetidae Baloch et al. (59) suggested that the evolution of life-styles and ascomatal morphologies in this group was very plastic, as shown by the multiple evolution of peritheciod ascomata in the Gyalectaceae. Early evolutionary splits in the Ostropomycetidae, in fact the second-most species-rich subclass of lichenized Ascomycota, were considered by Resl et al. (60) in their analysis of trapelioid fungi. The uncertain phylogenetic resolution of the c. 170 species prevented a clear backbone concept for the subclass. A monophyletic group of nine core trapelioid genera was found, which do not form the sister group of Ostropales, but the hypothesis of a sister group relationship of trapelioid genera and
Baeomycetaceae or Hymeneliaceae could not be rejected. Recently, Miadlikowska et al. (61) provided the most comprehensive phylogenetic survey of the entire Lecanoromycetes using a multigene maximum likelihood analysis with a cumulative supermatrix approach. However the analysis of this massive data set (1139 taxa) revealed that the cumulative addition of taxa with an increasing amount of missing data leads to relatively stable representation of relationships for many families and orders, but also in substantial loss of phylogenetic resolving power and support for deep phylogenetic relationships.

In the Graphidaceae, a very large family of >2000 species, Lumbsch et al. (62) studied character evolution and found that certain characters (secondary metabolites, in particular) had a high frequency of reversible phenotypic state changes, whereas others, such as photobiont, hymenial persistence or ascoma aggregation, exhibited low frequency of transformations. But even in the character with the highest number of state changes (changes in secondary metabolite composition), the shifts were highly structured phylogenetically, suggesting that the evolution of the character, rather than the character state itself, can be used to predict phylogenetic relationships with certain accuracy.

**Leotiomycetes.** *Trizodia acrobia* is a borderline lichen and the sister group of Leotiomycetes. The lineage was discovered by Stenroos et al. (63) in a phylogenetic analysis of bryophilous ascomycetes. *Trizodia* is ecologically unique by its association with cyanobacterial colonies (mostly *Nostoc*) growing on the tips of peat mosses (*Sphagnum spp.*). *Trizodia* was consistently present in all *Sphagnum–Nostoc* associations studied. It envelops the cyanobacterial colonies both on the moss surface as well as inside the leaf but does not form organized thallus structures.
4. The Upcoming Genomics Perspective For Understanding Lichen Symbioses

Increasing the number of loci used to determine phylogenetic relationships is now possible through characterization of entire genomes of lichens. Genome-scale datasets may lead to the development of consistent, well-supported hypotheses about the evolution of lichenized fungi. A first step towards a phylogenomic analysis of lichen-forming fungi was undertaken in an exemplary phylogenomic study of the genus *Rhizoplaca* (Lecanoromycetes) by Leavitt et al. (64).

Current high-throughput sequencing technology has opened new opportunities for studying organisms that grow slowly and are difficult to establish and grow in axenic cultures. These difficulties explain why comparative genomics of lichen fungi lags behind the advances made so far in other fungal groups. Meanwhile, genetic manipulation, such as transformation, of lichens has been attempted (65). Numerous lichen genome projects have been started in the past years, and some preliminary data are already available. Basically, two strategies are followed in lichen genomics. The more traditional approach is the acquisition of genetic information from the individually cultured symbiotic partners. With the alternative metagenomic approach the total symbiotic association is first sequenced and the genetic information is afterwards assigned to symbiotic partners by bioinformatic analysis. Such progress is possible by exploiting technologies that have significantly improved the quality of sequence assemblies (e.g., by “mate-pair” sequencing) and by powerful bioinformatics pipelines. Apart from gaining information on basic genomic features such as genome size and predicted numbers of genes (Tab. 1), several studies provided more detailed analyses of interesting functions.
Some of these analyses focused on the biosynthetic pathways of secondary metabolites of lichenized fungi (including depsides and depsidones, as classes of coupled phenol carboxylic acids). Sequencing the 34 Mb genome of the *Cladonia grayi* mycobiont revealed new insights in the production of the lichen depsidone grayanic acid (66), and suggests that a single polyketide synthase (PKS) synthesizes two aromatic rings on tandem acyl carrier proteins and links them into a depside, and that the transition from depside to depsidone requires only a cytochrome P-450 mono-oxygenase. Sequencing of the *Cladonia uncialis* mycobiont revealed a putative biosynthetic gene cluster leading to usnic acid, a dibenzofuran derivative (67).

Although no typically crystallized lichen substances have been recorded for *P. membranacea*, a large number of mycobiont and photobiont genes and gene clusters associated with secondary metabolite biosynthetic pathways have been identified in its metagenome, and an unusual trans-AT polyketide biosynthetic pathway of a type known only from other bacterial-eukaryote symbiosis has been identified in the *Nostoc* photobiont (68).

In addition, the genome sequencing provided evidence that the tight association of fungi and prokaryotes might have favored horizontal gene transfer (HGT) events. One such event was detected in the methylammonium permease family between prokaryotes and the *Cladonia grayi* mycobiont (69). Subsequently, McDonald et al. (70) suggested that lichen-forming fungi are losing this gene family at slower rate than other fungal lineages.

The integration of further available (meta)omics approaches, such as (meta)transcriptomics or (meta)proteomics, provides new and complementary insights into the lichen symbiosis. In this context, transcriptomics reveals which of the total set of genes are activated under certain conditions, whereas proteomics suggests which
functions are actually translated into protein functions. An initial full-length cDNA library as a reflection of the transcriptome was provided by Wang et al. (71), using the cultured mycobiont of the desert lichen *Endocarpon pusillum*. However, because a symbiotic context is missing, the significance of the detected gene expression for symbiosis is unclear. In a subsequent study of the same lichen, Wang et al. (72) also analysed dehydrated thalli and confirmed expression of 23 candidate stress responsive genes, selected from a larger set found with mycobiont cultures exposed to PEG-induced drought stress. There is also ongoing RNA-Seq work aiming to compare gene expression in the mycobiont alone in pure culture versus the mycobiont in the symbiotic state to identify genes that are differentially expressed and might therefore be correlated with symbiotic interactions. Gene expression studies elucidate relative levels of active genes (73). Also, epigenetic modifications such as the presence of 5-methylcytosine in lichen genomes can be determined in conjunction with next-generation sequencing platforms.

Moving a step further, Juntilla and Rudd (74) used high-throughput next generation sequencing and expressed sequence tag (EST) sequence data to present a first transcriptome of the eukaryotic partners in the thalli of the reindeer lichen *Cladonia rangiferina* (with 62.8% reads of fungal and 37.2% of algal origin). Even though a higher percentage of algal reads was found in the wetted thalli used, gene ontology (GO) terms (http://geneontology.org/) and identified KEGG pathways (www.genome.jp/kegg/) largely agreed with eukaryotic metaproteome patterns found by Schneider et al. (75). Juntilla et al. (76) tracked the expression profiles during dessiccation and rehydration using microarray analyses, but the data do not provide detailed insights into the regulatory processes. Most of the differentially expressed
genes do not show sequence similarity to known genes. It is, however, remarkable
that largest changes of gene expression are observed only minutes after rehydration.

Given the complexity of lichens it is not surprising that the functional
contributions of genes are organ-specific and modified by pertinent ecological and
developmental conditions. The partially annotated *Peltigera membranacea*
metagenome revealed the presence of mycobiont genes encoding galectin-like
proteins, which are family of proteins defined by their binding specificity for β-
galactoside sugars (77). RNA-Seq data further showed that one of these genes, *lec-1*,
was differentially expressed in rhizines, a purely fungal tissue, when compared to the
remainder thallus, composed of both mycobiont and photobiont (73).

While forthcoming comparative genomics studies will inform us about the
evolutionary dynamics of lineages diverging in lichen-symbiotic stages,
transcriptomics, proteomics, and metabolomics will improve our understanding of the
symbiotic regulation and processing. *Lobaria pulmonaria*, a tripartite lichen
(including both a green algae and a cyanobacterium) widely distributed in the
Northern hemisphere, has been featured in publications that have explored
metaproteomic issues (75, 78). Most algal proteins were assigned to energy
production and conversion. Carbohydrate transport and metabolism were significant
in both eukaryotic partners, but fungal functions were more diverse, with substantial
read numbers suggesting biogenesis and posttranslational modification. The bacterial
fraction (see also further below) in the metaproteome was dominated by proteins from
Alphaproteobacteria. The identified proteins are primarily involved in energy
conversion and carbohydrate metabolism, as well as responses to stress. Also, some of
the bacterial proteome spectra suggested a role of bacteria in secondary metabolite
synthesis, but this could not be resolved in full detail so far.
5. Fungal-Algal Connections And Interactions In The Thallus.

The main role of the algal partners is the provision of photosynthetically fixed carbohydrates as the energetic basis for the self-sustained life style. The lichen-forming fungi take up osmotically active monomeric sugars from the algae, which are then further metabolized. Typically, green algae supply polyols (ribitol in chlorococcal algae and erythritol in filamentous Trentepohliales), whereas glucose is provided by cyanobacterial algae. Apparently the process of lichenization also stimulates upregulation of photo-protective mechanisms in the photobiont (79). In turn, the photobiont stimulates the antioxidant system of the mycobiont (8, 9).

Currently it is not well known, which chemical signals are transferred during the initiation and onset of lichenized stages, and what effects they have in the symbiosis. By comparison of algal strains, Meeßen and Ott (80) detected characteristic metabolites in lichen-forming algae, such as the cyclic dipeptides cyclo-L-leucyl-L-tyrosine and cyclo-(L-tryptophyl-L-tryptophyl), rhamnose and indole-3-carbaldehyde, a precursor or a degradation product of the phytohormone indole-3-acetic-acid.

Because hyphal branching is not stimulated with these substances alone, but when unicellular algae grow in proximity (81), further metabolites seem to be involved in the signalling of lichenization. Such molecules probably include phytohormones abscisic acid and ethylene, as well as others (82, 83, 84).

The fungal partner frequently produces attachment or penetration structures (appressoria or haustoria, respectively) of different kinds at the mycobiont-photobiont interface. Water and dissolved nutrients are can move readily between the bionts at the contact zone, within a hydrophobic coat that the fungus produces over the algal cell surface (85, 86).
Lichen fungi associate with only about 120 known species of algae, most of them green algae, and a few cyanobacterial algae (86), although the species taxonomy is so poorly understood in lichenized cyanobacteria that these currently cannot be identified to species level (87). The photosynthetic partners are green algae in about 90% of lichen species. Hence 10% of the lichens take advantage of bacterial nitrogen fixation and associate with cyanobacterial algae, most commonly with filamentous *Nostoc* strains. Another strategy is the additional association with *Nostoc* strains in specialized organs (cephalodia; Fig. 6A) in or on a green-algal thallus (88), resulting in tripartite lichens, which apparently evolved as a segregation of functions. The higher proportion of heterocysts suggests a main role of cephalodia in nitrogen fixation (89). On the other hand, several cyanobacterial lichens may also have green algae in addition to the cyanobacteria in their photosynthetic layer (90), which might widen their ecological amplitude in cool habitats. Few fungal species are even able to use either eukaryotic green or prokaryotic blue-green algae to develop independent symbiotic thalli (91). These phycosymbiodemes, primarily found in the order Peltigerales of Lecanoromycetes either have similar morphologies, or are shaped profoundly differently (Fig. 6B). When growing separately they were described in independent genera, which obviously has blurred the understanding of their phylogenetic relationships (92).

With very few exceptions, the green algae propagate clonally and do not form sexual structures in the lichenized stage. The apparent suppression of the sexual capacity of the algal partner by the lichen-forming fungi could be interpreted as a selfish strategy to avoid genetic diversification of the partner and to maintain efficient control over the algal physiology.
Refined molecular analyses meanwhile have helped to improve the knowledge about lichen photobiont diversity as well, both their phylogenetic relationship as well as their patterns of association with their fungal hosts. Major mycobiont lineages seem to vary by their overall spectra of preferences for algal groups. While the majority of Arthoniomycetes have a preference for Trentepohliaceae (except for some species assigned to Arthonia), Lecanoromycetes and particular members of Chaetothyriomycetes (above all Verrucariales) associate with a broader range of green algae (43, 61, 93).

Recent work suggests that species of lichenized fungi frequently vary in their algal specificity and selectivity. This correlates with the range of the species and climatic differences, i.e. the same lichens occurring in different habitats often associate with related algal species with different ecological preferences (94, 95). This flexibility supports the hypothesis of habitat-adapted symbioses proposed by Rodriguez et al. (96) which suggests that the environment determines the optimal partnership of symbionts. Wedin et al. (97) showed that Diploschistes muscorum has an even more flexible photobiont strategy than earlier believed. Diploschistes starts as a parasitic fungus infecting the unrelated lichen Cladonia (Fig. 7), eventually taking over and forming an independent thallus. Although the Cladonia used the same photobiont at all investigated sites, Diploschistes associated with different photobionts at all three sites, keeping the original Cladonia alga at one site but replacing it with others in the two other sites (97). This suggests a very generalistic photobiont strategy in this lichenicolous (=lichen-inhabiting) lichen.

Rikkinen et al. (98) suggested that lichen fungi in the Peltigerales form ecological photobiont guilds, within which the fungi share related photobiont cyanobacteria. Such associations can be seen both among an ecological assemblage
of epiphytic macrolichens, the *Nephroma*-guild, and among a group of predominantly terricolous cyanolichens, the *Peltigera*-guild, where the photobionts with each guild are closely related *Nostoc*-strains. Several authors have likewise observed similar photobiont-mediated guilds in green-algal lichens (99, 5).

6. Additional Fungi In The Lichen Symbiosis

As lichen thalli are very persistent and variable morphologically, they provide a rich diversity of small-scale niches for various microorganisms, in particular fungi and bacteria (treated below). Lichenicolous (lichen-inhabiting) fungi comprise all fungal species living in or on lichens, apart from the thallus-forming fungus itself (the mycobiont). This biological group has been studied extensively, and was even recognized before the symbiotic nature of their lichen hosts was established. More than 1,800 species of lichenicolous fungi have been described (100), but as numerous new species are still being discovered, their precise number is unknown but is clearly currently much underestimated. The evolutionary origin of the lichenicolous life style is diverse, but a substantial number of lichenicolous fungi apparently evolved after delichenization of originally lichenized lineages. As most lichenicolous fungi seem to exploit their hosts without rapid destruction, they are highly specific for their hosts, and for particular symbionts of the hosts.

Typically lichenicolous fungi are recognizable by conspicuous reproductive structures or by their symptoms such as discolorations or gall-like hypertrophications. Some other species associate with lichens but remain cryptic. Isolation techniques and axenic cultivation revealed that these species either reside on the surfaces of the thalli (101, 102, 103), or occur internally in lichen host thalli (also known as “endolichenic fungi”, 104). Given the abundance and the potential of
culturable endolichenic fungi to produce secondary metabolites, it might be argued whether these fungi might have a biological effect on their host, or influence the phenotype in various ways. However, even though microscopic evidence already demonstrates the endolichenic growth of fungi beside the mycobiont in lichens (86), it is not clear which of these fungi grow in lichens or reside as spores or otherwise in dormant stages.

Culture techniques have retrieved a surprisingly large number of fungal species from lichens (105), but these numbers only represents a subset of the total fungal diversity present in environmental samples. Therefore, culture-independent methods are now also employed to characterize the mycobiome of lichen thalli. Using DNA-fingerprinting techniques, Fleischhacker et al. (106) found a high diversity of lichen-associated fungi without correlation with the presence of externally visible lichenicolous fungi. Zhang et al. (107) provided an overview of diversity and distribution of fungi in lichens from an Arctic habitat using next generation sequencing. Their study of the lichen mycobiome indicated that lichens harbor fungi related to those with diverse ecological context. Lichens thus represent a reservoir or an evolutionary hotbed for fungi that may have a role in other habitats, but the specific conditions in fungi with recurrent and prolonged cryptobiotic stages do not favor the rise of, for example, a biotrophic plant pathogen. Studies in Lobaria revealed the high specificity of Tremella lobariacearum (108), but whether the same is true for non-symptomatic occurrences of Tremellomycetes is unknown. Refined microscopic techniques can reveal to what extent the scattered occurrence of tremellalean fungi might influence the morphology of the host. Millanes et al. (109) described Cystobasidiomycetes as a new lineage of Pucciniomycotina. The
lichenicolous members of this lineage, which primarily comprises yeasts, cause conspicuous fertile hypertrophi-cations of their hosts.

7. Bacterial Participation In Lichen Thalli

Fungi associated with lichens have been studied extensively for a long time, but the ubiquitous presence of bacteria in lichen thalli received more attention only comparatively recently. Sequencing of lichen-associated bacterial communities revealed information about their diversity on lichen thalli (110, 111, 112, 113, 114, 115, 116, 117, 118). Alphaproteobacteria usually dominate the bacterial communities in lichens, while other bacterial groups (frequently Acidobacteria, Actinobacteria, and Betaproteobacteria) are also found in significant numbers. One group of Alphaproteobacteria, the lichen-associated Rhizobiales (LAR1) is a clade of bacteria so far known only from lichens (113; 115). Sequence-based data are complemented with microscopic information, in particular employing fluorescence in situ hybridization (FISH)(119). The distribution of bacteria belonging to certain groups can be visualized under the microscope using specific probes (confocal laser scanning microscopy, CLSM). Bacteria often form biofilm-like communities on the lichen thalli and are usually tightly connected with the fungal structures, in particular with hydrophilic surfaces of the lichens. The bacteria can also intrude to various depths in the intercellular matrix of the upper cortex, and are occasionally also found inside the hyphae of the fungal hosts (120). The lichen-associated bacterial communities so far investigated are host-specific in their composition (112; 115). A clear shift is microbiome profile is observed when the parasitic lichen Diploschistes muscorum infects, and eventually overcomes Cladonia symphycarpa, to form a thallus of its own (97; Fig. 7). Here, the Alphaproteobacteria population dominating
the *Cladonia* microbial community decreases during the transition, and numbers of Betaproteobacteria or Chloroflexi increase substantially when *Diploschistes* takes over, supporting the view that the microbial community is highly species-specific in lichen thalli.

**Conclusions**

Phylogenetic studies significantly improved the understanding of evolution and phenotypic diversification of lichenized fungi and their partners. Lichen thalli are dominated by the primary fungal symbiont, which controls the characteristic photosynthetic partners. Beside these commonly known symbionts, an unknown numbers of additional organisms can participate in the symbiont system (121). The analysis of massive amounts of molecular data from lichens, which has just started, will help to further understand the role of these complex and fascinating partnerships.

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**Figure legends**

Fig. 1. Diverse shapes of lichen thalli: A. Coral-like fruticose (shrub-like) thallus of *Cladia retipora*. Photo: Birgitta Strömbäck. B. Foliose (leaf-like) thalli of *Lobaria pulmonaria* and *Lobaria scrobiculata* covering the trunk of a *Salix caprea* in an old-growth spruce-dominated forest. Photo: Mats Wedin. C. Crustose (crust-like) thallus of *Acarospora fuscata* growing on a siliceous rock. Photo: Martin Westberg.

Fig. 2. Substantial biomass of lichens: A. Scandinavian costal lichen heath dominated by reindeer lichens (*Cladonia* spp.). Photo: Birgitta Strömbäck. B. Lichen-dominated soil crust community on the Great Alvar of Öland (Sweden). Photo: Martin Westberg. C. Abundant lichen cover dominated by *Pseudocyphellaria homoeophylla*, in a New Zeeland cool temperate rain forest. Photo: Birgitta Strömbäck.

Fig. 3. Vertical section through a thallus of *Parmelia saxatilis*, a foliose lichen. A distinct layer of green algal cells is clearly visible under the uppermost cortex; all other structures are made up by fungal hyphae. Photo: Einar Timdal.

Fig. 4. Vegetative dispersal in lichens: A. Soralia formed along the thallus margins in *Vulpicida pinastri*. These structures produce soredia, small granules of algal cells surrounded by fungal hyphae. Photo: Einar Timdal. B. Simple, cylindrical isidia formed on the thallus upper surface by *Parmelia saxatilis*. Photo: Einar Timdal.

Fig. 5. Optional lichenization: *Schizoxylon albescens*. A. Apothecium of a saprotrophic colony on dead aspen (*Populus tremula*) wood. Photo: Lucia Muggia. B.
Lichenized morph on aspen bark. Note the green algal colonies around the young apothecium. Photo: Lucia Muggia.

Fig. 6. A. Cephalodia visible as dark structures that include cyanobacteria, on *Peltigera aptosa*. Photo: Einar Timdal. B. Phycosymbiodemes with different morphologies: *Sticta* with joined photomorphs. Note green-algal foliose parts growing out from the basal cyanobacterial fruticose parts. Photo: Mats Wedin.

Fig. 7. Symbiotic invasion: The transition of *Cladonia* thallus into *Diploschistes* thallus, by invasion of the latter. A. Uninfected *Cladonia symphycarpa* thallus. Photo: Einar Timdal. B. *Cladonia* thallus with clear *Diploschistes* infection (whitish areas) with typical *Diploschistes* apothecia (dark patches in the whitish areas). Photo: Einar Timdal. C. An almost complete take-over by *Diploschistes*, with only small remnants of *Cladonia*. Photo: Martin Westberg.
Table 1. Lichenized fungal species for which genome information is available (as of March 2016).

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