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Diversity and phylogeny of symbiotic partners in zeorin-containing red-fruited *Cladonia* species

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Ph.D. Thesis

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Declaration

I hereby declare that I have written this thesis independently, using the listed references; or in cooperation with other paper co-authors. I have submitted neither this thesis, nor any of its parts, to acquire any other academic degree.

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Prague, June 2018 Jana Steinová

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PAPERS INCLUDED IN THE THESIS

This thesis is based on four papers, mentioned in the text as „Papers 1, 2, 3 and 4“:

Paper 1

Cardinale, M., **Steinová, J.**, Rabensteiner, J., Berg, G., & Grube, M. (2012). Age, sun and substrate: triggers of bacterial communities in lichens. *Environmental Microbiology Reports*, *4*(1), 23-28.

Paper 2

Steinová, J., Stenroos, S., Grube, M., & Škaloud, P. (2013). Genetic diversity and species delimitation of the zeorin-containing red-fruited *Cladonia* species (lichenized Ascomycota) assessed with ITS rDNA and β-tubulin data. *The Lichenologist*, *45*(5), 665-684.

Paper 3

Škaloud, P., **Steinová, J.**, Řídká, T., Vančurová, L., & Peksa, O. (2015). Assembling the challenging puzzle of algal biodiversity: species delimitation within the genus *Asterochloris* (Trebouxiophyceae, Chlorophyta). *Journal of Phycology*, 51(3), 507-527.

Paper 4

Steinová, J., Škaloud, P., Yahr, R., Bestová, H., & Muggia, L. Reproductive and dispersal strategies shape the diversity of mycobiont-photobiont association in lichen symbioses – manuscript.

Authors contributions

Paper 1: M. Cardinale and M. Grube designed the study. **J. Steinová**, M. Cardinale and M. Grube collected the lichen samples. **J. Steinová** and M. Cardinale performed the FISH and CLSM and evaluated the results. M. Cardinale wrote the manuscript and the co-authors helped with proofreading and editing.

Paper 2: **J. Steinová** planned the design of the study in consultation with the coauthors. **J. Steinová** collected all the material and performed the lab work. **J. Steinová** and P. Škaloud jointly

analysed the results. **J. Steinová** wrote the manuscript and the coauthors helped with proofreading and editing.

Paper 3: P. Škaloud designed the study. The algal strains were isolated by O. Peksa. O. Peksa, **J. Steinová** and L. Vančurová obtained the molecular data. O. Peksa and P. Škaloud performed optical and CM observations. P. Škaloud analysed the results and wrote the manuscript. The co-authors helped to proofread and edit the manuscript.

Paper 4: **J. Steinová** planned the study and consulted over its design with P. Škaloud and L. Muggia. **J. Steinová** obtained the lichen material and performed the lab work. **J. Steinová**, P. Škaloud and H. Bestová jointly analysed the results. **J. Steinová** and L. Muggia wrote the manuscript. The co-authors proofread and contributed to editing.

On behalf of all the co-authors, we declare the keynote participation (as first author) of Jana Steinová in completing the research and writing the papers, as described above

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Pavel Škaloud

ABSTRACT

Lichens are a classic example of mutualistic symbiotic associations, yet the views on lichen symbiosis have changed considerably during the last fifty years. Nowadays, lichens are generally understood to be microecosystems consisting of several symbiotic partners which contribute in different ways to the prosperity of the whole system and which differ by the strength of their bond to other symbiotic partners. The level of knowledge of the individual partners (mycobionts vs. photobionts vs. bacteria) varies greatly in terms of their specificity, diversity and in the forces that shape this diversity.

The main aim of this work was to reveal the diversity of organisms participating in lichen symbiosis and to better understand the biological forces which shape this diversity. We worked with a relatively common lichen group, zeorin-containing red-fruited *Cladonia* species, and specifically, we focused on the mycobionts, photobionts and bacteria that participate in this association. During the course of the study, it became apparent that species delimitation, which is a fundamental requirement for accurate diversity estimates, is another topic that requires further research.

Our analyses revealed that species circumscription of most of recently recognized *Cladonia* mycobionts cannot by supported by molecular data. The genetic diversity of the mycobionts was relatively high and we detected several lineages that we were not able to characterize phenotypically, these probably correspond to cryptic species. These lineages are most likely a result of either hybridization or incomplete lineage sorting. The photobionts associated with these mycobionts also showed a relatively high level of diversity. Furthermore, in contrast to the mycobionts, the majority of photobiont lineages represent phenotypically distinguishable species.

We demonstrated that the reproductive and dispersal strategies of the mycobiont are the key factors influencing the diversity of the *Asterochloris* species in zeorin-containing red-fruited *Cladonia* species. We found that the sorediate *Cladonia* species were strongly selective towards their photobiont, whereas esorediate *Cladonia* species were photobiont generalists. In the case of bacteria, the age of the thallus was the main factor influencing the structure of the bacterial community. In the older thallus parts the bacterial community displayed a drastic change due both to the reduction of the otherwise dominant Alphaproteobacteria and to the increased abundance of other bacterial groups.

ABSTRAKT

Ačkoliv lišejníky představují jeden z nejklasičtějších příkladů symbiotických asociací, tak se pohled na jejich symbiózu během posledních sto padesáti let výrazně změnil. V současnosti jsou lišejníky obvykle vnímány jako mikroekosystémy skládající se z několika symbiotických partnerů, kteří různým způsobům přispívají k fungování celého systému a kteří se vzájemně liší silou vazby k dalším partnerům. Úroveň poznání specificity, diverzity a faktorů, které tuto diverzitu ovlivňují, se u jednotlivých partnerů (mykobiont, fotobiont a bakterie) výrazně liší.

Hlavním cílem této práce bylo odhalit diverzitu organismů, které se na lišejníkové symbióze podílejí a lépe porozumět biologickým silám, které tuto diverzitu ovlivňují. Pracovali jsme s červenoplodými zeorin obsahujícími dutohlávkami, což je poměrně běžná skupina lišejníků. Konkrétně jsme se zaměřili na studium organismů (mykobiontů, fotobiontů a bakterií), které se v těchto dutohlávkách vyskytují. V průběhu studie vyšlo najevo, že dalším tématem, které si zaslouží naši pozornost, je problematika vymezení druhů (species delimation).

Z výsledků vyplynulo, že současné vymezení většiny druhů mykobiontů není podpořeno molekulárními daty. Genetická diverzita mykobiontů byla poměrně vysoká a bylo zjištěno několik linií, které nebylo možné fenotypově charakterizovat a které pravděpodobně představují kryptické druhy. Tyto linie vznikly pravděpodobně hybridizací anebo jsou následkem neúplného sortování linií (incomplete lineage sorting). U fotobiontů, kteří jsou symbiotickými partnery těchto mykobiontů, byla zjištěna také poměrně vysoká úroveň diverzity. Na rozdíl od mykobiontů však většina zjištěných fotobiontích linií představuje fenotypově odlišitelné druhy.

Z výsledků dále vyplynulo, že diverzita fotobiontího rodu *Asterochloris* v zeorin obsahujících červenoplodých dutohlávkách je určována především reprodukčními a disperzními strategiemi mykobionta. Zatímco sorediózní dutohlávky byly výrazně selektivní vůči svým fotobiontům, tak nesorediózní druhy byly fotobiontími generalisty. V případě bakterií bylo zjištěno, že nejpodstatnějším faktorem ovlivňujícím strukturu mikrobiální komunity je stáří stélky. Ve starších částech stélky docházelo k výrazným změnám mikrobiální komunity – především k poklesu jinak dominujích alfaproteobakterií a dále k nárůstu jiných skupin bakterií.

CONTENT

1 INTRODUCTION

1.1 Lichen symbiosis and the development of the views on it

Lichens are iconic examples of symbiosis and in several aspects they maintain a unique status among other symbiotic associations. First, their **self-construct and self-replicate characteristic body plan arises only with a symbiotic lifestyle** and differs strongly from life-forms of the organisms participating in this symbiosis when they live individually (Spribille, 2018). Moreover, once the symbiotic phenotype is established, **the lichen thallus can be maintained for several thousand years.** Another striking characteristic of lichens is their **ability to live in extreme environments** where few other macroscopic organisms can survive. Lichens can colonise almost any type of substrate ranging from bare soils and rocks to freshwater streams and marine intertidal zones. However, despite all of these unique features of lichens now being mostly attributed to their symbiotic lifestyle, opinions on the nature of this association have developed dynamically over the past 150 years.

Originally, lichens were thought of as **single organisms** and accordingly were treated as a systematic unit, Lichenes, separated from fungi, mosses, and algae (e.g., Acharius, 1803). **The symbiotic nature of lichen symbiosis was revealed by Schwenderer in 1867** (Schwendener, 1867), but this was rejected by most of the leading lichenologists of the time, and many years passed before his discovery was broadly accepted by the scientific community (e.g., Santesson, 1952). In 1950, the *Code of botanical nomenclature* anchored the name of the lichen to the fungus (Spribille, 2018) and, since that time, lichen systematics and taxonomy has been based on the mycobiont.

Most recent biological textbooks treat lichens as **an obligate symbiotic association between a heterotrophic fungus and an autotrophic chlorophyll-containing partner, where the partner is either green algae or cyanobacteria, or both. The photobiont provides the mycobiont with carbohydrates** – sugar alcohol in the case of most algal photobionts and glucose in the case of cyanobacteria (Friedl and Büdel, 2008). **The production of energy by photobionts is enhanced by outer layers of fungal hyphae** (with secondary metabolites produced by a mycobiont) **which function like a shelter.** Additionally, **cyanobacteria supply a symbiotic system with fixed nitrogen.** This is especially important in tripartite lichens which contain both green algae and cyanobacteria. In these lichens the green algae is the photosynthetic component whilst the cyanobacteria, which are generally restricted to delimited portions of the thallus (cephalodia), mainly function in N_2 fixation (Friedl and Büdel, 2008).

The view of lichen symbiosis has developed further during the last 15 years, as the importance of the other organisms associated in lichen symbiosis has been recognised. **Lichens are currently understood to be meta-organisms hosting many diverse cohabitants which contribute in different ways to the prosperity of the holobiont** (Aschenbrenner et al., 2016; Spribille, 2018). This is in contrast to the former dual concept of lichen symbiosis (sensu a heterotrophic fungus living together with an

autotrophic photobiont). This concept, however, had already been questioned by the failures of resynthesis experiments using axenic mycobiont and photobiont cultures. In this context, attention concentrated mainly on the role of two large groups of microorganisms: bacteria and microscopic fungi (e.g., Aschenbrenner et al., 2016; Sigurbjörnsdóttir et al., 2016; Spribille, 2018).

The presence of **bacteria** in the lichen thallus was reported for the first time by Uphof (1925). Other studies followed, but there were no major advances in the research on the diversity and role of bacteria in lichen symbiosis until DNA-became commonplace in the first decade of 21st century. Both culture-dependant and culture-independent approaches were used to assess the taxonomical structure of the bacterial community and, to a certain extent, also to deduce the possible contribution of the bacterial microbiome to the lichen symbiosis. Recently developed omics approaches (metagenomics, metatranscriptomics and proteomics) and related bioinformatical tools enabled researchers to focus specifically on the functional aspects of the association between bacteria and fungal and/or algal partners (Cernava et al., 2017; Grube et al., 2015; Sigurbjörnsdóttir et al., 2015). The results of these works suggest that the **microbiome has several important ecophysiological roles for a healthy lichen thallus, namely nutrient provision and the degradation of old thallus parts, biosynthesis of vitamins and hormones, detoxification processes and the protection against biotic and abiotic stress** (Fig. 1; Aschenbrenner et al., 2016; Grube et al., 2015; Sigurbjörnsdóttir et al., 2015).

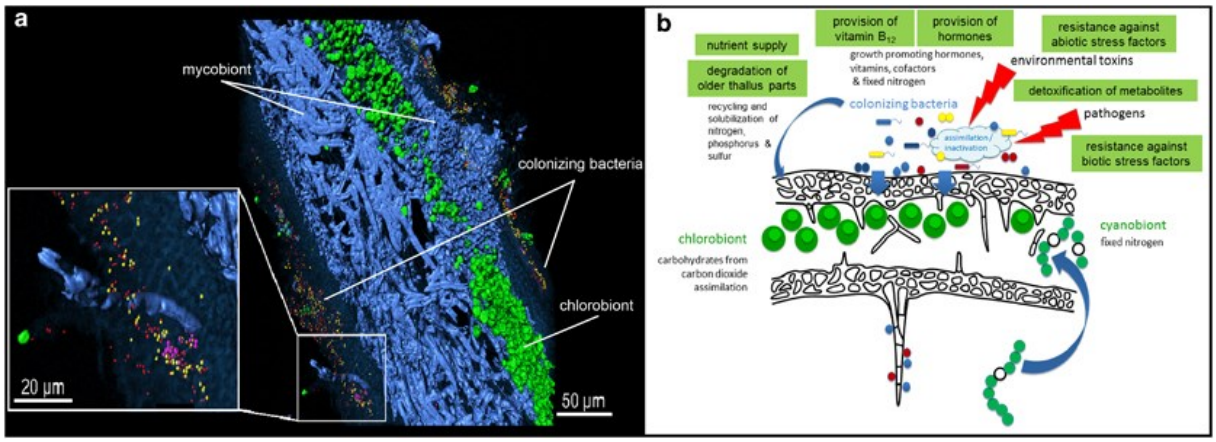

Fig. 1 Leaf-like thallus visualization of bacteria on a cross-section of *Lobaria pulmonaria* by 3D reconstruction of FISH image stacks. Eubacteria (red) and Alphaproteobacteria (yellow) were found widespread on both the upper and the lower cortex, while Betaproteobacteria (pink) were less abundant and locally contained. Fungal hyphae (blue) and algae located under the upper cortex (green) were visualized without specific FISH probes, due to the naturally occurring fluorescence of the internal structures. (b) Model of the lichen symbiosis depicting the functional network of the participants. The model includes relevant functions of the colonizing bacteria, which are derived from metagenomic/metaproteomic analysis, as well as cultivation-dependent experiments. From Grube et al. (2015).

The lichen thallus also provides a habitat for different fungi (besides a mycobiont) and their role in the lichen symbiosis has stimulated enthusiastic discussion in recent years. Fungi present in the lichen thallus can either influence lichen morphology e.g. by developing recognizable reproductive structures or causing conspicuous symptoms (lichenicolous fungi), or may stay asymptomatic (endolichenic fungi). In 2016, Spribille and collaborators found yeasts from a previously unknown order of basidiomycetes, the Cyphobasidiales, in the lichen cortex of *Bryoria fremontii* and *B. tortuosa* (Spribille et al., 2016). These yeasts were about 10 times more abundant in *B. tortuosa* than in *B. fremontii* and occurred in close proximity to concentrations of vulpinic acid crystals (the presence of this substance is characteristic for *B. fremontii*). **The authors related the phenotypical differences between the two species to the different numbers of yeasts in the cortex and concluded that "more than one fungus may be involved in its (cortex) construction"** (Spribille et al., 2016). Although the authors did not explicitly use the term **"third partner"**, this had already been used in several recent works (e.g., Calcott et al., 2018; de la Torre Noetzel et al., 2018; Potera, 2017; Suryanarayanan et al., 2017; Suryanarayanan and Thirunavukkarasu, 2017). **The robust statement about the fundamental role of the yeasts in lichen symbiosis was, however, criticised by Oberwinkler who wrote in his monography on yeasts in Pucciniomycotina: "In summary, it is obvious that basidiomycetous yeasts in lichen thalli are not a third component of symbiosis, but rather the vegetative propagules of mycoparasites"** (Oberwinkler, 2017). Some other scientists are also more reluctant when evaluating the role of endolichenic fungi in the lichen symbiosis (e.g., Fernández-Mendoza et al., 2017; Muggia et al., 2016). In his recent paper, Spribille emphasizes **the role of the complex cortical biofilm** (composed of fungi and bacteria) rather than the role of the yeasts (Spribille, 2018).

1.2 Species concepts and delimitation approaches

Accurate species delimitation is a basic requirement for ecological, evolutionary or conservation studies as well as for biodiversity estimates. Species concepts differ among the kingdoms of life and even at lower taxonomical levels. To date, a wide range of species concepts have been proposed. For example, Wilkins (2012) listed 26 different species concepts, many of which are incompatible to each other, and, therefore, can lead to different conclusions concerning the boundaries and number of species (De Queiroz, 2007).

As described above, lichen symbiosis involves organisms belonging to different evolutionary lineages (Fungi, Viridiplantae, and Bacteria). Species recognition of different symbionts is therefore influenced by the specific characteristics of individual groups of symbiotic organisms.

Species delimitation in lichenized fungi

A phenotype-based approach to species recognition is still widely used for lichenized fungi, although a rising number of papers show that the currently used interpretation of phenotypic characters is inadequate to accurately describe diversity (Lumbsch and Leavitt, 2011). Phenotype-based species recognition relies on using **morphological and chemical features** to characterise the species in the case of lichenized fungi.

The morphological characters used for classification of lichenized fungi include ascomatal characters and a broad spectrum of vegetative characters. The most common characters used to distinguish species are: the thallus form and size, the presence/form/colour of attachment organs (e.g., rhizines, holdfast) or other supplementary organs (cilia, hairs, etc.), the presence and form of pseudocyphellae and maculae, the type of reproduction and the form and the location of both generative and vegetative reproductive structures (Lumbsch and Leavitt, 2011).

The presence or absence of **specific secondary metabolites,** or their replacement by another substance, are other phenotypic characters that are commonly used to distinguish species of lichen-forming fungi. Over 800 secondary metabolites are known from lichens (Huneck, 1999), these are mostly unique to lichens, with only a small minority occurring in other fungi or higher plants. Until recently, all of these secondary substances were considered to be produced exclusively by the lichen-forming fungi (Elix and Stocker-Wörgötter, 1996). However, it has now been suggested that, in *Bryoria tortuosa,* associated yeasts can either directly participate in the production of vulpinic acid, or induce lecanoromycete fungi to synthesise it (Spribille et al., 2016). **Species delimitation in groups of lichenized-fungi that lack clear distinctive morphological characters is primarily based on differences in their chemistry** (e.g., genus *Lepraria* - Fehrer et al., 2007; Lendemer, 2011; Lendemer and Hodkinson, 2013; Slavíková-Bayerová and Orange, 2006, some species complexes in *Cladonia* - Pino-Bodas et al., 2010, 2012.; Stenroos, 1989; Timsina et al., 2014). In cases where chemically different populations are similar or identical in terms of their morphology, ecology and/or distribution, these lineages are regarded either as

species or as chemical races, depending on the opinion of the author (e.g., Christensen, 1987; Culberson and Culberson, 1994; Huovinen et al., 1989; Orange, 2001; Slavíková-Bayerová and Orange, 2006).

The biological species concept (Mayr, 1969, 1942) has never been widely applied for lichenized fungi (Lumbsch and Leavitt, 2011) due to the problems associated with carrying out mating tests in fungi. In this concept, species are understood to be "groups of interbreeding natural populations that are reproductively isolated from other such groups" (Mayr, 1942). This definition implies that distinct species do not necessarily need to have diagnosable morphological differences due to a selective advantage of maintaining a specific phenotype, parallel or convergent evolution (Lumbsch and Leavitt, 2011). The terms "cryptic" or "sibling" species are used for these phenotypically undistinguishable species.

Traditional species concepts have been challenged by the development of techniques that allowed the study of genetic information. However, while traditional phenotypically-based delimitation was supported by molecular data for some groups of lichenized fungi, it turned out to be inadequate for other groups. Molecular phylogenetic reconstructions revealed the existence of so called "semi-cryptic" species that can be characterised by previously overlooked characters. The term **semi-cryptic species** was introduced by Vondrák et al. (2009) who defined it as "species which cannot be clearly diagnosed by their morphology, but which are determined by other characters, mainly by their ecology and distribution". Lumbsch and Leavitt (2011) also use this term for distinct lineages that can be distinguished by "subtle morphological or previously overlooked chemical differences". These semi-cryptic species were discovered in various groups of lichenized fungi (e.g., Hodkinson and Lendemer, 2011; Kraichak et al., 2015; Kroken and Taylor, 2001; Leavitt et al., 2011; Molina et al., 2013; Orange, 2012; Vondrák et al., 2009; Wirtz et al., 2003).

Molecular studies revealed also the existence of **real cryptic species** which cannot be correlated to any morphological, chemical, ecological or geographical characters (Leavitt et al., 2011a; Muggia et al., 2014; O'Brien et al., 2009; Otálora et al., 2010). There are different views how to treat the 'cryptic' species within the traditionally defined nominal species.

Some authors (e.g., Kotelko and Piercey-Normore, 2010) have suggested a more conservative attitude in maintaining the traditional delimitation of the species, even when they are not supported by molecular data. They have argued that the acceptance of cryptic species would have implications for ecophysiological studies, and more generally, for the detection and preservation of rare or unusual species. Conversely, other authors (e.g., Grube and Kroken, 2000) have proposed applying the phylogenetic species concept and thus defining the well-supported phylogenetic lineages as cryptic species. They mentioned that morphological characterization of the species is often facilitated after finding cryptic lineages with molecular data, as it may then become apparent which characters are significant. They also claimed that the knowledge of cryptic species is useful in investigations at fine

scales of taxonomic resolution, such as for interpretations of ecophysiological differences and microhabitat preferences (Grube and Kroken, 2000).

Leavitt et al. (2015) proposed using a **general lineage concept** (**GLC**; De Queiroz, 1999) for lichenized fungi as a practical solution to the species concept. This species concept, sometimes called also unified species concept (De Queiroz, 2007), defines species as „**segments of separately evolving metapopulation lineages**". This definition is common to all alternative species concepts and it helps to overcome the conflicts among them. Most of previously used definitions of alternative species concepts are proposed not to be defining properties of the species but rather contingent properties which may or may not be acquired by species during the course of their existence. This is based on the assumption that different properties separating lineages arise at different times during the speciation process and they do not even necessarily occur in a regular order (Fig. 2; De Queiroz, 2007). **Speciation should be regarded as a process and not as a single event in time** (Hey and Pinho, 2012). The **'general lineage concept' shifts the discussion from the conceptual problems of defining species category to searching for appropriate methods to infer the boundaries and numbers of species.** The GLC allows researchers to use several independent empirical properties (e.g., morphology, chemistry, geographic range, host preference) to provide robust hypotheses of species boundaries (Leavitt et al., 2015c).

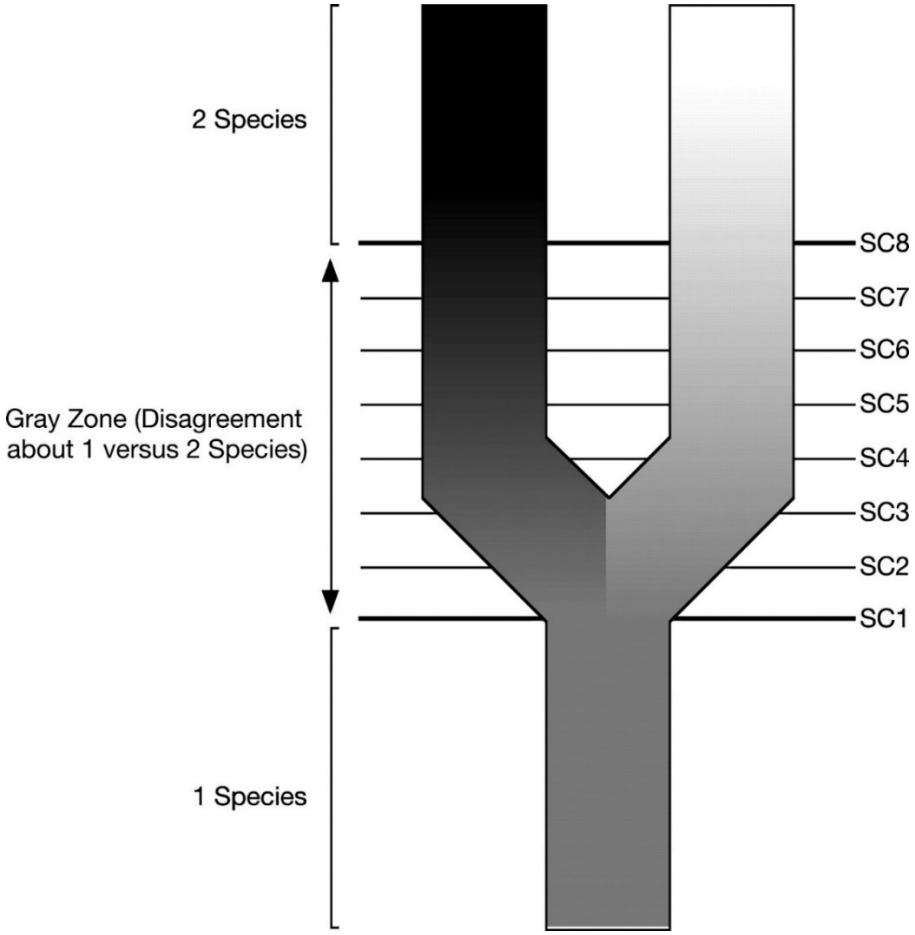

Fig. 2 A highly simplified representation of the process of metapopulation lineage divergence (speciation) illustrating the conflicts caused by adopting different contingent properties of metapopulation lineages as necessary properties of species. Progressive darkening and lightening of the daughter lineages represent their progressive divergence through time (bottom to top), and the numbered lines labelled SC (species criterion) 1-8 represent the times at which the daughter lineages acquire different properties relative to one another (e.g., when they become phenetically distinguishable, diagnosable by a fixed character difference, reciprocally monophyletic, reproductively incompatible, ecologically distinct, etc.). Before evolution of the first property (SC1), authors will agree there is a single species, and after evolution of the last property (SC8), they will agree there are two. Between these events, however, there will be disagreement among authors about whether one vs. two species are involved. Those disagreements result from authors adopting different contingent properties (species criteria) as the basis for their species definitions. From De Queiroz (1999).

The dynamic development of molecular-biological methods during last 30 years led to the **routine use of genetic data for assessing the species boundaries** of lichenized-fungi. Leavitt et al. (2015) provided an overview of relevant DNA-based species delimitation methods. Several methods use **single-locus data** as an input, e.g., general mixed Yule coalescent method (GMYC; Pons et al., 2006), Poisson tree processes model (PTP; Zhang et al., 2013), Automative Barcode Gap Discovery (ABGD; Puillandre et al., 2012) or "Species Delimitation" plug-in to the Geneious software (Masters et al., 2011). These methods are computationally relatively fast and provide a **valuable starting point for determining species boundaries, but they might fail to recognize significant proportions of species-level biodiversity**, or provide spurious inflations of estimated diversity based on genetic differences that do not correspond to species-level lineages (Leavitt et al., 2015c)

It has been repeatedly shown that **genetic data based on multiple independent loci can provide robust hypotheses for testing species boundaries** (e.g., Altermann et al., 2014; Carstens et al., 2013; Kroken and Taylor, 2001; Leavitt et al., 2011b; Zhao et al., 2017). There are three widely used approaches for evaluating candidate species using multilocus sequence data. First, **genealogical concordance of unlinked markers**, which relies on the identification of lineages that exhibit genealogical exclusivity across unlinked neutral loci genealogies (Hudson et al., 2002). However, species with recent divergence histories may stay undiscovered because a substantial amount of time is required after the initial divergence before ancestral polymorphisms can be fully sorted (Hudson et al., 2002; Knowles and Carstens, 2007). The genealogical concordance criterion has been used in a number of studies of lichen-forming fungi (Kroken and Taylor, 2001; Leavitt et al., 2012a, 2012b, 2013; Molina et al., 2011). Second, **coalescent-based species recognition** represents another commonly used approach (Fujita et al., 2012) which overcomes the problem of conflicting signals from different genes (Degnan and Rosenberg, 2009) that can be caused by different evolutionary processes (e.g., Degnan and Rosenberg, 2009; Mallo and Posada, 2016; see Fig. 3). A number of coalescent-based species delimitation methods have recently been introduced, namely SpeDeSTEM (Ence and Carstens, 2011), Bayesian Phylogenetic and Phylogeography (BP&P; Yang and Rannala, 2010), Brownie (O'Meara, 2010) and these have already been tested for assessing the species boundaries of lichen-forming fungi (Del-Prado et al., 2016; Leavitt et al., 2016, 2018; Nicolas Magain et al., 2017; Singh et al., 2015). The third group of recent and commonly used methods includes **tests originally proposed to infer population structure and assigning individuals to populations without a *priori* information**, namely algorithms BAPS (Corander and Marttinen, 2006), Gaussian clustering (Fraley and Raftery, 2007), Guillot's unified model (Guillot et al., 2012), STRUCTURE (Falush et al., 2003) and STRUCTURAMA (Huelsenbeck et al., 2011). Some of these algorithms have recently been used for species boundaries assessment of lichenized fungi (Altermann et al., 2014; Nicolas Magain et al., 2017).

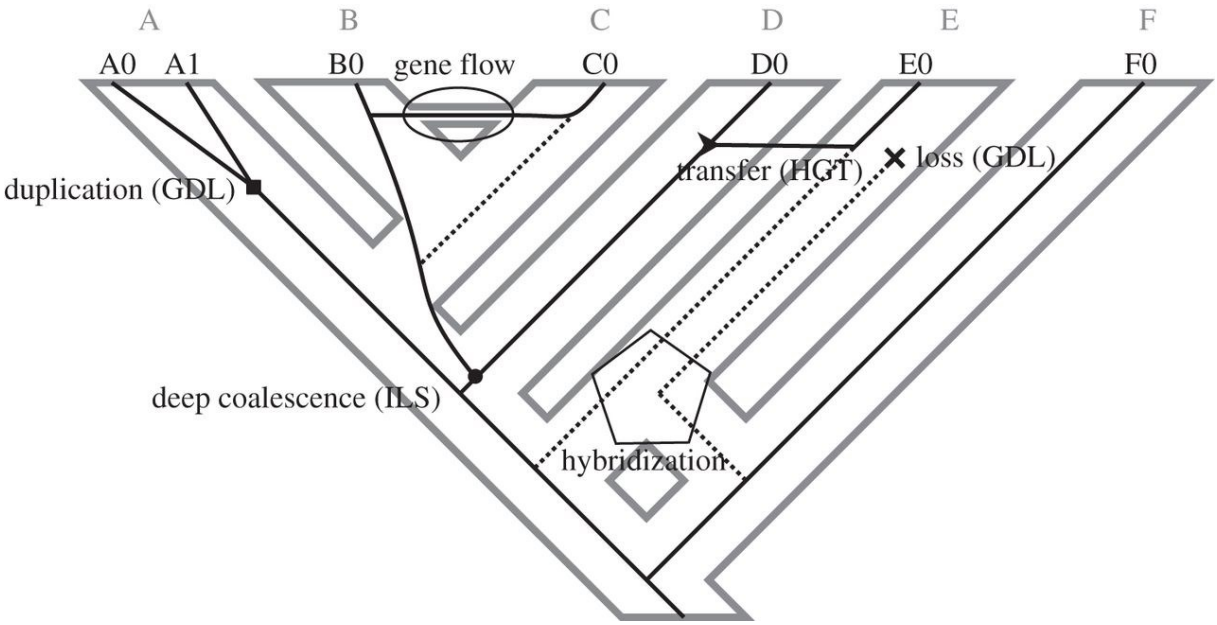

Fig. 3 Evolutionary processes that generate species tree/gene tree incongruence. The figure shows the species tree (grey tree in the background) and a gene tree (black tree) tracking the evolutionary history of six species (A, B, C, D, E and F) and nine gene copies (A0α, A0β, B0, C0, C1, D0, E0, E1 and F0) in eight individuals (A0, B0, C0, C1, D0, E0, E1, F0). Each evolutionary process is indicated by a label and a specific figure in the node where it is mapped (duplication, square; loss, cross; transfer, arrow; deep coalescence, circle; hybridization, pentagon; gene flow, ellipse). Dashed lines indicate superfluous lineages that do not reach the present due to gene loss. From Mallo and Posada (2016).

In the future, species delimitation of lichenized fungi is also likely to include **high-throughput sequencing approaches**, such as anchored phylogenomics, transcriptome sequencing or reduced representation genomic library sequencing (restriction-site-associated DNA sequencing: RAD-Seq and genotype-by-sequencing: GBS). None of these robust methods has yet been tested on lichenized fungi, although they have provided an important insight into species boundaries for a number groups (Beheregaray et al., 2017; Domingos et al., 2017; Herrera and Shank, 2016; Léveillé-Bourret et al., 2018; Pante et al., 2015; Pyron et al., 2016).

Although the use of modern computationally based algorithms can be a powerful tool for a species boundaries assessment, the results should be interpreted with caution. D**iscrepancies among species delimitation approaches** are a common phenomenon, both in lichenized fungi (e.g., Alors et al., 2016; Leavitt et al., 2015a; Magain et al., 2017) as well as in other groups of organisms (e.g., Eyer et al., 2017; Miralles and Vences, 2013). Leavitt et al. (2015) advocate to applying the so called '**iterative taxonomy' to circumscribe and refine species limits using multiple lines of evidence**. A similar (Integrative Taxonomic approach - ITAX) approach has been proposed and successfully used for other groups of organisms (Miralles and Vences, 2013). The authors provide "a non-exhaustive list of species delimitation criteria to be integrated in the ITAX approach, including (a) sympatric occurrence without admixture as revealed by consistent differences in morphological or molecular characters at the same geographic location; (b) strong differences in a behavioral, morphological, or genetic character known to mediate premating isolation; (c) unviability or infertility of hybrids; (d) lack of gene flow across a geographical hybrid zone; (e) congruent diagnostic differences between sister lineages in various

unlinked morphological character; (f) absence of haplotype sharing in several unlinked nuclear loci; and (g) a combination of criteria e–f' (Miralles and Vences, 2013). This integrative approach is in agreement with the general lineage concept proposed by De Queiroz (1999).

Species delimitation in green-algal photobionts associated with lichenized fungi

The main attributes playing an important role in the species delimitation of lichenized fungi are also typical for algae. Algae are also characterised by **relatively simple cell organisation and by the near absence of morphological features that could be used to clearly distinguish one species from another. Similarly, to fungi,** algae also exhibit a cosmopolitan distribution and a great ecological amplitude. Species delimitation in lichenized fungi and their symbiotic green algal partners have therefore undergone a similar historic line of development. In the following text I will, therefore, mention only the important issues specific to the green-algal photobionts species delimitations that differ substantially from the species delimitation in lichenized fungi.

In the past, species delimitation of green-algal lichen photobionts was based almost exclusively on the **morphological and cytological characters** (Darienko et al., 2015). These primarily included mainly the cell shape (Ettl and Gärtner, 1995), the presence and the structure of pyrenoids (Ettl and Gärtner, 1995; Pröschold and Leliaert, 2007; Vančurová et al., 2015) and the chloroplast morphology (Škaloud and Peksa, 2010; Vančurová et al., 2015).

Although the **biological species concept** has been applied more often to assess species boundaries in some algal groups than for lichenized fungi (Leliaert et al., 2014), it has not been used for lichen photobionts.

In the molecular-biological era, numerous DNA-based algorithms (described in the previous section) for species delimitation have been applied also for green-algal photobionts, for instance by (Darienko et al., 2015; Malavasi et al., 2016; Sadowska-Deś et al., 2014; Škaloud et al., 2016).

Species delimitation in prokaryotic organisms

Prokaryotes differ in many aspects from the eukaryotic organisms and this has important implications for different approaches to species concepts and delimitations. First and foremost, the **biological species concept defining species as groups of interbreeding natural populations that are reproductively isolated from other such groups (Mayr, 1942) cannot be applied because of the absence of sexual reproduction in prokaryotes.** However, **prokaryotic organisms are characterised by the ability to exchange their genetic material through a different mechanism, so called horizontal gene transfer** (HGT; or lateral gene transfer, LGT; e.g., Vos et al., 2015). HGT is well known also from eukaryotic organisms, though at much lower rates. In contrast to eukaryotic interbreeding, HGT includes an exchange of the genetic material not only within the same or closely related species but also within species that are phylogenetically very distant. The existence of such a homogenisation of the genomes among bacteria even led scientists to doubt whether species really exist in prokaryotes (e.g., Doolittle, 2012; Konstantinidis et al., 2006).

Similar to eukaryotic organisms, **the traditional species concepts of prokaryotes were based on morphological traits**, which were later demonstrated to be wrongly tailored (Rosselló-Mora and Amann, 2001). Later, physiological and biochemical features were also used to delimit bacterial species.

Recently, the so called **polyphasic approach** is the commonest method used for species delimitation of prokaryotes (Rosselló-Mora and Amann, 2001; Rosselló-Móra and Amann, 2015; Vandamme et al., 1996; Vos, 2011). This integrates genotypic, phenotypic and chemotaxonomic information about an organism and delineates the microbial species based on a consensus of available data. This polyphasic approach relies on a toolbox of methods including DNA–DNA hybridization (DDH), G+C content variation, sequence comparison of selected DNA markers (including 16S rDNA), identification of certain metabolites such as fatty acids, polar lipids, cell wall composition and exopolysaccharides, as well as morphological, biochemical and enzymological characterization (Varghese et al., 2015). The incautious application of some of these methods may, however, lead to misleading results. For instance, Rosselló-Móra and Amann (2015) marked DNA–DNA hybridization as "cumbersome and error prone" and suggested that it should be abandoned. Also the originally proposed (and often still used) threshold value of 97 % similarity of 16S rDNA sequences (Stackebrandt and Goebel, 1994) has been challenged by studies recommending that higher thresholds should be applied (e.g., Kim et al., 2014; Meier-Kolthoff et al., 2013; Stackebrandt, 2006).

Compared to eukaryotic organisms, whole genome sequencing of prokaryotes (as well as other methods based on high throughput sequencing) is currently far more common due to the small sizes of prokaryotic genomes. To illustrate this, in 2015, Rosselló-Móra and Amann (2015) reported that 3500 type strains have an almost full or a draft genome available (out of approximately 12000 classified species of Bacteria and Archaea). Different parameters have been developed to compare genomes for species circumscription purposes, such as Average Nucleotide Identity (ANI; Konstantinidis and Tiedje, 2004; Richter and Rosselló-Móra, 2009), digital DDH (Meier-Kolthoff et al., 2014), or Maximal Unique Matches (MUM; Deloger et al., 2009). **Some microbiological journals already strongly recommend that any description of a novel species needs to be accompanied by, at least, the almost complete genome sequence of the type strain** (Rosselló-Móra and Amann, 2015).

Other methods based on high throughput sequencing have also been used to study species boundaries, such as Whole Genome Shotgun (WGS) from environmental DNA. In some cases, the **sequencing of metagenomes helped to uncover a population structure that other techniques (including 16S rRNA gene sequencing) were not able to differentiate** (e.g., Caro-Quintero and Konstantinidis, 2012). This is explained by the fact that current methods for species demarcation do not take into account the extent of the ecological and genomic adaptations of the strain within its habitat of isolation. Current methods also struggle to detect discernible species among strains sharing more than 95% ANI due to lack of resolving power (Caro-Quintero and Konstantinidis, 2012).

The commonly used **polyphasic approach has recently been criticised** for being too conservative and orthodox, and for not addressing the contemporary needs of its users in microbial ecology and clinical microbiology (Thompson et al., 2015). This approach favours describing new species of cultivable microorganisms, which results in a growing gap between the field of microbial community diversity (in many environments capturing mostly uncultivable prokaryotes) and microbial taxonomy. The conservative description of new prokaryotic species is often cost- and time-consuming even though the taxonomic value of some of the characteristics required for describing novel species is questionable (Thompson et al., 2015). **More important, and a fundamental failure of the polyphasic taxonomy, is that it aims to reflect the pragmatic taxonomical need to classify prokaryotes, rather than aiming to capture the real biological nature of prokaryotic species as separately evolving lineages.** This approach was guided by the historic view that biological species delimitation was considered to be inapplicable for asexual prokaryotes and that they were subsequently shown to be able to homogenise their genomes by HGT (e.g., Bobay and Ochman, 2017; Thompson et al., 2015). Recently, however, several authors discovered **the existence of barriers for HGT, suggesting that bacteria can also form genetically isolated lineages** and cannot be therefore understood as a simple genetic continuum (e.g., Bobay and Ochman, 2017; Majewski et al., 2000; Planet et al., 2017; Popa et al., 2011). A biological species definition based on gene flow is, therefore, also applicable to prokaryotic organisms (e.g., Bobay and Ochman, 2017; Thompson et al., 2015).

Although the species delimitation of bacteria associated with lichens is not a topic discussed in detail by any paper included in this thesis, I still considered it sufficiently relevant to incorporate it here. This is because describing both the 'state of the art' and currently used methods for species delimitation of prokaryotes might (in a simplified way) indicate the directions that the species boundaries assessment of eukaryotes may develop in the future.

1.3 Factors shaping the diversity of organisms associated with lichenized fungi in lichen symbiosis

Factors shaping the diversity of lichen photobionts

Lichens differ by the degree of mycobiont specificity towards its photobiont, with the possibilities spanning from **generalist associations to strong specificity**, or any of a range of intermediate outcomes, including local ecological specialization (e.g., Leavitt et al., 2015b; Magain et al., 2017; Muggia et al., 2014; Otálora et al., 2010; Yahr et al., 2004). Numerous studies have focused on identifying the main forces underpinning the photobiont associations in different lichen-forming fungi. **Environmental factors** seem to play a key role in shaping the photobiont diversity, namely the **macroclimate** (e.g., Fernández-Mendoza et al., 2011; Singh et al., 2017), the **altitude** (Blaha et al., 2006; Dal Grande et al., 2017; Muggia et al., 2008), the **habitat** (e.g., Rikkinen et al., 2002; Thüs et al., 2011; Werth and Sork, 2010) or the **rain and sun exposure** (Peksa and Škaloud, 2011).

Another factor that possibly affects photobiont diversity is the **lichen reproduction and dispersal**

strategy (e.g., Cao et al., 2015; Fedrowitz et al., 2012, 2011; Otálora et al., 2012; but see also Wornik and Grube, 2009). Lichen fungi can reproduce either sexually or asexually and the reproduction mode has broad implications for the evolutionary advantages and drawbacks of the symbioses. Lichens can form **asexual propagules** (in some species even thallus fragments), which represent **clonal diaspores in which the mycobiont and its compatible algal partner are co-dispersed.** Asexual reproduction circumvents problems of low symbiont availability (Wornik and Grube, 2009) but reduces the opportunities for adaptive evolution (Eckert, 2002). Alternatively, **sexually reproducing lichen fungi disperse independently by spores and hence have to re-establish the symbiosis *de novo* every time.** The thallus re-synthesis requires the presence of compatible algae in the environment where the spore germinates and is triggered by the degree of preference by the fungi towards the available photobionts (e.g., Beck et al., 1998; Honegger, 2008, 1993; Ott, 1987; Yahr et al., 2004). Sexual reproduction of mycobionts increases the genotypic diversity and the successful dissemination by long range dispersal (Bailey, 1976; Belinchón et al., 2015; Pyatt, 1973; Werth et al., 2006). None of these reproduction modes is usually exclusive, most lichenized fungi exhibit a combination of sexual and asexual reproduction.
In the *Cladonia* lichens different patterns of selectivity and specificity of the mycobionts towards the photobiont have already been documented (Piercey-Normore and DePriest, 2001; Yahr *et al.*, 2004, 2006). Yahr *et al.* (2006) used *Cladonia subtenuis* to evaluate the role of different factors shaping the algal-fungal association in *Cladonia* symbiosis, demonstrating that geographic position and habitat are the best predictors of algal genotype distribution.

Factors shaping the diversity of bacteria associated with lichens

Many diverse factors influence the structure of the microbial communities associated with lichens. First, several studies have proved that **the composition of the lichen-associated microbiota is host-specific** (Bates et al., 2011; Grube et al., 2009; Wedin et al., 2015). Second, the **photobiont type** (chlorolichens vs. cyanolichens) was demonstrated to influence the structure of microbial communities (Hodkinson et al., 2012). This is possibly attributed to differences in the availability of fixed nitrogen and the type of fixed carbon (Hodkinson et al., 2012). Also the lichen **growth type** (crustose vs. foliose or fruticose lichens) was shown to affect the microbial community structure (Grube et al., 2009; Hodkinson et al., 2012).

The composition of associated microbial communities was shown to depend on **geography, climate and habitat** (Aschenbrenner et al., 2014; Cardinale et al., 2012; Hodkinson et al., 2012; Printzen et al., 2012). Printzen et al. (2012) studied the composition of Alphaproteobacteria in the bipolar lichen species *Cetraria aculeata*. The results revealed that the alphaproteobacterial communities of high latitudes are depauperate and more closely related to each other than to those of extrapolar habitats. Interestingly, **this is in concordance with the geographical patterns found in the green-algal photobiont of *C. aculeata*, *Trebouxia jamesii*** (Fernández-Mendoza et al., 2011), **which is best explained by climatic differences and codispersal with the mycobiont rather than geographical distances alone** (Printzen et al., 2013, 2012).

Different parts of the lichen thallus are colonised by bacteria in different abundances and patterns. This **internal spatial structuring** of lichen-associated microbiome is usually studied by confocal laser scanning microscopy (Fig. 4). The lichens with a **dorsiventral thallus structure are equally colonised by bacteria on both the upper and lower cortexes** (Cardinale et al., 2012; Grube et al., 2009). In contrast, **crustose lichens exhibit the highest numbers of associated bacteria in the cracks of areoles of the thalli** (Grube et al., 2009). Cardinale et al. (2008) studied the spatial pattern of the bacterial community hosted by the **shrub-like reindeer lichen *Cladonia arbuscula* and found out that bacterial cells were embedded in a biofilm-like, continuous layer on the internal surface of the podetia**, whereas the other parts of the lichen showed a lower level of bacterial colonization.

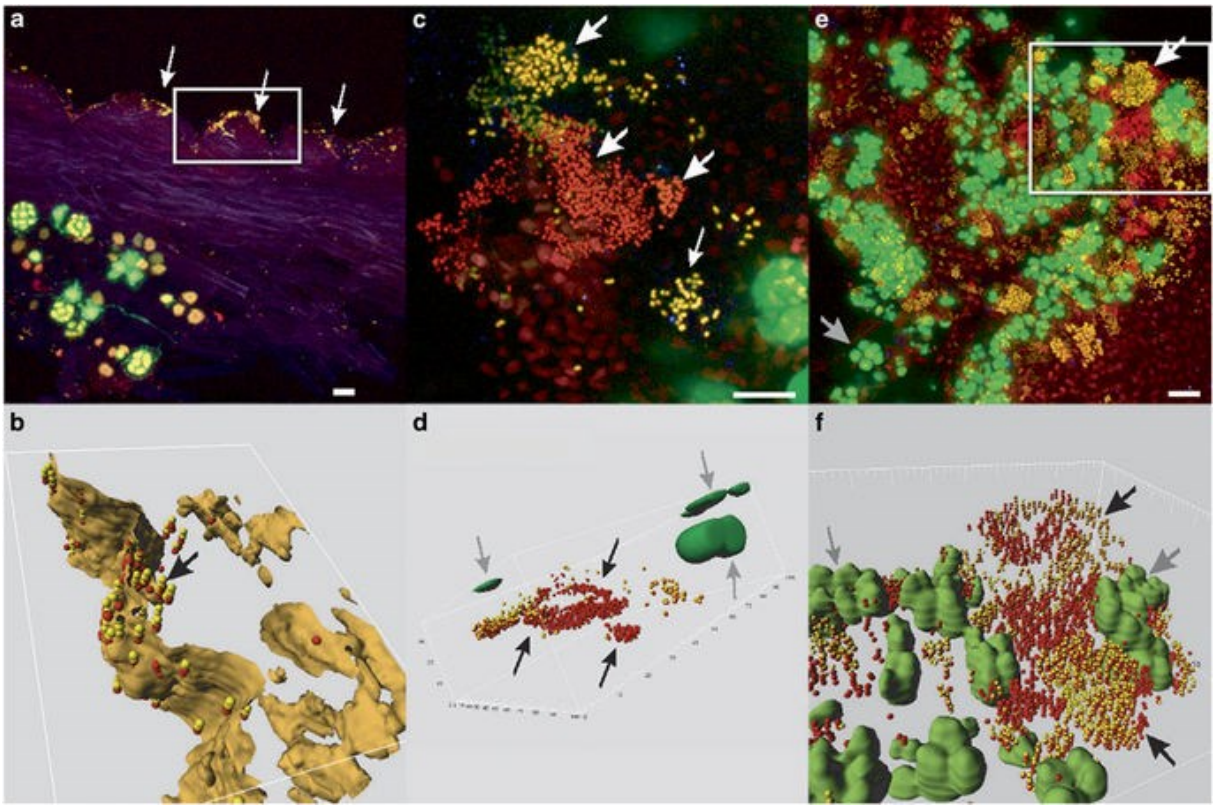

Fig. 4 Localization of bacteria in lichens by confocal laser-scanning microscopy (CLSM) and fluorescence in situ hybridization (FISH). Yellow: Alphaproteobacteria, red: other bacteria, green: eukaryotic algal cells, blue/pink: fungal hyphae. (**a**) Colonization of inner surfaces in *C. arbuscula* (arrow). (**b**) 3D reconstruction from the framed region in (**a**) shows bacteria below the surface of extracellular polysaccharides (arrow). (**c**) Lower surface of *U. cylindrica* with dense clusters of bacteria (arrow). (**d**) 3D reconstruction of (**c**) reveals bacterial community (black arrow) growing well separated from the algal cells (gray arrow). (**e**) Basal parts of *L. polytropa* with mixture of algal cells (gray arrow) and bacterial colonies (white arrow). (**f**) 3D reconstructions of the framed region in (**e**) with bacteria growing (black arrow) abundantly among the algal colonies (gray arrow). Scale bar=15 µm. From Grube et al. (2009).

1.4 Zeorin-containing red fruited *Cladonia* species as model organisms

Zeorin-containing red-fruited *Cladonia* species are conspicuous lichens, two of which had been **described already by Linnaeus** (1753). Similar to most other *Cladonia* species, members of this aggregate usually **grow in habitats with a low rate of competition from vascular plants** (e.g., on

sandy or rocky soils, on thin soil over rock, on bark, or on rotten wood; Fig. 5). Currently, the aggregate of zeorin-containing red-fruited and scyphose (cup-forming) *Cladonia* species consists of **five species worldwide**, of which four are known from Europe and North America [***C. coccifera*** (L.) Willd., ***C. deformis*** (L.) Hoffm., ***C. diversa*** Asperges ex S. Stenroos, **and *C. pleurota*** (Flörke) Schaer]. The fifth species, ***C. sinensis*** S. Stenroos & J. B. Chen (Stenroos et al., 1994), has a limited distribution in South-East Asia. This group of species is characterized by **similar chemical patterns** (presence of usnic acid derivates and zeorin, occasionally accompanied by porphyrilic acid), and **species within this group are delimited morphologically. The size, shape and location of the vegetative propagules on the podetia are traditionally considered to be the most important diagnostic characters** for separating these species (e.g., Asperges, 1983; Stenroos, 1989). The shape and width of the podetium is another relevant morphological feature commonly used to distinguish the species belonging to this group (e.g., Ahti and Stenroos, 2012; Asperges, 1983; Osyczka, 2011).

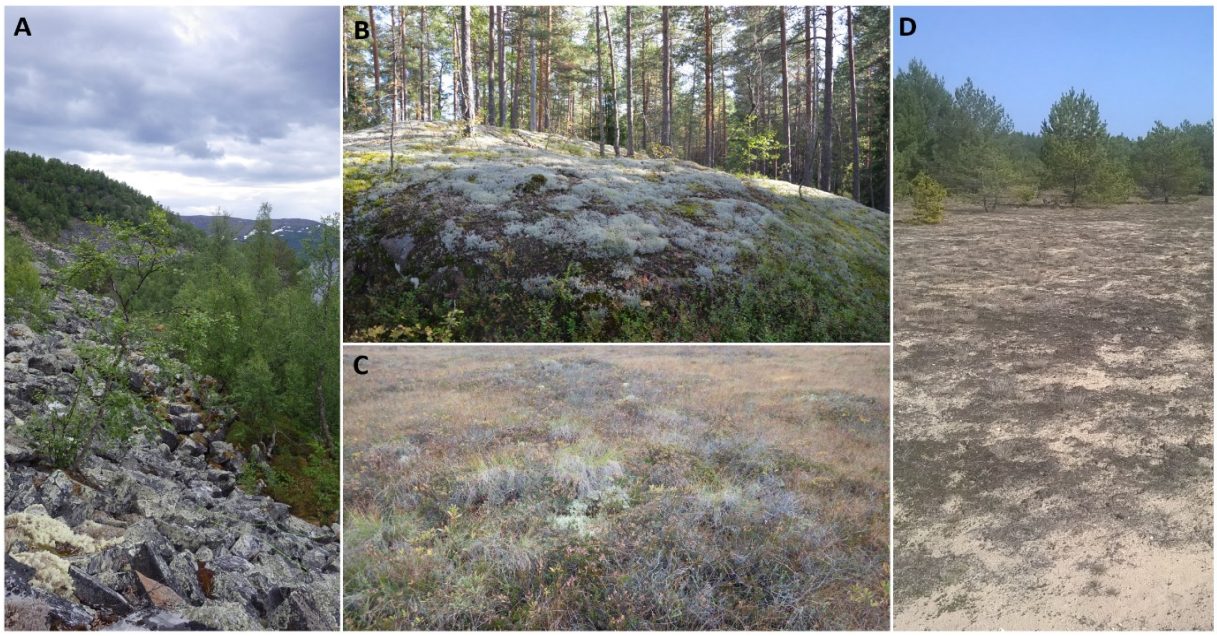

Fig. 5 Biotopes in which zeorin-containing red-fruited *Cladonia* species can often be found: (A) boulder scree (Norway), (B) granitic outcrops in pine forest (Finland), (C) raised mire (Wales), (D) early successional sands (Germany).

Cladonia coccifera (Fig. 6A) is an esorediate species with gradually expanded cups. The surface of the podetium is areolate corticate, covered by bullate and scaly plates. This species has often been confused with *C. diversa*, *C. pleurota* and *C. borealis* (Osyczka, 2011; Stenroos, 1989). ***Cladonia deformis*** (Fig. 6B) is, by comparison with the other species in this group, easy to recognize when well developed. Podetia are usually tall, relatively narrow and farinose sorediate. However, it can also be short-podetiate and then difficult to distinguish from *C. pleurota* (Ahti et al., 2013; Osyczka, 2011). ***Cladonia pleurota*** (Fig. 6C) is morphologically very variable (Stenroos, 1989). Young individuals are usually completely granulose sorediate, but when fertile the surface may turn almost totally corticate and is partly covered by granules or verruculae (Ahti et al., 2013; Stenroos, 1989). ***Cladonia diversa*** (Fig. 6D) is the most controversial species. The podetia of this species are usually slender and microsquamulose-granulose.

Because of its obvious morphological similarity to *C. coccifera* (esorediate podetia covered by irregular plates and/or granules), the natural status of this species was disputed by Stenroos (1989). However, although it has gradually been accepted (e.g., Ahti et al., 2013; Ahti and Stenroos, 2012; Pino-Bodas and Stenroos, 2017) it is still often included in *C. coccifera*.

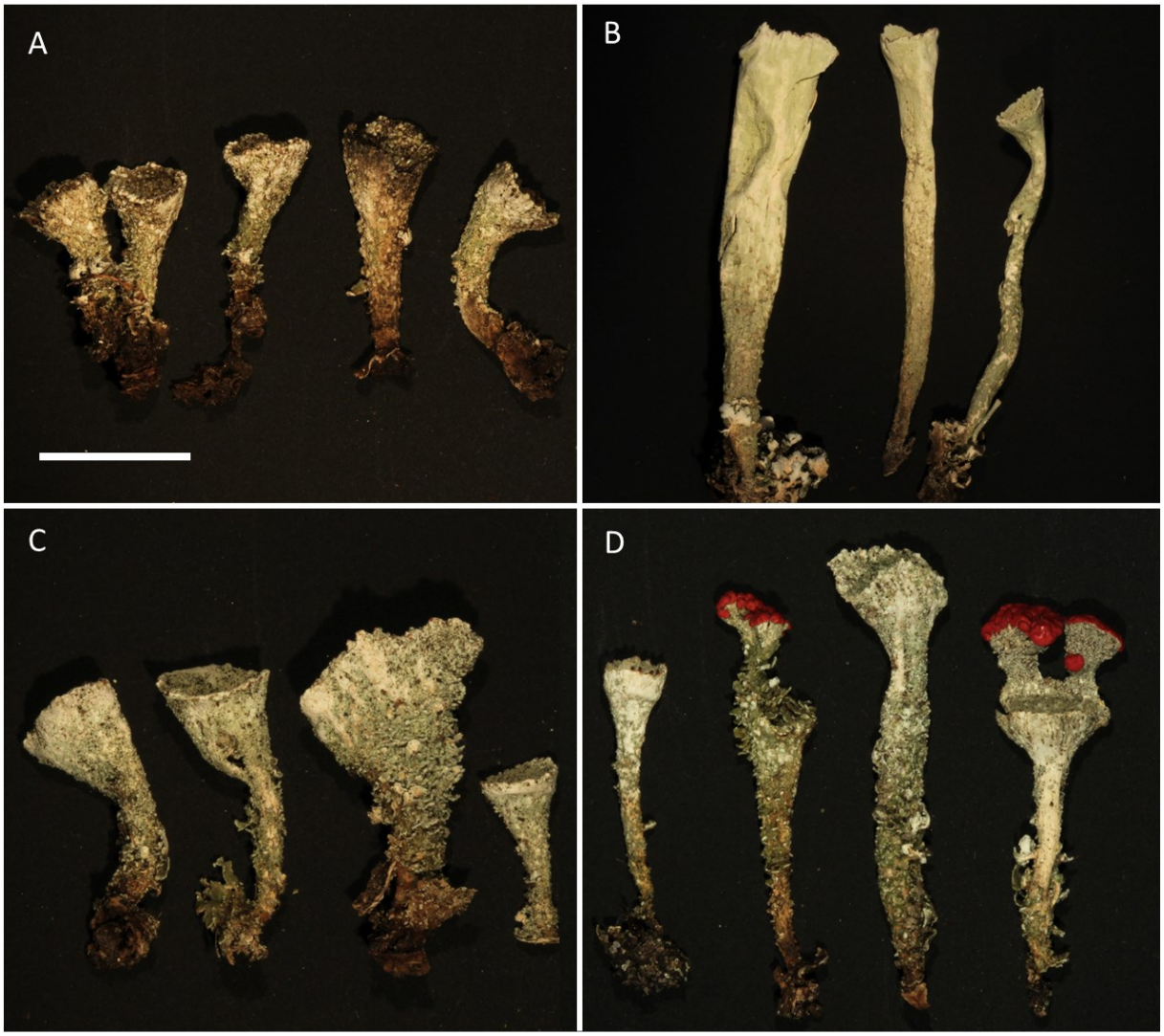

Fig. 6 *Cladonia* species included in the study. (A) *C. coccifera*, (B) *C. deformis*, (C) *C. pleurota*, (D) *C. diversa.* Scale is 1 cm.

Cladonia species are known for their **high specificity towards the green photobiont genus *Asterochloris*** (Trebouxiophyceae; Bačkor et al., 2010; Beiggi and Piercey-Normore, 2007; Nelsen and Gargas, 2006; Piercey-Normore et al., 2010; Yahr et al., 2006, 2004). This genus was described by Tschermak-Woess (1980), who differentiated it from the closely related genus *Trebouxia*. Subsequently, several authors proposed splitting the genus *Trebouxia* into two genera, *Asterochloris* and *Trebouxia*, (e.g., Helms et al., 2001; Rambold et al., 1998) based on the suggestion made by Tschermak-Woess (1989). Škaloud and Peksa (2010) transferred six former *Trebouxia* species into *Asterochloris* and established a new genus delimitation that was supported also by molecular data.

Currently, the **genus *Asterochloris* includes 15 described species** (Kim et al., 2017; Moya et al., 2015; Škaloud et al., 2015). **The *Asterochloris* species are distinguishable morphologically** (especially by

chloroplast ontogeny), **genetically** (by ITS r DNA and actin intron sequences) **and ecologically. They also differ in terms of their specificity towards the fungal partner.** A few of these species are highly specific and have been reported to associate only with a single mycobiont genus (e.g., *A. leprarii* or *A. gaertneri*; Škaloud et al., 2015). The others can associate with several mycobiont genera (e.g., *A. glomerata*, *A. irregularis*, *A. lobophora*, *A. woessiae*; Bačkor et al., 2010; Beiggi and Piercey-Normore, 2007, 2007; Peksa and Škaloud, 2011; Škaloud et al., 2015). **Besides *Cladonia*, the genera *Diploschistes, Lepraria*, *Pycnothelia*, *Stereocaulon* or *Sphaerophorus* represent the most common fungal partners** that can associate with *Asterochloris* species. Interestingly, these genera share similar ecological requirements and are often reported from the same habitats.

Until recently, *Cladonia* mycobionts were considered to associate exclusively with *Asterochloris* photobionts, however Elshobary et al. (2015) reported **another green algal species belonging to the genus *Stichococcus* from thallus of *C. macrophylla*** collected in Manitoba. The identity of the associated photobiont was determined by both cultivation and sequencing techniques. However, the authors admit that this anomalous finding might be also explained by the coexistence of two different algal strains in one lichen thallus (as already reported before; see e.g., Bačkor et al., 2010) or that the detected *Stichococcus*-like alga might have lived on the thallus surface.

The composition of **microbial communities associated with *Cladonia* species** was studied by Cardinale et al. (2008), Hodkinson et al. (2012), and Wedin et al. (2015). The results of these studies were consistent and showed the **dominance of Alphaproteobacteria** in *Cladonia* lichens. Betaproteobacteria, Actinobacteria and Acidobacteria are other relatively common bacterial groups present in *Cladonia* lichens. Wedin et al. (2015) studied the shift in the microbial community structure during an infection of *C. symphycarpa* by *Diploschistes muscorum* and recorded consistent changes across several study sites that included a notable decrease in the relative abundance of Alphaproteobacteria with a concomitant increase in Betaproteobacteria.

2 AIMS OF THE STUDY

The main aim of this study was to reveal the diversity of organisms participating in lichen symbiosis and to understand the biological forces which shape this diversity. As a model organism I chose **zeorin-containing red-fruited *Cladonia* species** because they are relatively common and they can be found across a broad geographical scale. During the study, it became apparent that **species delimitation**, which is a fundamental requirement for accurate diversity estimates, is another topic that requires closer attention. The specific aims were defined as follows:

1) **to determine the diversity of symbiotic partners associated in zeorin-containing red-fruited *Cladonia* lichens** (Paper 1, Paper 2, Paper 4)

2) **to assess the factors shaping the diversity of symbiotic partners associated in zeorin-containing red-fruited *Cladonia* lichens** (Papers 1 and 4)

3) **to study whether the current species delimitation of the two main partners (*Cladonia* mycobionts and *Asterochloris* species) is supported by molecular data** (Papers 2 and 3)

3 SUMMARY AND COCLUSIONS

3.1 The diversity of symbiotic partners associated in zeorin-containing red-fruited *Cladonia* lichens

Mycobionts

In Paper 2, the **genetic diversity and species delimitation** of the zeorin-containing red-fruited *Cladonia* species was assessed with ITS rDNA and β-tubulin loci. **β-tubulin and ITS loci trees had clearly different topologies.** The β-tubulin gene tree revealed three well- and one moderately supported lineages. In contrast, the ITS gene tree contained eight lineages and a single *Cladonia* sequence which was recovered on individual branch and did not correspond to any other lineage. Five major evolutionary mechanisms can cause the observed incongruence between phylogenetic markers: presence of pseudogenes, horizontal gene transfer, gene paralogy, incomplete lineage sorting, and hybridization. We consider **incomplete lineage sorting and/or hybridization to best explain the incongruence** in the case of zeorin-containing red-fruited *Cladonia* species. These two mechanisms are difficult to distinguish from each other and both may occur simultaneously (Meng and Kubatko, 2009; Seehausen, 2004). Hybridization has not yet been reported from lichens, but it has been proved to occur in most fungal phyla (e.g., Brasier et al., 1999; Craven et al., 2001; Xu et al., 2000). Similar incongruences have also been detected among genetic markers in other *Cladonia* groups (Fontaine et al., 2010; Kotelko and Piercey-Normore, 2010; Myllys et al., 2003) and these were usually attributed to ongoing recombination and sexual reproduction, incomplete lineage sorting, or, alternatively, the presence of the fungal chimera where two or more fungal strains make up a single podetium (Fontaine et al., 2010; Myllys et al., 2003).

Photobionts

The diversity of *Asterochloris* species associated in zeorin-containing red-fruited *Cladonia* lichens in Europe was studied in Paper 4. A total of **eight *Asterochloris* lineages** plus two *Asterochloris* sequences, which were recovered on individual branches and did not correspond to any currently recognized lineage, were found to associate with four *Cladonia* taxa. ***Asterochloris glomerata*, *A. italiana* and *A. irregularis* were the most common photobionts** in this lichen group.

We detected **clear differences of photobiont distribution across Europe**. *A. italiana* was mostly recovered from localities in the North-Western oceanic part of Europe (Great Britain, Denmark, Belgium, The Netherlands and the Norwegian coast), but also from Central Europe (Austria, Czech Republic and Germany), Portugal and Spain. In North-East Fennoscandia, we only detected *A. glomerata* and *A. irregularis*. Samples collected in Central and Southern Europe associated with a considerably higher number of *Asterochloris* lineages. Although we included only six *Cladonia* samples from the Iberian Peninsula, we found four *Asterochloris* lineages associated with the mycobionts in this region (*A. echinata*, *A. irregularis*, *A. italiana* and *A. woessiae*). Samples collected in Central Europe contained seven *Asterochloris* lineages and the two unique *Asterochloris* sequences. The Austrian Alps

and the Krkonoše Mts. in the Czech Republic were the regions with the richest *Asterochloris* diversity detected in Central Europe.

Bacteria

In Paper 1, **the bacterial diversity of several lichen species, including *Cladonia coccifera,* was studied by fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM).** In keeping with other works that have studied the composition of bacterial communities present in *Cladonia* lichens (Cardinale et al., 2008; Hodkinson et al., 2012; Wedin et al., 2015), **Alphaproteobacteria were found to be a dominant bacterial group** in both the squamules (79% of the total bacterial count) and the podetium (82% of the total bacterial count) of *C. coccifera*. **Other detected bacterial groups** (Actinonobacteria, Gammaproteobacteria and Deltaproteobacteria) **were present in much lower** abundancies (less than 1% of the total bacterial counts for both the squamules and the podetium). This also corresponds to the results of other studies (e.g., Bates et al., 2011; Grube et al., 2009; Wedin et al., 2015).

3.2 Factors shaping diversity of symbiotic partners in *Cladonia* lichens

Photobionts

Paper 4 focused on seeking the **factors responsible for shaping the diversity of photobionts associated in zeorin-containing red-fruited *Cladonia* lichens.** This species complex includes **species which share the same chemical patterns but are differentiated by the type of vegetative propagules present (soredia, granules, plates or microsquamules) and by the incidence of producing sexually reproductive structures.** Esorediate species have been considered to reproduce mainly sexually because, although a small number of heavy, corticated, vegetative propagules are produced which are firmly attached to the podetial surface, they also regularly build sexual reproductive structures. In contrast, the primary dispersal mode of the sorediate species has been hypothesized to be asexual and to depend on the small, light and ecorticate soredia that are produced in large amounts, because these species are seldom recovered with apothecia. We tested the **photobiont diversity** from 72 European localities against the **genetic distance of the mycobionts, as well as the geographic, climatic and reproductive variances.**

Our results clearly indicate that the **reproductive mode had the highest explanatory power in shaping photobiont diversity.** The **sorediate *Cladonia* species** associated only with two *Asterochloris* species and **were found to be strongly selective towards their photobiont.** In contrast, the **esorediate *Cladonia* species were shown to be photobiont generalists** by associating with seven *Asterochloris* lineages and two *Asterochloris* sequences recovered on individual branches.

The importance of reproductive and dispersal modes as key factors in shaping photobiont diversity has also been demonstrated in some other lichen groups (Cao et al., 2015; Fedrowitz et al., 2011; Otálora et

al., 2012; Yahr et al., 2006), although usually on a smaller geographic scale. The algal genetic diversity in populations of lichenized fungi having distinct propagation strategies is, however, not always necessarily different (Wornik and Grube, 2009). It was suggested that, depending on the viability of the soredial algae, the soredial fungi could choose between establishing the new thallus with the co-propagated alga or with another photobiont, likely better adapted to the local conditions. The main role of the original photobiont would be to prolong the survival of the co-propagated fungal hyphae (Wornik and Grube, 2009).

Bacteria

In Paper 1, a microscopic approach was used **to test the significance of both lichen-intrinsic and extrinsic environmental factors on the bacterial communities** associated with 11 lichen samples, belonging to six species (two of which belonged to genus *Cladonia*). **In *Cladonia coccifera*, the highest level of bacterial colonization was found on the surface of the internal cavities in the podetium.** The results also showed that the **lichens growing on rock** (*C. coccifera*, *Lecanora polytropa* and *Umbilicaria cylindrica*) **harbour fewer bacteria than lichens from other substrate types** (e.g. soil and bark). There was a statistically significant effect on the bacterial community structure related to the **age of the thallus**: in the older thallus parts the bacterial community displayed a drastic change due both to the reduction of the otherwise dominant Alphaproteobacteria and to the increased abundance of other groups, which include Actinobacteria, Gamma- and Betaproteobacteria. Interestingly, Mushegian et al. (2011) found a similar pattern in *Xanthoparmelia* lichens, in which the central (older) parts of the lichen thallus harboured a richer bacterial community than marginal (younger) parts. The reason for this difference is unclear but one of the possible explanations is that **the growing apices may act as anabolic centers, whereas the senescing parts might represent catabolic sinks of the lichen system.** The diverse bacteria of these older parts may help to convert the old biomass into simple molecules, which might then be released into the substrate or be recycled by translocation to the growing parts of the lichens.

3.3 Species delimitation

Mycobionts

The main objective of Paper 2 was to explore whether the **current delimitation of zeorin-containing red-fruited *Cladonia* species is supported by molecular data** (namely ITS rDNA and β-tubulin sequences). This group of species is characterized by similar chemical patterns, and species within this group are delimited morphologically. The width and the shape of the podetium and the type of the vegetative propagules on the podetia and are traditionally considered as the most important diagnostic characters separating species (e.g., Ahti and Stenroos, 2012; Asperges, 1983; Osyczka, 2011; Stenroos, 1989).

The results of the phylogenetic reconstruction indicate that three species belonging to the species complex (*Cladonia coccifera*, *C. deformis* and *C. pleurota*) are polyphyletic and hence **the traditional morphologically-based species circumscription cannot be supported**. Only *C. diversa* formed a monophyletic group in the ITS phylogeny, but it was not supported statistically.

Other studies focusing on *Cladonia* (Fontaine et al., 2010; Kotelko and Piercey-Normore, 2010; Pino-Bodas et al., 2010) also found a similar incongruence between the phylogenetically inferred lineages and the traditional, morphologically and/or chemically delimited species. In the case of zeorin-containing red-fruited *Cladonia* species, **no phenotypic feature was found to unambiguously define majority of the lineages** (except *Cladonia diversa*, and chemical patterns in two lineages). Such phylogenetic units that cannot be characterised phenotypically could be either regarded as populations of a morphologically variable species or accepted as cryptic species. Because some lineages of red-fruited zeorin-containg *Cladonia* species in the present study were characterised phenotypically (morphologically or chemically) **we considered the well-supported phylogenetic lineages as separate cryptic species, that cannot be characterised phenotypically at this time.**

Photobionts

Paper 3 focused on the **species delimitation within the photobiont genus *Asterochloris*.** This genus was described by Tschermak-Woess (1980), who distinguished it from the closely related genus *Trebouxia* by the different chloroplast morphology. A recent investigation of *Asterochloris* photobionts across Europe revealed a number of new lineages occurring in lichen thalli. The main aim of this paper was **to assess whether these lineages represent well-defined species and to determine which features can be used for their reliable recognition.**

Six new species (*A. echinata*, *A. friedlii*, *A. gaertneri*, *A. leprarii*, *A. lobophora*, and *A. woessiae*) were described and characterised. These species **differ genetically, morphologically, ecologically, and with respect to their mycobiont partners.** Chloroplast morphology was shown to be the best morphological marker for species delineation. Surprisingly, **all currently recognised 13 *Asterochloris* species share identical small subunit rDNA sequences.** This marker is commonly used for the assessment of protist diversity by both the traditional Sanger as well as the next generation sequencing approaches (Countway et al., 2005; Kilias et al., 2013; Majaneva et al., 2012; Massana et al., 2015). The fact that the small subunit rDNA sequences can be shared by several well-characterised species **can lead to diversity underestimates.** Therefore, we suggest sequencing the variable ITS rDNA gene in addition to 18S rDNA is needed to capture the true protist diversity.

3.4 Conclusions

The view of lichen symbiosis has changed dramatically during the last two hundred years. Nowadays, lichens are generally understood as **microecosystems consisting of several symbiotic partners which contribute in different ways to the prosperity of the whole system** (e.g., Grube et al., 2015, 2009;

Spribille, 2018). The organisms that participate in this symbiosis differ by the strength of their bond to other symbiotic partners, ranging from highly specific to very unspecific relationships. I adopted this holistic view of the lichen symbiosis and studied the diversity of the symbiotic partners in zeorin-containing red-fruited *Cladonia* species and the forces that shape these symbiotic associations. This work, hence, represents an attempt to describe the complexity of the lichen symbiosis, though the level of knowledge of the individual partners (mycobionts vs. photobionts vs. bacteria) is incomplete and varies greatly.

In Papers 2 and 4, I studied the genetic diversity of the mycobionts and photobionts in the same lichen group. In the case of the **lichenized fungi** (Paper 2), **the genetic diversity was higher than expected** from the morphological variation and we detected several phenotypically yet uncharacterizable lineages that probably correspond to the **cryptic species**. These lineages are most likely a result of either hybridization or incomplete lineage sorting. **The photobionts associated with these mycobionts showed also a relatively high level of diversity** (Paper 4). We detected eight *Asterochloris* lineages and two *Asterochloris* sequences, which did not correspond to any currently recognized lineage. However, in contrast to the mycobionts, the majority of photobiont lineages represent **phenotypically distinguishable species** (Paper 3).

The results indicate that the same factor is important for shaping the diversity of both, the algal and fungal partners. In Paper 4, we demonstrated that the **reproductive and dispersal strategies of the mycobiont are the key factors** influencing the diversity of the *Asterochloris* species in zeorin-containing red-fruited *Cladonia* species. A closer look at the results of the mycobiont study (Paper 2) reveals that all detected lineages comprised specimens with the same dispersal strategy (sorediate or esorediate species) although the species delimitation of the individual mycobiont species was not supported. From these results we can conclude that the ability to produce soredia is the key factor influencing the lichen association of zeorin-containing *Cladonia* species. Interestingly, it has been reported that ascospores of other sorediate lichens (*Physconia distorta*) can have a strongly reduced reproductive function compared to esorediate species (*P. grisea*) (Molina et al., 2013).

The level of specificity varies greatly among the symbiotic partners. Whereas the **mycobionts of the sorediate species** (*C. deformis* and *C. pleurota*) **were shown to be highly selective towards their photobionts** by associating with only two *Asterochloris* lineages, **the esorediate *Cladonia* species were far less selective** (Paper 4). The ***Asterochloris* species** also differ from *Cladonia* mycobionts in terms of their specificity towards the fungal partner, **being generally less selective and associating with several fungal genera** (with the exceptions of *A. leprarii* and *A. gaertneri,* which have been reported to associate only with a single mycobiont genus) (Paper 3). As the diversity of the bacteria associated with lichens is not usually studied at the species level, comparison of the level of specificity with other symbiotic partners is difficult. In Paper 1 we illustrated that the lichen species is not the key factor shaping bacteria diversity (at higher taxonomical levels), therefore the **bacteria present in lichens are probably not strongly specific towards their partners.** This is in contrast with the results of Wedin

et al. (2015) who studied the shift in the microbial community structure during an infection of *Cladonia symphycarpa* by *Diploschistes muscorum* and recorded consistent changes across several study sites. However, the authors attribute this transition to the different physical parameters of their thallus structure, and more specifically to the hydrophilic character of the thallus surface of *C. symphycarpa* as opposed to the hydrophobic surface of *D. muscorum*.

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5 ORIGINAL PAPERS

5.1 Paper 1

Cardinale M., **Steinová J.**, Rabensteiner J., Berg G. and Grube M. (2012):

AGE, SUN AND SUBSTRATE: TRIGGERS OF BACTERIAL COMMUNITIES IN LICHENS

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Age, sun and substrate: triggers of bacterial communities in lichens

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SUMMARY

Bacterial communities colonize the surfaces of lichens in a biofilm-like manner. The overall structure of the bacterial communities harboured by the lichens shows similarities, in particular the dominance of not yet cultured Alphaproteobacteria. Parameters causing variation in abundance, composition and spatial organization of the lichen-associated bacterial communities are so far poorly understood. As a first step, we used a microscopic approach to test the significance of both lichen-intrinsic and extrinsic environmental factors on the bacterial communities associated with 11 lichen samples, belonging to six species. Some of these species have thalli with a distinct age gradient. A statistically significant effect can be attributed to the age of the thallus parts, which is an intrinsic factor: growing parts of the lichens host bacterial communities that significantly differ from those of the ageing portions of the thalli. The substrate type (rock, tree, understory) and (at a lower extent) the exposition to the sun also affected the bacterial communities. Interestingly, the abundance of bacterial cells in the lichens was also influenced by the same structure-triggering factors. No effect on the composition with main bacterial groups was attributed to different lichen species, differentiated thallus parts or thallus growth type. Our results are important for the experimental designs in lichen-bacterial ecology.

INTRODUCTION

The lichen association represents one of the most 'eyecatching' fungal symbioses. Lichens are often dominating aspects of vegetation in otherwise hostile habitats such as high mountains or subpolar regions. Lichenized fungi form long-living and compact thallus structures to position their phototrophic partners in a controlled manner towards light (the name of a lichen holobiont applies in fact to the shape-determining fungal partner). Recent studies suggest that lichens are more complex symbiotic systems than previously assumed. Diverse bacteria were characterized in lichens by rRNA gene sequencing (Cardinale *et al*., 2006; 2008; Liba *et al*., 2006; Hodkinson and Lutzoni, 2009) and we consider the concept of lichens be expanded by including the associated bacterial community (Grube *et al*., 2009). Fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM) revealed details about the abundance and localization of the bacterial communities (Cardinale *et al*., 2008). Bacteria preferentially colonize hydrophilic surfaces of the lichens, varying from individual colonies to

biofilm-like communities. Alphaproteobacteria usually form a predominant fraction, while diverse other bacteria are present at lower abundances (Grube *et al*., 2009; Bates *et al*., 2011; Schneider *et al*., 2011). Functional studies of the culturable bacteria display a range of lytic activities, including chitinolysis, glucanolysis and proteolysis (Grube *et al*., 2009), while metaproteomic analysis suggested a multifunctional role of the bacterial partner in the lung lichen symbiosis (Schneider *et al*., 2011). Sequence data confirm presence of *nif*H genes in lichen-associated bacteria and suggest a contribution to the lichen's nitrogen budget. It appears that lichen symbioses resemble miniature ecosystems (Farrar, 1985; Grube *et al*., 2009), comprising algae as producers, fungi as consumers, as well as bacteria contributing to nutrient acquisition, recycling and antagonism.

The view of lichens as micro-ecosystems spurs new research questions about stability and variation of the associated microbial community. The long-living thalli of lichens grow in vastly different habitats and represent rather complex niches. Lichens can essentially maintain metabolic activity over the whole thallus area, but species can present a more or less distinct gradient of ageing, with proliferating young parts and slowly decaying distal parts (Grube, 2010). Because FISH presents mostly physiologically active subpopulation, we can thus analyse in the lichen miniature ecosystem the succession of active microbial communities and the effects of habitat variation at a microscopic level.

In this work, we studied the bacterial communities associated with 11 lichen samples, representing six species, by FISH coupled with confocal laser scanning microscopy (FISH-CLSM) with the aim to analyse and compare the abundance and diversity. For this purpose, we developed a new method to measure the ratio between the volume and the weight of the lichens, defined as Delta volume method (Dvol). We also investigated the effect of lichen-intrinsic (lichen species, thallus age, differentiated thallus parts, growing type) and extrinsic environmental factors (sun exposure and substrate) on the taxonomic structure of the bacterial communities in lichens.

RESULTS AND DISCUSSION

Lichen samples were collected from above and near the timber line in subalpine altitudes in the Styria region (Austria), and from broad-leaved montane forests, in the years 2007-2009. At least four different healthy lichen thalli for each sample were collected from sites specifically chosen to study the effects of several lichen-intrinsic and extrinsic environmental factors on the lichen-associated bacterial communities. Cryosections of paraformaldehyde-fixed lichen samples were pretreated with lysozyme and then hybridized with different FISH probes (Table S1), following the protocol of Cardinale and colleagues (2008). Initial experiments showed no difference between the bacterial cell counts of Gram-positive bacteria in paraformaldehyde- and ethanol-fixed lichens, when pretreated with lysozyme. A negative control for systemic errors within FISH experiments consisted of a mixture of NONEUB FISH probes labelled with all the fluorochromes present in the positive probes. Typical signals from probe-stained bacteria were not detected in the negative control, whereas autofluorescence of algae and fungi

showed the same intensity, which confirmed the absence of both probe- and fluorochrome-unspecific labelling. The FISH-stained samples were viewed under a confocal microscope and the stacks were recorded to perform both bacterial counts and 3D-reconstructions. Bacterial cell count was performed on 3D image stacks semi-automatically, supported by the counting options in the software Imaris 7.0 (Bitplane, Zurich, Switzerland). Specifically, the bacteria were tagged automatically and tags were then inspected by eye. Particular attention was paid to dense cell clusters to avoid underestimation of counts due to signal overlap. The stacks showing low signal/noise ratio or ambiguous signals were discarded from the analysis. At least 30 confocal stacks from at least five independent FISH experiments per lichen sample were recorded and analysed.

To determine the density of the EUB338MIX-stained bacterial cells per gram of lichen we developed a new measurement method, which we call Delta volume (Dvol). First, lichen thalli were saturated with water in a humid chamber until a constant wet weight (ww) was reached. The volume of the wet thalli was measured by dipping it into a graduated tube (Dvol). Then the thalli were dried at 50°C and the dry weight (dw) was assessed. The ratio Dvol/dw represents the volume of hydrated lichen thallus containing one gram of lichen biomass (dw). Results are shown in Table 1.

To check the detection efficiency of FISH, we used the nucleic acid stain Acridine Orange, which was shown to be superior to DAPI in lichens (Cardinale *et al*., 2008). On average, 82% of the Acridine orange-detected cells were stained by the EUB338MIX probe. The number of EUB338MIX-stained cells associated with the analysed lichens were in the range of $2.11 \pm 0.24 \times 10^9$ cells/g_{dw} (squamules of *Cladonia coccifera*) and $7.56 \pm 1.09 \times 10^9$ cells/g_{dw} (senescing part of the *Cladonia arbuscula* thallus, sun-exposed site). The means between lichen samples (Log_{10}-transformed values) were significantly different, $F_{10,518} = 11.598$, $P < 0.001$, $\eta^2 = 0.183$, 95% $CI_{\eta^2} = 0.113$-0.227, $1 - \beta_{\alpha=0.05} = 1.000$. All analysed lichens hosted a very high number of bacteria, comparable with the abundance in the rhizosphere (Berg and Smalla, 2009), and similarly represents bacterial hot spot.

According to our data *Lobaria pulmonaria* and the old thalli of *C. arbuscula* harboured the highest number of active bacteria, at a confidence level of 0.05 (Tukey test). Lichens grown under shaded conditions harboured a higher total number of bacteria than the sun-exposed ones, $t_{527} = 3.242$, $P = 0.001$, $d = 0.28$. Interestingly, the old senescing parts of lichen thalli host a significantly higher number of bacteria, than the young growing parts, $t_{527} = 7.890$, $P < 0.001$, $d = 0.70$. This difference suggests a succession or gradient of the lichen-associated bacterial community. The ageing structures allow growth of diverse bacterial taxa, whereas young parts with prolifering fungal and algal partners constrain the spectrum of bacteria to highly adapted ones, mostly Alphaproteobacteria. ANOVA followed by Tukey test also showed that the lichens growing on rock (*C. coccifera, Lecanora polytropa* and *Umbilicaria cylindrica*) harbour fewer bacteria than lichens from other substrate types (e.g. soil and bark), $F_{3,525} = 22.762$, $P < 0.001$, $\eta^2 = 0.115$, 95% $CI_{\eta^2} = 0.066$-0.164, $1 - \beta_{\alpha=0.05} = 1.000$. Alphaproteobacteria dominated the vital structures in all lichens (Fig.1), ranging from $37.6 \pm 10.5\%$ in *U. cylindrica* to $82.1 \pm 3.8\%$ in the squamules of *C. coccifera* (Fig.2A). Only in the older basal part of *C. arbuscula* collected

from a shaded site the dominant group was Betaproteobacteria (53.5 ± 5.1%) and the Alphaproteobacteria represented 15.4 ± 2.6% (Fig.2A). Other bacterial groups were relatively rare in the lichens (Fig.2A).

Canonical correspondence analysis showed that morphologically different lichens harbour communities, which are similar with respect to the principal bacterial groups according to FISH/CLSM analyses (Fig.2B). Testing the environmental variables for significance demonstrated that the lichen species does not exert an exclusive effect on the overall community structure (P = 0 640) (Fig.2B). This observation does not exclude variation between the bacterial communities at the species/strain level in different lichen species; specificity was already shown in different lichens for the composition of microbial fingerprints (Grube *et al*., 2009) and corroborated recently by 16S rRNA gene deep-sequencing (Bates *et al*., 2011).

A statistically significant effect on the bacterial community structure was found for the age of the thallus (P = 0.002): in the older thallus parts the bacterial community displayed a drastic change due both to the reduction of the otherwise dominant Alphaproteobacteria and to the increased abundance of other groups including Actinobacteria, Gamma- and Betaproteobacteria. On the other hand, Deltaproteobacteria and Firmicutes were not significantly affected (Fig.2A). It is worth noting that no Alphaproteobacteria were isolated in culture experiments from the same lichens as used for FISH-CLSM (data not shown). This suggests that the dominant Alphaproteobacteria have special requirements for growth, possibly expressed by the physiologically active lichen. The exposition to sun was also found to be of significant effect on the bacterial communities (P = 0.010), as well as the substrate type (P = 0.004) (Fig.2B). Other apparently important factors such as differentiated thallus forms and growth types also did not affect the main taxonomic structure of the bacterial communities (P = 0.244 and 0.490, respectively).

The analysed lichens show an interesting duality in their associated bacteria, with a constrained and stable community in the healthy areas and more variable one in the ageing, or senescing, parts. The divergence occurs at distances of millimetres to centimetres depending on the size of the thalli. The contributions by additional bacterial diversity in ageing portions could be interpreted as transient bacterial fraction in lichens. Further research using deep amplicon sequencing data needs to take into consideration the differences within a single lichen thallus that result from the considerable age of the structures. As lichen thalli can live for decades to hundreds of years, we conclude that the lichens maintain a dynamic equilibrium. The growing apices with alphaproteobacterial dominance act as anabolic centres, whereas the senescing parts might represent catabolic sinks of the lichen system. We assume that the diverse bacteria of these parts help to convert the old biomass into simple molecules, which might be released into the substrate or be recycled by translocation to the growing parts of the lichens. Such recycling of nutrients has been demonstrated previously in lichens (Ellis *et al*., 2005).

Confocal laser scanning microscopy is a valuable tool to complement diversity studies based on fingerprinting or sequencing methods, by providing localized information on microbial diversity and circumventing biased views of diversity at small scales (Bent and Forney, 2008). The limited resolution

of the probes, however, precludes comments on differences at the level of species/strains. Such level of detail was not necessary to demonstrate that thallus vitality is a principal factor for intra-individual variation of lichen-associated bacterial communities. Two additional triggers were found, namely the substrate type and the sun exposition, whereas growth type does not play a significant role in modifying the main composition of lichen-associated bacterial communities. Interestingly, the same factors affecting the structure of the bacterial community also affected the abundance of FISH-detected cells in the analysed lichens. This work represents a study of the ecology of lichen-associated bacterial communities and provides first evidence for environmental triggers of their main composition. These results will be informative and could serve as a guideline for experimental design in lichen-bacterial ecology, and more generally for exposed and long-living terrestrial symbiotic systems.

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TABLES

Tab. 1 Correspondence between the volume and the weight of the lichens, assessed by the Delta volume method – Δvol.

Lichen species	Thallus part	Dvolume (mm^3)	Wet weight (g)	Dry weight (g)	Volume (mm^3) corresponding to 1 g of lichen dry weight
Cetraria islandica	Younger	4.0×10^3	3.7	0.77	5.19×10^3
Cetraria islandica	Older	6.0×10^3	4.4	0.72	8.33×10^3
Lobaria pulmonaria	Whole	6.5×10^3	5.13	1.58	4.11×10^3
Lecanora polytropa	Whole	5.5×10^3	5.18	3.02	1.82×10^3
Cladonia arbuscula	Younger	8.0×10^3	12.39	3.1	2.58×10^3
Cladonia arbuscula	Older	5.0×10^3	3.4	0.91	5.49×10^3
Umbilicaria cylindrica	Whole	2.6×10^4	22.94	7.04	3.69×10^3
Cladonia coccifera	Podetia	2.0×10^3	1.72	0.67	2.99×10^3
Cladonia coccifera	Squamules	6.5×10^2	0.46	0.19	3.42×10^3

FIGURES

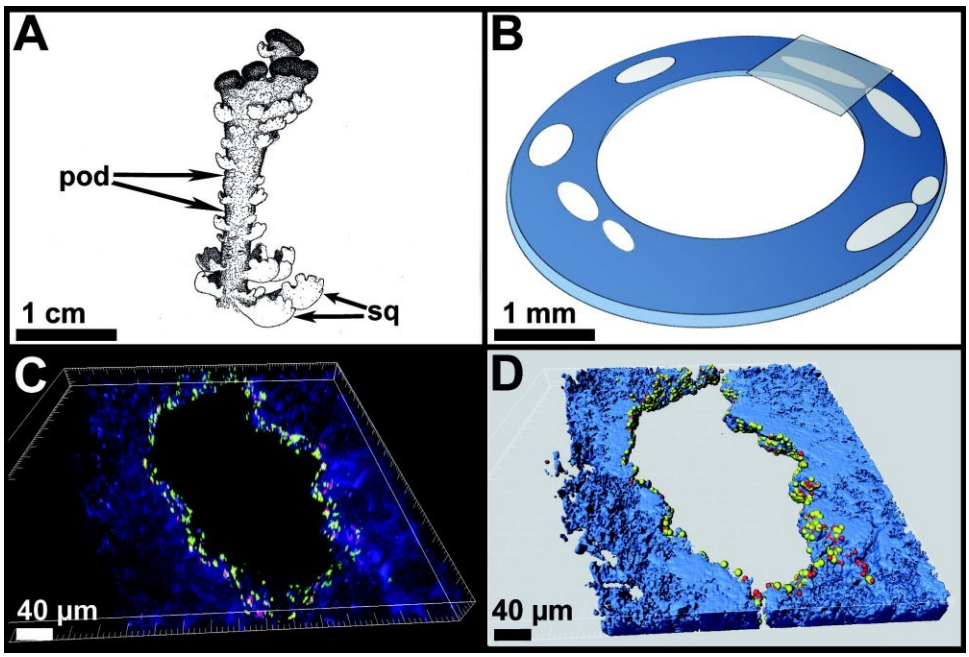

Fig.1 A: The lichen *Cladonia coccifera* with the differentiated plectenchyma forming squamules (sq) and podetia (pod). **B:** A transversal section of a *C. coccifera* podetium, showing the characteristic cylindric shape, and with cavities within the fungal thallus structure. **C:** Volume rendering of a confocal stack showing the bacterial colonization of the lichen *C. coccifera*; the bacteria densely colonize the surface of the internal cavities in the podetium [see rectangle in (B); blue: lichen-fungal plectenchyma; yellow: Alphaproteobacteria; red: other bacteria (algae not present in this section)]. **D:** Three-dimensional model of the image in (C); blue surface: lichen-fungal plectenchyma; yellow spheres: Alphaproteobacteria; red spheres: other bacteria. Bacteria were stained by FISH as described by Cardinale and colleagues (2008) using the universal bacterial probe EUB338MIX (Cy3-labelled) and the Alphaproteobacteria-specific probe ALF968 (Cy5-labelled). The confocal microscope Leica TCS SPE (Leica microsystems GmbH, Mannheim, Germany) was used for the image acquisition; the Z-step size, laser intensity and detector settings (gain and offset) were optimized to get the optimal resolution and the best signal/noise ratio; the software Imaris 7.0 (Bitplane, Zurich, Switzerland) was used to create the 3D models.

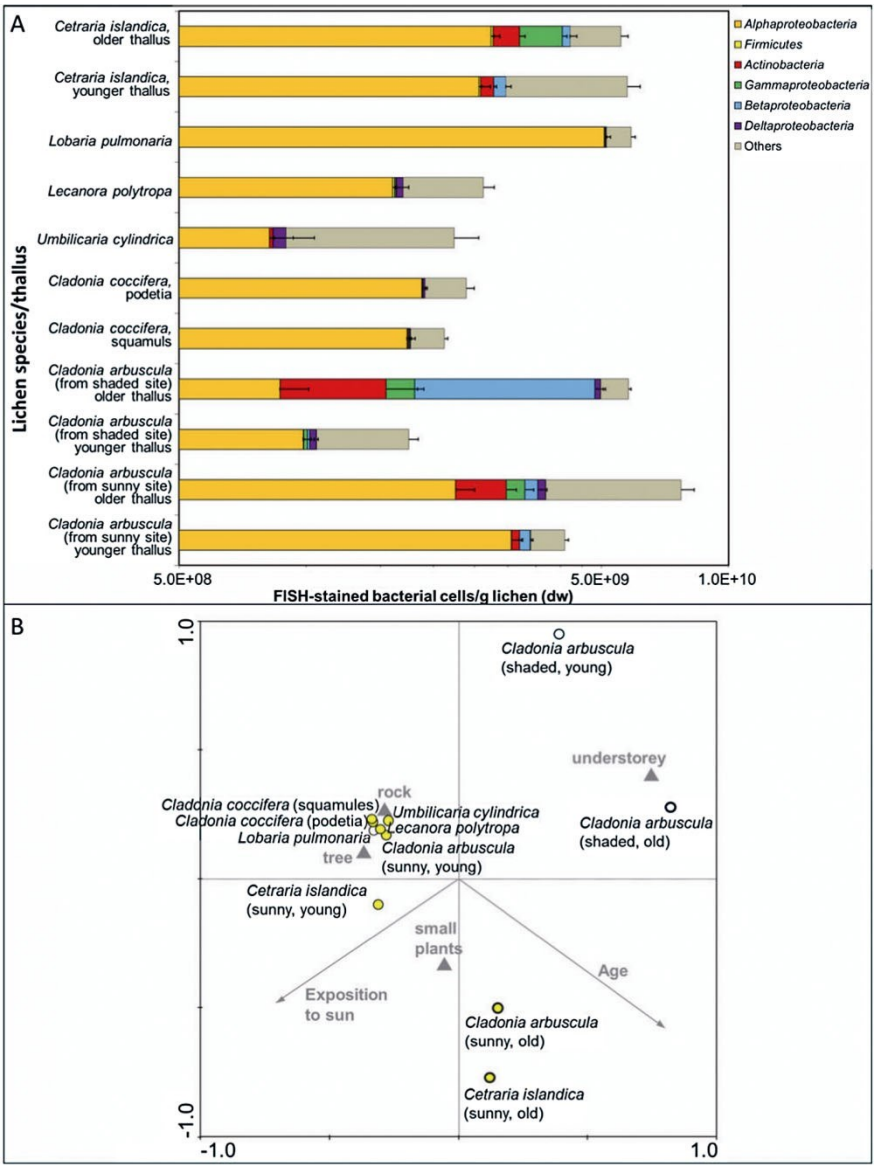

Fig.2 A: Density of principal bacterial groups associated with different lichen species/thalli assessed by fluorescence in situ hybridization-confocal laser scanning microscopy (FISH-CLSM). FISH was performed as described by Cardinale and colleagues (2008) on 30-50 mm thick cryosections of PFA-fixed lichens; for details on the used FISH probes, see Table S1. **B:** Canonical correspondence analysis (CCA) performed with CANOCO for Windows (ter Braak and Šmilauer, 2002), showing the similarity between the bacterial community structures, assessed by FISH-CLSM; yellow-filled circles indicate bacterial communities of lichens samples collected from sun-exposed sites; circles with bold lines indicate bacterial communities of the older parts of the thalli. Statistically significant environmental factors are shown (grey labels); for the nominal variables 'substrate type' the centroid (triangle) is shown. Significance of environmental factors was evaluated by Monte Carlo permutation test, as implemented in CANOCO.

SUPPORTING INFORMATION

Table S1. Characteristics of the FISH probes used in this work.

Probe name	Sequence (5'- -3')	Fluorescent	Target	% FA (41°C)	Reference
ALF968	GGT AAG GTT CTG CGC GTT	Cy5	Alphaproteobacteria	40	Neef 1997
LGC354A*	TGG AAG ATT CCC TAC TGC	FITC	Firmicutes	40	Meier *et al.*, 1999
LGC354B*	CGG AAG ATT CCC TAC TGC	FITC	Firmicutes	40	Meier *et al.*, 1999
LGC354C*	CCG AAG ATT CCC TAC TGC	FITC	Firmicutes	40	Meier *et al.*, 1999
EUB338**	GCT GCC TCC CGT AGG AGT	Cy3	Most bacteria	10	Amann *et al.*, 1990
EUB338II**	GCA GCC ACC CGT AGG TGT	Cy3	Planctomycetales	10	Daims *et al.*, 1999
EUB338III**	GCT GCC ACC CGT AGG TGT	Cy3	Verrucomicrobiales	10	Daims *et al.*, 1999
NONEUB	ACT CCT ACG GGA GGC AGC	FITC, Cy3, Cy5, ATTO488	/	#	Wallner *et al.*, 1993
HGC236	AAC AAG CTG ATA GGC CGC	Cy5	Actinobacteria	20	Erhart *et al.*, 1997
BET42a***	GCC TTC CCA CTT CGT TT	FITC	Betaproteobacteria	40	Manz *et al.*, 1992
GAM42a***	GCC TTC CCA CAT CGT TT	Cy5	Gammaproteobacteria	40	Manz *et al.*, 1992
Delta495a****	AGT TAG CCG GTG CTT CCT	ATTO488	Deltaproteobacteria	40	Lücker *et al.*, 2007
Delta495b****	AGT TAG CCG GCG CTT CCT	ATTO488	Deltaproteobacteria	40	Lücker *et al.*, 2007
Delta495c*****	AGT TAG CCG GCG CTT CCT	ATTO488	Deltaproteobacteria	40	Lücker *et al.*, 2007
Delta495ab competitor*****	AGT TAG CCG GCG CTT CKT	/	NON Deltaproteobacteria	40	Lücker *et al.*, 2007
Delta495c competitor*****	AAT TAG CCG GCG CTT CTT	/	NON Deltaproteobacteria	40	Lücker *et al.*, 2007

* to be used mixed in equimolar concentration;
** to be used mixed in equimolar concentration;
# the same % of the hybridization with the positive FISH probe, in the same experiment;
*** to be used together (the probes are competitors each other);
**** to be used mixed in equimolar concentration;
***** to be used mixed in equimolar concentration.

5.2 Paper 2

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GENETIC DIVERSITY AND SPECIES DELIMITATION OF THE ZEORIN-CONTAINING RED-FRUITED *CLADONIA* SPECIES (LICHENIZED ASCOMYCOTA) ASSESSED WITH ITS rDNA AND β-TUBULIN DATA

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Genetic diversity and species delimitation of the zeorin-containingred-fruited Cladonia species (lichenized Ascomycota) assessed with ITS rDNA and β-tubulin data

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ABSTRACT

Zeorin-containing red-fruited *Cladonia* species, the so-called *C. coccifera* group, are widespread terrestrial lichens which share most of their secondary substances but differ morphologically. The main objective of this study was to explore whether the current delimitation of these species is supported by molecular data. A total of 52 European and North American specimens of *C. coccifera*, *C. deformis*, *C. diversa*, and *C. pleurota* were examined. The internal transcribed spacer regions of the nuclear ribosomal DNA and the β-tubulin gene loci were sequenced for phylogenetic analyses. Traditional morphological species circumscriptions in zeorin-containing members of the *C. coccifera* group are not supported by molecular data. *Cladonia coccifera*, *C. deformis*, and *C. pleurota* were recovered as polyphyletic in both gene topologies; *C. diversa* formed a lineage in the ITS phylogeny but this was not statistically supported. We detected chemical patterns of the presence/absence of porphyrilic and/or isousnic acid which may help to characterize two lineages. Our results also show incongruence between the two molecular markers studied. Therefore, we focused on possible explanations of this phenomenon. Five major evolutionary mechanisms can potentially result in phylogenetic discordance between genes: presence of pseudogenes, horizontal gene transfer, gene paralogy, incomplete lineage sorting, and hybridization. These mechanisms are briefly discussed. We consider incomplete lineage sorting and/or hybridization to best explain the incongruence.

Key words: bootscanning, *Cladoniaceae*, Cocciferae, Lecanoromycetes, lichens, taxonomy

INTRODUCTION

The genus *Cladonia* P. Browne represents one of the largest genera of lichen-forming fungi, with more than 400 described species (Ahti 2000). *Cladonia* species are often major contributors to overall biomass in the groundlayer vegetation in arctic and alpine tundra, in lichen woodlands, on rock outcrops, on heaths, and on peatlands (Lechowicz & Adams 1974). Several *Cladonia* species, commonly termed reindeer lichens, serve as a winter food source for animals. Other species are found in habitats where higher plants are not competitors, such as wood or burned habitats. These lichens usually develop two distinct kinds of

thallus morphology: a horizontal primary thallus (foliose or crustose, largely absent in reindeer lichens) and a vertical secondary thallus called a podetium (fruticose, bearing the hymenia). These thalli are among the most complex and aesthetic in lichens and, not surprisingly, there is a tremendous variation in morphological details, which provides many characters for classification. As frequently found with lichens, the interpretation of phenotypic variation of the thallus has been controversial (Stenroos & DePriest 1998; Stenroos et al. 2002; Divakar et al. 2006; Grube & Hawksworth 2007).

The traditional species circumscription of *Cladonia* is based on morphological and chemical characters. However, several recent molecular studies have revealed a lack of correlation between morphological and molecular data, and many traditionally delimited species are problematic or even artificial in light of these data (Myllys et al. 2003; Kotelko & Piercey-Normore 2010; Piercey-Normore et al. 2010; Pino-Bodas et al. 2012a, b). The incongruence between morphological and genetic data is usually attributed either to significant intraspecific variation of the species as a response to environmental conditions, or to genetic recombination (e.g., Fontaine et al. 2010; Kotelko & Piercey-Normore 2010).

Zeorin-containing red-fruited *Cladonia* species are conspicuous lichens, two of which were distinguished by Linnaeus (1753). Similar to most other *Cladonia* species, members of this aggregate usually grow in habitats with a low rate of competition from vascular plants (e.g., on sandy or rocky soils, on thin soil over rock, on bark, or on rotten wood). Currently, the aggregate of zeorin-containing red-fruited and scyphose (cup-forming) *Cladonia* species consists of five species worldwide, of which four are known from Europe and North America [*C. coccifera* (L.) Willd., *C. deformis* (L.) Hoffm., *C. diversa* Asperges ex S. Stenroos, and *C. pleurota* (Flörke) Schaer]. The fifth species, *C. sinensis* S. Stenroos & J. B. Chen (Stenroos et al. 1994), has a limited distribution in South-East Asia, and was not included in the analysis.

This group of species is characterized by similar chemical patterns (presence of usnic acid derivates and zeorin, occasionally accompanied by porphyrilic acid), and species within this group are delimited morphologically. The size, shape and location of the vegetative propagules on the podetia are traditionally considered as the most important diagnostic characters separating species (e.g., Asperges 1983; Stenroos 1989). The shape and width of the podetium is another relevant morphological feature commonly used to distinguish the species belonging to this group (e.g., Asperges 1983; Osyczka 2011; Ahti & Stenroos 2012).

Cladonia coccifera (Fig. 1A & B) is an esorediate species with gradually expanded cups. The surface of the podetium is areolate corticate, covered by bullate and scaly plates. This species had often been confused with *C. diversa*, *C. pleurota* or *C. borealis* (Stenroos 1989; Osyczka 2011). *Cladonia deformis* (Fig. 1C & D) is easy to recognize when well developed. Podetia are usually tall, relatively narrow and farinose sorediate. However, it might also be short-podetiate and then difficult to distinguish from *C. pleurota* (Osyczka 2011). *Cladonia pleurota* (Fig. 1G & H) is morphologically very variable (Stenroos 1989). Young individuals are usually completely granulose sorediate, but when fertile the surface may turn almost totally corticate and is partly covered by granules or verruculae (Stenroos 1989). *Cladonia diversa* (Fig. 1E & F), described by Asperges (1983), is the most controversial species. The podetia of

this species are usually slender and microsquamulose-granulose. Because of its obvious morphological similarity to *C. coccifera* (esorediate podetia covered by irregular plates and/or granules), the natural status of this species was disputed by Stenroos (1989). However, recently Ahti & Stenroos (2012) became "more convinced that it is an acceptable taxon".

Until now, no comprehensive attempt has been made to assess phenotypically circumscribed red-fruited *Cladonia* species within a molecular phylogenetic context. However, some species belonging to this aggregate were included in previous studies which focused on the generic phylogeny of *Cladonia* (Stenroos et al. 2002), a study of lichen diversity in some Antarctic regions (Lee et al. 2008), or a DNA-barcoding study of taxonomically diverse lichens in the UK (Kelly et al. 2011). Stenroos et al. (2002) examined four species belonging to this aggregate (from 1 to 4 specimens per species) and the possible polyphyly of *C. coccifera*.

Many phylogenetic surveys using sequence data inferred the evolution of *Cladonia* at higher taxonomical levels. Myllys et al. (2003) investigated the genetic diversity of two closely related putative species, *C. arbuscula* and *C. mitis*. The analysis involved four markers: ITS rDNA, a group I intron in SSU rDNA at position 1516 (according to Escherichia coli numbering), two introns in β-tubulin gene, and a single intron in the GAPDH gene. Surprisingly, a significant conflict between the four gene regions was detected. According to their conclusions, Myllys et al. (2003) regarded either incomplete lineage sorting or recombination as the most likely reason for the incongruences among the markers. Recently, Fontaine et al. (2010) studied the *C. gracilis* complex by using ITS rDNA and polyketide synthase (PKS) genes and obtained similar results, but they suggest paralogy in both genes as an alternative explanation of the incongruence identified between individual gene trees.

In the present study, inferences from ITS rDNA and an intron-containing portion of the β-tubulin gene were used to explore the genetic diversity of currently recognized zeorin-containing red-fruited *Cladonia* species. We examined numerous collections of all the four currently accepted zeorin-containing red-fruited *Cladonia* species known from the European continent and North America. In addition, we address possible explanations for the incongruence between individual gene trees detected in this study, similar to other multilocus studies of *Cladonia* (Myllys et al. 2003; Fontaine et al. 2010).

MATERIALS AND METHODS

Species sampling and determination

The material for this study was either collected by the authors or obtained from the following herbaria: BG, CBFS, GZU, NY, PL, PRA, PRC, and PRM. A total of 52 samples were collected, largely in Europe (44 specimens); eight collections were made in North America (Table 1). All the specimens were examined by the first author and revised by S. Stenroos and T. Ahti. Patterns in secondary metabolite variation were identified by thin-layer chromatography (TLC) on Merck silica gel 60 F254 pre-coated

glass plates in solvent systems A, B and C, according to Orange et al. (2001). *Cladonia crispata* and *C. squamosa* were used as an outgroup, based on the study of Stenroos et al. (2002).

DNA extraction, PCR, and DNA sequencing

Fine ground lichen material was used for total genomic DNA extraction with the CTAB protocol (Cubero et al. 1999) or the Invisorb Spin Plant Mini Kit (Invitek). The fungal nuclear ITS region and an intron-containing portion of the β-tubulin gene were amplified with the following primers: ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990), and Bt3-LM and Bt10-LM (Myllys et al. 2001). In most cases, PCR reactions were prepared for a 30 µl final volume containing 4.05 µl double-distilled water, 3 µl 10 × Taq polymerase reaction buffer (10 mM Tris; pH 8.3), 1.8 µl $MgCl_2$ (25 mM), 3 µl of 2.5 mM dNTPs, 0.15 µl Taq DNA polymerase, 1.5 ml of each of the 10 mM primers. Amplifications consisted of an initial 2 min denaturation at 95ºC followed by 30 cycles of 1 min at 95ºC, 1 min at 54ºC (ITS)/51ºC (β-tubulin), 1 min at 72ºC, and a final extension of 7 min at 72ºC.

The PCR products were quantified on a 1% agarose gel stained with ethidium bromide and cleaned either with QIAquick PCR Purification Kit (Qiagen) or JetQuick PCR Product Purification Kit (Genomed), according to the manufacturer's protocols.

Sequencing of PCR products was performed with an Applied Biosystems (New York, USA) automated sequencer (ABI 3730xl) at Macrogen Corp. in Seoul, Korea. The PCR primers were also used for sequencing.

Sequence alignment and model selection

Sequences were initially aligned using Clustal X 1.83 (Thompson et al. 1997) and MUSCLE (Edgar 2004). ITS sequences (comprising ITS1, 5.8S, and ITS2 regions) were aligned on the basis of their rRNA secondary structure information (see below) with Mega 4 (Kumar et al. 2008). For subsequent phylogenetic analyses, the alignments were minimalized to contain the unique sequences only. Alignments can be downloaded at http://botany.natur.cuni.cz/algo/align/03_ Cladonia_ITS.nex (ITS) http://botany.natur.cuni.cz/ algo/align/03_Cladonia_BT.nex (β-tubulin).

For both ITS and β-tubulin datasets, suitable partitioning strategy and partition-specific substitution models were selected in a multi-step process (Verbruggen et al. 2010). Initially, guide trees were obtained by carrying out a second-level maximum likelihood (ML) search on the unpartitioned dataset with an HKY + Γ_8 model with TreeFinder (Jobb et al. 2004) by using the Bayesian information criterion (BIC). Then, the datasets were divided by five (ITS) and six (β-tubulin), respectively, different partitioning strategies. For each partition present in these partitioning strategies, 12 different nucleotide substitution models were evaluated (F81, HKY, GTR, and their combinations with Γ, I, and Γ+I). Subsequently, Bayesian information criterion (BIC) calculations were performed for all potential partitioning strategies, assuming the guide tree and evaluated models for each partition. For both datasets, three partitioning strategies with the best fit to the data (lowest BIC scores) were retained for further analysis. In the next step, the best models of sequence evolution were selected for individual partitions by using the BIC. Finally, the partitioning strategies were re-evaluated using the selected models for particular partitions.

This BIC-based model selection procedure selected the following models. For the ITS rDNA dataset, the strategy with 3 partitions was selected: i) ITS1 region (HKY + Γ_8), ii) 5.8S rDNA (HKY), and iii) ITS2 region (HKY + Γ_8). In the case of the β-tubulin dataset, the strategy with two partitions was selected as the best: (i) first and second codon positions of exon (HKY), and (ii) third codon position of exon and intron region (HKY).

Molecular data and phylogenetic analyses

Possible substitution saturation of both markers studied that would imply a low reliability of phylograms (Lopez et al. 1999; Muschner et al. 2003) was assessed by two different approaches. Firstly, we plotted the uncorrected distances against the corrected distances, determined with the respective model of sequence evolution estimated by the BIC-based model selection as described above (HKY + Γ_8 for ITS rDNA and HKY for the β-tubulin dataset). Secondly, the phylogenetic signal present in the data partitions was estimated by ML mapping (Strimmer & von Haeseler 1997) using the Tree-puzzle 5.2 program (Schmidt et al. 2002).

The phylogenetic trees were inferred with Bayesian inference (BI), Maximum Likelihood (ML) and Maximum Parsimony (MP). A Bayesian analysis was implemented using MrBayes version 3.1 (Ronquist & Huelsenbeck 2003). Two parallel MCMC runs were carried out for 2 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value between simultaneous runs was 0.004683 in ITS and 0.002003 in the β-tubulin. ML and MP phylograms were obtained using Garli version 2.0, and PAUP version 4.0b10 (Swofford 2002), respectively. The same programs were used for bootstrap analyses. ML analyses consisted of rapid heuristic searches (100 pseudo-replicates) by using automatic termination (the genthreshfortopoterm command set to 100 000). The weighted parsimony (wMP) bootstrapping (1000 replications) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences (the number limited to 10 000 for each replicate), and gap characters treated as a fifth character state. The weight to the characters was assigned using the rescaled consistency index on a scale of 0 to 1000. New weights were based on the mean of the fit values for each character over all of the trees in memory.

The secondary structures of ITS sequences were constructed in order to detect presumed sexual barriers between studied species. The incompatibility for sexual reproduction between species can be ascertained by the presence of compensatory base changes (CBSs; so-called CBS approach) (Coleman 2000; Müller et al. 2007). The secondary structures of ITS sequences were constructed using the Mfold computer program version 2.3, (Walter et al. 1994; Zuker 2003), with the folding temperature set to 25ºC. The structures were compared with published ITS secondary structures of *Cladonia* species (Beiggi & Piercey-Normore 2007). Common secondary structures were created by using RnaViz (version 2; De Rijk et al. 2003) and used to identify compensatory base changes (CBCs) and hemi-CBCs.

Analyses of hybridization

Two different attempts were used to detect hybridization events in the diversification of *Cladonia* species. Firstly, incongruence between the ITS and β-tubulin derived trees was examined using NeighborNet analysis as implemented by the program Splits Tree 4 (Huson & Bryant 2006). This method provides a visualization of the extent to which a collection of gene trees suggests contradictory taxon relationships. If a collection of gene trees has congruent topologies, consensus networks will be tree-like, and where the relationships are incongruent, the graphs will be net-like (McBreen & Lockhart 2006). To explain the incongruent relationships displayed by a network analysis in terms of reticulation events, a consensus network was constructed.

Secondly, the evidence of hybridization was evaluated by the bootscanning method (Salminen et al. 1995) on the concatenated sequence dataset. We used two different programs to run bootscanning analyses: 1) the alignment was analyzed by SimPlot version 3.5.1 (Lole et al. 1999), using the bootscan option and default settings; 2) several different algorithms (RDP, GENECONV, Chimaera, MaxChi, BootScan, SiScan and 3Seq) were run to determine the presence of recombination using the Recombination Detection Program, Rdp3, v. 3.22 (Martin et al. 2010). In the case of positive recombination detection, bootstrap support curves were visualized to locate hybrid sequences, and to reveal potential parent sequences present in the alignment.

RESULTS

Secondary chemistry

All samples studied contained zeorin and usnic acid as major lichen substances. We detected four chemotypes which differ by the presence/absence of accessory substances isousnic and porphyrilic acids. Isousnic acid (chemotype 1) was present in 19 specimens, porphyrilic acid in 12 specimens (chemotype 2), both were present (chemotype 3) in 10 specimens and neither of the two mentioned (chemotype 4) were found in 11 specimens (Table 2).

Analysis of the molecular data

Amplification products of the ITS1, 5.8S, and ITS2 regions of the ribosomal rRNA gene were ca. 600 bp long, while those of the two exon and one intron regions of the β-tubulin genes were ca. 700 bp in length. Although the number of nucleotides analyzed differed accordingly (ITS: 546 bp, β-tubulin: 674 bp), the datasets were comparable in the amount of phylogenetic signal. Although the ITS dataset contained more variable sites (ITS: 44 sites; β-tubulin: 31 sites), the number of parsimony-informative characters was the same for both loci (31). No ambiguous positions that could bias the inference of phylogeny were detected.

Testing the data partitions for substitution saturation (distribution of the uncorrected vs corrected distances) revealed a practically linear correlation, indicating no saturation in both ITS and β-tubulin data

(see Appendix 1). Similarly, the results of likelihood mapping demonstrated a strong phylogenetic signal detected in both ITS and β-tubulin loci (89.2% and 94.1% of the fully resolved quartets, respectively).

Phylogenetic analyses

The Bayesian, MP and ML analyses yielded trees with similar topology. Figure 2 shows the β-tubulin phylogram obtained from the Bayesian analysis. It revealed three well-supported (#1, #2, and #4) and one moderately supported (#3) lineages. Lineage #1 comprised 24 identical sequences belonging to *C. deformis* and *C. pleurota*. *Cladonia pleurota* strains also formed lineage #2. In contrast, lineage #4 contained sequences belonging to both *C. coccifera* and *C. diversa*, and lineage #3 comprised three *C. coccifera* strains.

Compared to the β-tubulin gene tree, ITS phylogenetic analysis inferred a clearly different topology. Three of the four well-resolved lineages of β-tubulin phylogeny were not resolved, but separated into different and distantly related clades (Fig. 2). Lineage #1 was separated into four lineages (#1a, #1b, #1c, and #1d). Whereas lineages #1a, #1c, and #1d contained both *C. pleurota* and *C. deformis* specimens, lineage #1b comprised only the *C. pleurota* strains. Lineage #4 from the β-tubulin gene tree formed lineages #4a and #4b in the ITS phylogram. Although receiving low support, they were obviously unrelated. Clade #4a was composed of all the analyzed *C. diversa* strains, whereas lineage #4b contained sequences belonging to *C. coccifera*. Finally, lineage #3 was split into two lineages: unsupported lineage #3a and lineage #3b containing only one sequence. Lineage #2 was the only one that was inferred with high statistical support by both ITS and β-tubulin phylogenetic analyses.

Since ITS and β-tubulin phylogenies were obviously not congruent, concatenated analysis was not performed.

Hybridization tests

A visual comparison of ITS and β-tubulin phylograms indicated a discrepancy in relationships among some taxa in both markers. For example, *C. diversa* formed a highly supported monophyletic clade together with some *C. coccifera* strains in the β-tubulin tree (lineage #4), but it created a separated lineage #4a in the ITS phylogram (which was, however, not statistically supported). A consensus network constructed from the trees obtained from the Bayesian analysis of the β-tubulin gene and ITS suggested contradictory taxon relationships. This network (Fig. 3) explains the conflict between source-tree topologies as a consequence of the hybridization event. Based on the investigation of concatenated datasets, the Phi test did find statistically significant evidence for recombination ($P = 1.1 \times 10^{-7}$).

The presence of the recombination event was also examined by two tests. Rdp3 analysis detected two hybridization events within both ITS and β-tubulin loci, which led to two hybrid lineages: #4a (all *C. diversa* strains) and #4b (some *C. coccifera* strains) (Fig. 3). The recombination was detected by three different tests implemented in Rdp3: MaxChi ($P = 0.0084$), Chimaera ($P = 0.0030$) and 3Seq ($P = 0.0006$). These hybrid lineages formed highly supported monophyletic clade #4 in the β-tubulin tree. Based on the bootscanning analysis conducted by the SimPlot program, we discovered the probable ancestral lineages

of both hybrids. In fact, only one parent lineage was unequivocally inferred for both hybrids. In the case of *C. diversa* (#4a), lineage #1a was identified as ancestral, whereas in the second case (#4b), the ancestral lineage was found to be lineage #1c. The second ancestral lineage in both cases of hybridization was unknown; however, SimPlot identified a hybrid as the ancestor of the other one, and vice versa. From this, we concluded that both of the hybrid lineages had a common, as yet unknown, parent lineage.

ITS1 and ITS2 secondary structure

A common organization of ITS1 and ITS2 secondary structures was found in all *Cladonia* strains. The ITS1 secondary structure comprised two main paired regions (helices I and II), with two additional lateral helices (IIa and IIb) on helix II. Helix I was more divergent than helix II. The ITS2 structure was more conserved than that of ITS1. It contained three paired regions (helices I, II, and III). Helix I was identical in all the specimens examined, whereas helices II and III showed a small degree of divergence. The ITS secondary structures were compared with the lineages inferred in the ITS phylogram (Fig. 4) to check the occurrence of compensatory base changes (CBCs, nucleotide changes at both sides of the paired bases) and hemi-CBCs (change on only one side of the nucleotide pair, but still preserving pairing), according to Coleman (2000, 2003). We revealed no CBC and 161 hemi-CBCs in all the lineages. The number of hemiCBCs varied from zero (#3b) to six (#2) between the different lineages. Altogether, 15 hemi-CBC sites were identified in both the ITS regions. ITS1 contained 11 hemi-CBCs, of which seven were located in helix I. Four hemi-CBCs identified in ITS2 were situated in helices II and III. The highest number of hemi-CBCs (seven) was determined between lineages #1d–1a, #1d–4a, #1c–1a, #1c–2, and #1c–4a. In contrast, no hemi-CBC was identified between lineages #1a–4a.

DISCUSSION

Phenotypic and genetic variability of red-fruited zeorin-containing *Cladonia* species

The four species considered in this paper are chemically very similar but differ morphologically. The most obvious differences characterizing these species are the size and shape of the podetium, the character of the podetium surface, and the character and size of the vegetative propagules. However, three of these species were shown to be polyphyletic. Only *Cladonia diversa* formed a monophyletic group in the ITS phylogeny, although it was not supported statistically. Traditionally, this species was regarded as a member of the *C. coccifera* group. Stenroos (1989) doubted the status of *C. diversa* and found that its total variation is still obscure. Recently, Ahti & Stenroos (2012) accepted the species as a valid taxon. All the specimens studied have slender, narrow scyphi and their surfaces are covered by microsquamules, irregular plates and granules (Fig. 2). Furthermore, we can also confirm its preference for sandy substrata (out of six specimens, four were collected from sandy dunes, one from a sandstone), as previously reported by Asperges (1985), Christensen & Johnsen (2001), Hasse (2005), and Osyczka (2009). Also, chemical patterns are consistent with other sources (e.g. Osyczka 2011; Ahti & Stenroos 2012). James (2009)

proposed porphyrilic acid to be a stabile compound of this species, but according to our results this cannot be confirmed (porphyrilic acid was detected in only one specimen of *C. diversa*).

The specimens morphologically identified as *C. coccifera* were distributed in three lineages (#3a, #3b, #4b). Two of these (#3a and #3b) were phylogenetically distant from lineage #4b. Ten specimens representing lineage #4b showed wide morphological variability (Fig. 2), particularly in the features of the vegetative propagules and the shape of podetia. The surface of podetia in some specimens was largely covered with scaly and bullate plates, whereas microsquamules dominated on podetia of other specimens studied. The shape also varied from narrow to very broad cups. In contrast, lineages #3a and #3b contained specimens that were morphologically more uniform and consistent with the traditional delimitation (however, only three *C. coccifera* specimens were inferred in these lineages). According to our results, clade #4b differed chemically from clades #3a and #3b. Whereas specimens involved in the lineage #4b are characterized by the presence of porphyrilic acid and the absence of isousnic acid, the three specimens from the other two lineages lacked porphyrilic acid. Interestingly, whereas these specimens were collected in Norway and Missouri, the specimens from the lineage #4b were sampled from Central Europe and Spain. This suggests a possible geographical pattern in the distribution (and also chemical variation) of these two lineages, which should be studied more carefully with wider sampling.

Although *C. deformis* is regarded as a distinct species, the specimens were found in three lineages: #1a, #1c, as well as #1d together with *C. pleurota*. These two species are traditionally distinguished by the size and shape of the podetium, and by the size of the soredia. *Cladonia deformis* usually forms elongated farinose-sorediate podetia, whereas *C. pleurota* is characterized by shorter and more coarsely sorediate podetia. The four studied specimens of *C. deformis* did not show any uniform chemical pattern. In one case (specimen Cl102 from the Czech Republic) we detected porphyrilic acid, which has not been reported for this species previously.

On the basis of the findings of Stenroos et al. (2002), *C. pleurota* appeared to be a monophyletic species. However, our results with a more detailed sampling of the species disprove the monophyly of this taxon. Specimens of *C. pleurota* as currently understood are spread across five lineages (#1a, #1b, #1c, #1d, and #2). Although the specimens were studied thoroughly, we did not detect any morphological criteria that would properly characterize any of these lineages (Fig. 2). The podetial surface of all specimens used for the analysis was covered with granulose soredia, sometimes accompanied by farinose soredia in varying amounts. The shape of podetia exhibited considerable variation (from very narrow to short and extremely broad cups) that, however, does not correspond with the genetic diversity of the material studied. Discordance between the high morphological variation and sequence data has recently been discussed by Pérez-Ortega et al. (2012), with respect to vagrant forms in *Cetraria aculeata*. However, the induction of growth variation as suggested for vagrant vegetative offspring cannot apply in *Cladonia*. It seems that, on the contrary, there are at least some chemical patterns or tendencies which may help us to define some clades. Lineage #2 comprises nine samples with identical chemical characteristics (chemotype 1: presence of isousnic acid and absence of porphyrilic acid). Porphyrilic acid appears to be a constant substance in

samples of clade #1c, but with only six specimens studied we refrain from drawing taxonomic conclusions.

Discordance among gene-tree genealogies

In our study, we analyzed two commonly used and well-established molecular markers for Ascomycota: ITS rDNA and an intron-containing portion of the β-tubulin gene. The strong conflict between the ITS and β-tubulin topologies seems to be a recurrent phenomenon already found by other authors investigating *Cladonia* phylogenies (Myllys et al. 2003). Moreover, it could in fact represent a more widespread phenomenon than generally anticipated, as it has also been found in other lichen groups (e.g., Ertz et al. 2009). Phylogenetic incongruences among genes can occur for many reasons, including the presence of pseudogenes, gene paralogy, horizontal gene transfer, incomplete lineage sorting (ILS), and hybridization. Alternatively, the presence of hyphae from more species growing together in the same podetium could be another possible explanation for the conflict (Kotelko & Piercey-Normore 2010). We can clearly rule this out in our dataset, as we have unambiguous signals with the ITS primers.

Pseudogenes are dysfunctional relatives of known genes that have lost their protein-coding ability or are otherwise no longer expressed in the cell (Vanin 1985). Their base compositions are different from those of functional genes, and they evolve very rapidly (Buckler et al. 1997). Pseudogenous clones are characterized by occasional deletions in genes and spacers, by increased non-synonymous mutations in the otherwise almost identical rRNA-coding regions (Grimm & Denk 2008; Harpke & Peterson 2008), or by low predicted secondary structure stability in ribosomal genes or spacers (Buckler et al. 1997). However, the almost identical 5.8S rRNA sequences (only one substitution was detected), absence of long-branching artefacts in the phylograms, and the absence of deletions suggest the non-pseudogenous nature of any of the ITS sequences analyzed. In addition, conserved sequences of 3˙ and 5˙ ends also suggest that they are not pseudogenes.

Horizontal gene transfer (HGT) is the transfer of genes across species. This mechanism is well known mainly in bacteria, but it also occurs in the evolution of Eukaryota (Keeling & Palmer 2008; Khaldi et al. 2008; Marcet-Houben & Gabaldon 2010). However, an HGT event is an unlikely source for the conflict between tree topologies in our dataset because it would imply a recent HGT event between two eukarya lineages, which is rare (Won & Renner 2003) and generally involves the transfer of introns (Rot et al. 2006). Gene paralogy occurs if a gene in an organism is duplicated to occupy two different positions in the same genome and can also be responsible for the conflict between two phylogenies. Although paralogs of the β-tubulin gene are known from different groups of fungi (e.g., Begerow et al. 2004; Corradi et al. 2004; Msiska & Morton 2009), they have not been reported from lichenized Ascomycota. Moreover, in our case, intragenomic variation should be found in β-tubulin sequences to explain the incongruence in our dataset by potential gene paralogy. However, we did not find an ambiguous signal in the β-tubulin sequences.

Incomplete lineage sorting, also called deep coalescence, is a phenomenon that can cause conflicting gene and species trees. ILS represents the incomplete random sorting of alleles at many loci independently due to short intervals between divergence events (Blanco-Pastor et al. 2012). ILS has been reported in many different groups of organisms, more likely in those species which have a large population size and a short time between divergences (e.g., Morando et al. 2004; Jakob & Blattner 2006; Pollard et al. 2006). This process is difficult to distinguish from interspecific hybridization and both may even occur simultaneously (Seehausen 2004; Meng & Kubatko 2009). Although several methods distinguishing these two evolutionary processes have been recently proposed (e.g., Holland et al. 2008; Bloomquist & Suchard 2010), many independent loci are needed for their implementation and it is difficult to uncover multiple reticulation events (Blanco-Pastor et al. 2012).

Since we are not able to distinguish ILS and hybridization, we assume both could be responsible for the incongruence in our dataset. Here we propose the phylogenetic consequences of these two scenarios.

The presence of ILS would indicate that zeorin-containing *Cladonia* species probably diverged relatively recently (Leache & Fujita 2010). The ITS and β-tubulin phylogenies would represent gene trees, which would not correspond with the species tree. To be able to describe the phylogenetic relationships within this group, even in the presence of incomplete lineage sorting, it will be necessary to study more loci (e.g., Knowles & Carstens 2007), which will definitely be within reach with the ongoing *Cladonia* genome project hosted at Duke University.

Interspecific hybridization is regarded as one of the major factors responsible for conflicts among different loci (e.g., Taylor et al. 2000; Fehrer et al. 2007; Ertz et al. 2009). Similar incongruences have been detected in other lineages of *Cladonia* (Myllys et al. 2003; Fontaine et al. 2010; Kotelko & Piercey-Normore 2010). We assume hybridization should be considered as an important mechanism, possibly influencing the evolution of the lichen genus *Cladonia*, resulting in reticulate evolution that may contribute to the species diversification. Although hybridization is not yet known in lichens, it has been proved to occur in most fungal phyla (e.g., Brasier et al. 1998, 1999; Xu et al. 2000; Craven et al. 2001a, b).

If the effect of hybridization is considered, only one parent lineage could be identified in both hybridization events. The second ancestral lineage is unknown, which could have two alternative explanations: 1) the parent lineage is extinct and could therefore not be detected; 2) we did not sample and analyze the parent lineage. To better understand the species concept and delimitation in the group of zeorin-containing red-fruited *Cladonia* lichens, it will be important to address the question of frequency of hybridization events more carefully in the future. The suggested hybridization could represent either an exceptional ancient event or a common ongoing process. Sexual compatibility/incompatibility between two organisms can be detected by a comparison of the secondary structure of the ITS (so-called CBS approach). The presence of compensatory base changes (CBSs) indicates incompatibility for sexual reproduction between species (Coleman 2003; Müller et al. 2007). In our case, the absence of CBCs

reveals that there are presumably no reproduction barriers among the species studied, and hence, we can conclude that the second alternative is more feasible.

Species circumscriptions in *Cladonia*

Similarly to other recent studies focusing on *Cladonia* (Fontaine et al. 2010; Kotelko & Piercey-Normore 2010; Pino-Bodas et al. 2010, 2012a), our investigations clearly revealed the incongruence between the phylogenetically inferred lineages and traditional, morphologically and/or chemically delimited species. In fact, we were not able to find any phenotypic feature to unambiguously define the lineages in most cases (except *Cladonia diversa*, and chemical patterns in the lineages #2 and #4b). In general, there are two alternatives for interpreting this incongruence. The phylogenetic units could be either regarded as populations of a morphologically variable species or accepted as 'cryptic' or incipient species.

The question of how to treat the 'cryptic' species within the traditionally defined nominal species has been discussed by many authors, advocating two different attitudes. Some authors (e.g., Kotelko & Piercey-Normore 2010) have suggested a more conservative attitude in maintaining the traditional delimitation of the species, even when they are not supported by molecular data. They have argued for the possible implications of these species for ecophysiological studies, and more generally, for the detection and preservation of rare or unusual species. Conversely, other authors (Grube & Kroken 2000) have proposed applying the phylogenetic species concept and thus defining the well-supported phylogenetic lineages as cryptic species. They mentioned that morphological characterization of the species is often facilitated after finding cryptic lineages with molecular data, as it may then become apparent which characters are significant. They also claimed that the knowledge of cryptic species is useful in investigations at fine scales of taxonomic resolution, such as for interpretations of ecophysiological differences and microhabitat preferences (Grube & Kroken 2000).

Considering the findings in this study, we adopt the second opinion, understanding the well-supported phylogenetic lineages as separate species, but without describing them formally for the time being. One lineage indeed consisted of morphologically wellcharacterized specimens of *C. diversa*, traditionally recognized as a species. Moreover, *C. coccifera* samples belonging to clade #4b shared identical chemical characteristics (chemotype 2; presence of porphyrilic acid and absence of isousnic acid) and appeared to differ chemically from the other *C. coccifera* strains (lineages #3a and #3b). It is therefore very likely that the other lineages, even if morphologically indistinguishable, also represent separate species. Zeorin-containing red-fruited *Cladonia* species have wide morphological and ecological amplitudes, and thus, the correlation between phylogenetically separated lineages and different phenotypic characters should be studied more comprehensively in the future.

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TABLES

Table 1. List of lichen taxa used in this study with collection information and GenBank accession numbers. Asterisk (*) indicates private herbarium.

Taxon name	DNA extraction No.	Collection No. (herbarium)	Locality	GenBank No.	
				ITS	β-tubulin
Cladonia deformis	C8	Peksa 918, PL	Czech Republic, Chvaletice	HE611205	HE611257
	CL102	Steinová 110, PRC	Czech Republic, Brdy, Žďár	HE611184	HE611236
	CL175	Steinová 330, PRC	Finland, Suomossalmi	HE611190	HE611242
	CL176	Steinová 336, PRC	Finland, Varkaus	HE611186	HE611238
C. diversa	CL54	Bouda 777, *	Czech Republic, NP Českosaské Švýcarsko, Babylon	HE611164	HE611216
	CL106	Steinová 400, PRC	Portugal, Beira Alta, Parque Natural de Serra da Estrela	HE611165	HE611217
	CL130	Vondrák 6242, CBFS	Denmark, Bornholm, Jomfrugården	HE611166	HE611218
	CL172	Steinová 351, PRC	Belgium, Kalmthout, Van Ganzenven	HE611167	HE611219
	CL173	Steinová 352, PRC	Belgium, Kalmthout, Van Ganzenven - topotype	HE611168	HE611220
	CL174	Steinová 353, PRC	Netherlands, Grenspak De Zoom-Kalmthoutse Heide	HE611169	HE611221
C. coccifera	CL3	Peksa 84, PL	Czech Republic, Lužické hory, Studenec	HE611154	HE611206
	CL31	Hafellner 66608, GZU	Austria, Stubalpe, Größenberg	HE611155	HE611207
	CL32	Hafellner 66785, GZU	Austria, Stubalpe, Ofnerkogel	HE611156	HE611208
	CL39	Hafellner 66214, GZU	Austria, Stubalpe, Lichtengraben	HE611157	HE611209
	CL52	Bouda 778, *	Czech Republic, Novohradské hory, Kraví hora	HE611158	HE611210
	CL60	Peksa 359, PL	Czech Republic, Lužické hory, Studenec	HE611159	HE611211
	CL90	Steinová 43, PRC	Czech Republic, Krkonoše, Velká kotelní jáma	HE611160	HE611212
	CL93	Steinová 81, PRC	Czech Republic, Českosaské Švýcarsko, Křepelčí důl	HE611161	HE611213
	CL105	Steinová 401, PRC	Spain, Somosierra, arroyo de la Peña del Chorro	HE611162	HE611214
	CL141	Steinová 242, PRC	Austria, NP Nockberge, Erlacher Bockhütte	HE611163	HE611215
	CL120	Beeching 3100, NY	USA, Missouri, Iron Co., Pilot Knob National Wildlife Refuge	HE611170	HE611222
	CL178	Steinová 332, PRC	Norway, NP Rondane, Einsethøe	HE611171	HE611223
	CL179	Steinová 334, PRC	Finland, Heinola, Pirttijärvi lake	HE611172	HE61122
C. pleurota	B18	Peksa 820, PL	Slovakia, Veľká Fatra, Harmanec	HE611191	HE611243
	C6	Peksa 588, PL	Czech Republic, Chvaletice	HE611181	HE611233
	CL26	Palice 11305, PRA	Czech Republic, Dolní Loučky, Pásník	HE611193	HE611245
	CL36	Hafellner 65635, GZU	Austria, Stubalpe, Lahnhofen	HE611194	HE611246
	CL43	Peksa 562, PL	Czech Republic, Brdy, Hřebenec	HE611182	HE611234
	CL44	Peksa 564, PL	Czech Republic, Brdy, Hřebenec	HE611183	HE611235
	CL45	Peksa 563, PL	Czech Republic, Brdy, Hřebenec	HE611195	HE611247
	CL64	Vondrák 3631, CBFS	Romania, Retezat Mountains, Cheile Butii	HE611187	HE611239
	CL67	Vondrák 2868, CBFS	Czech Republic, Křivoklátksko, Na Andělu	HE611173	HE611225
	CL73	Peksa 574, PL	Czech Republic, Chvaletice	HE61117	HE611226
	CL77	Steinová 22, PRC	Austria, Zirbitzkogel, Linderhütte	HE611192	HE611244
	CL81	Lendemer 7139, NY	USA, New Jersey, Burlington Co., Rutgerds Pinelands Field Station	HE611175	HE611227
	CL84	Steinová 84, PRC	Czech Republic, NP Českosaské Švýcarsko, Křepelčí důl	HE611201	HE611253
	CL85	Steinová 103, PRC	Czech Republic, Brdy, Žďár	HE611196	HE611248
	CL98	Steinová 45, PRC	Czech Republic, Krkonoše, Kotel	HE611188	HE61124
	CL99	Steinová 99, PRC	Czech Republic, Brdy, Žďár	HE611202	HE611254
	CL100	Steinová 65, PRC	Czech Republic, Slavkovský Les, Křížky	HE611176	HE611228
	CL101	Steinová 108, PRC	Czech Republic, Brdy, Žďár	HE611203	HE611255
	CL104	Steinová 126, PRC	Czech Republic, Brdy, Hřebenec	HE611185	HE611237
	CL107	Harris 51548, NY	USA, Connecticut, Fairfield Co., Redding, Highstead Arboretum	HE611177	HE611229
	CL109	Lendemer 6720, NY	USA, Missouri, Iron Co. Pilot Knob National Wildlife Refuge	HE611178	HE611230
	CL111	Harris 52433, NY	USA, Missouri, Iron Co. Pilot Knob National Wildlife Refuge	HE611179	HE611231
	CL113	Lendemer 10223, NY	Canada, Island of Newfoundland, Big Otter Pond	HE611197	HE611249
	CL115	Lendemer 10384, NY	Canada, Island of Newfoundland, Burry Heights Center	HE611198	HE611250
	CL117	Lendemer 10563, NY	Canada, Island of Newfoundland, Ha-Ha Mountain	HE611199	HE611251
	CL128	Steinová 164, PRC	Czech Republic, Sedlčansko, Drbákov-Albertovy skály	HE611180	HE611232
	CL136	Steinová 215, PRC	Finland, Helsinki, Rastila	HE611200	HE611252
	CL148	Steinová 241, PRC	Austria, Gurktaler Alpen, Nassbodensee	HE611189	HE611241
	CL150	Steinová 187, PRC	Finland, Vantaa, Fagersta	HE611204	HE611256

Table 2. Number of *Cladonia* specimens studied of each chemotype. ISO = isousnic acid; POR = porphyrilic acid; USN = usnic acid; ZEO = zeorin.

	chemotype 1 ZEO, USN, ISO	chemotype 2 ZEO, USN, POR	chemotype 3 ZEO, USN, ISO, POR	chemotype 4 ZEO, USN
Cladonia coccifera	1	10	0	2
C. deformis	1	1	0	1
C. diversa	0	1	0	5
C. pleurota	17	0	10	3
Total	19	12	10	11

FIGURES

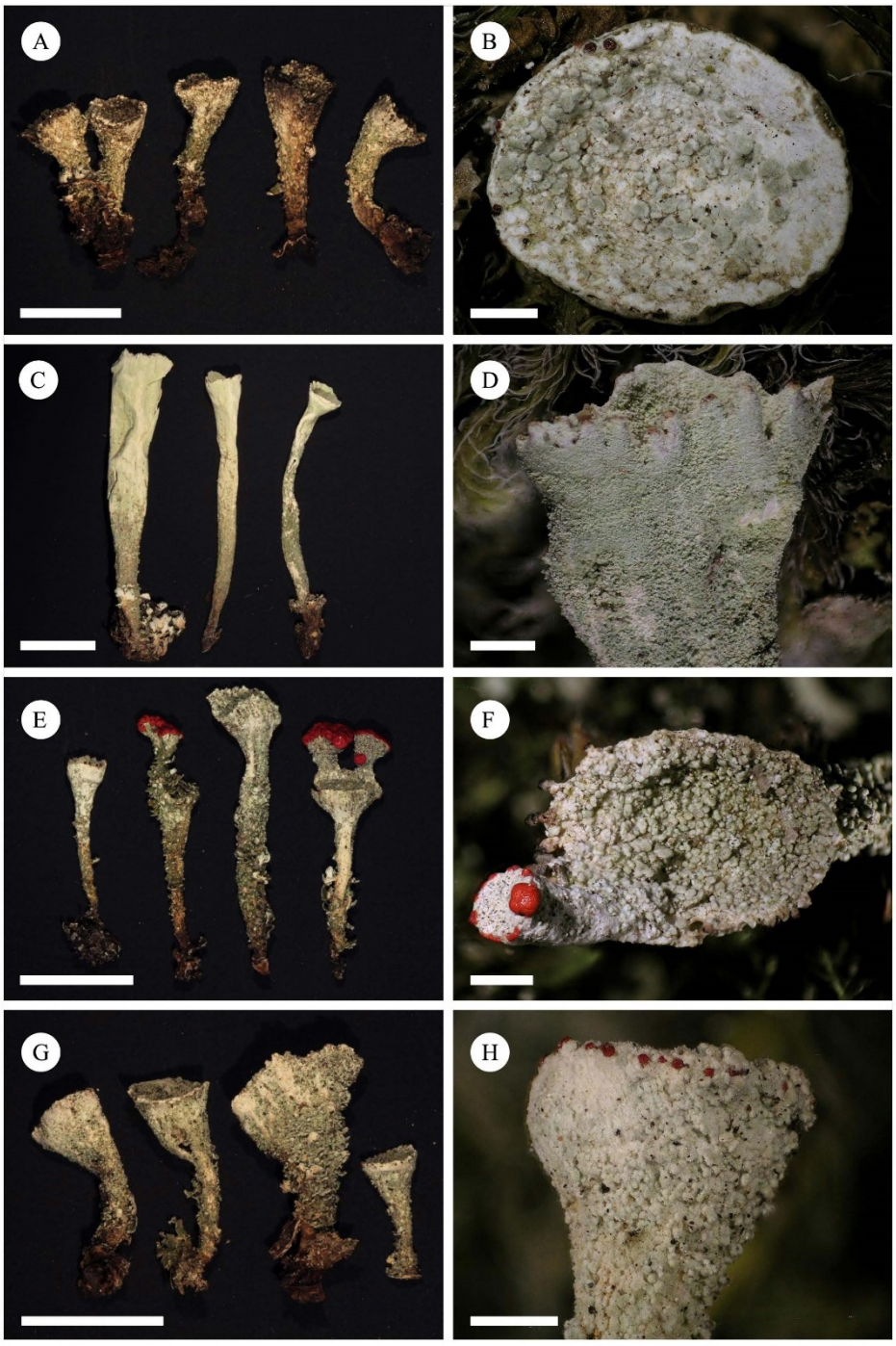


Fig. 1. Morphology of *Cladonia* species studied. A & B, *Cladonia coccifera* (CL179); C & D, *C. deformis* (CL176); E & F, *C. diversa* (CL173, topotype); G & H, *C. pleurota* (CL136). Scales: A, C, E & G = 5 mm; B, D, F & H = 1 mm.

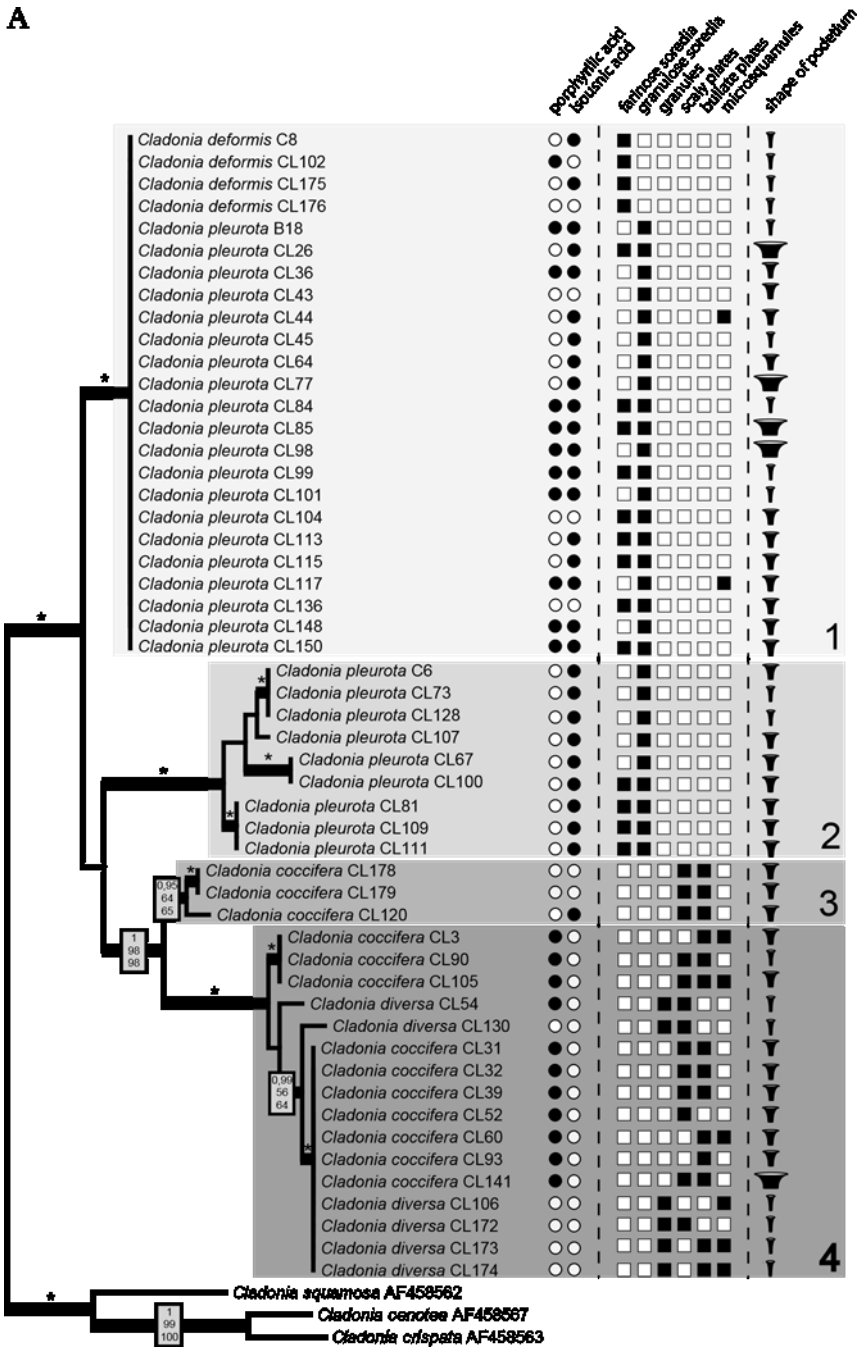


Fig. 2. Comparison of Bayesian topologies based on the β-tubulin (A) and ITS rDNA (B) datasets, together with observed chemical and morphological characters. For the analyses, an HKY + Γ_8 model for ITS1 and ITS2 regions, and HKY model for 5.8S rDNA, three codon positions and exon of β-tubulin gene was used. Values at the nodes indicate statistical support estimated by three methods: MrBayes posterior node probability (top), maximum likelihood bootstrap (in the middle) and maximum parsimony bootstrap (bottom). Thick branches represent nodes receiving PP support ≥ 0·90; asterisks (*) indicate statistical support 1/100/100. Only values receiving PP support ≥ 0·90 are shown. Species affiliation to four β-tubulin clades (including the corresponding sub-clades on the ITS rDNA tree) is indicated. Scale bar: estimated number of substitutions per site., displays narrow, slender podetia; , moderately broad cups; , extremely broad podetia.

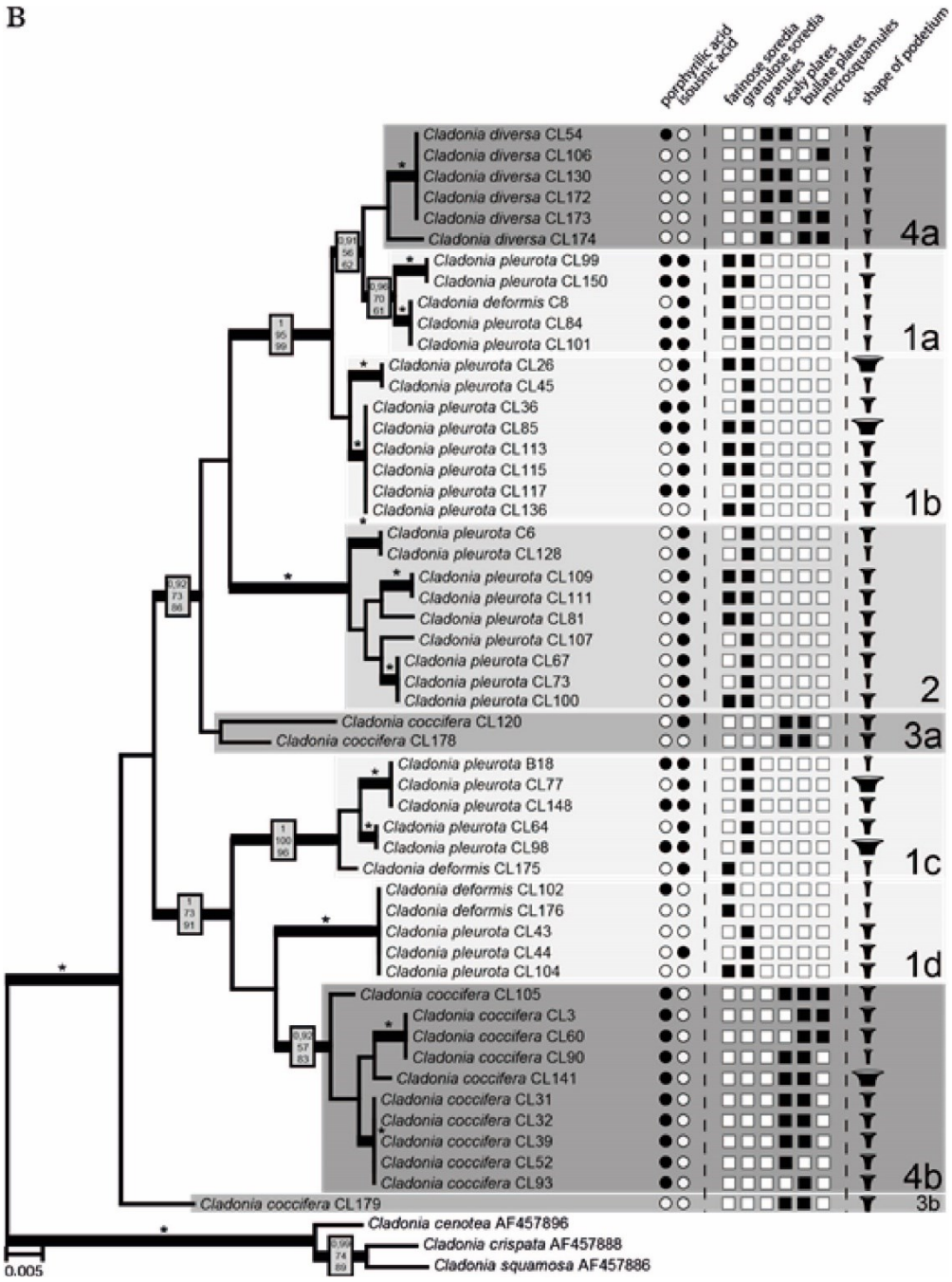


Fig. 2. Continued

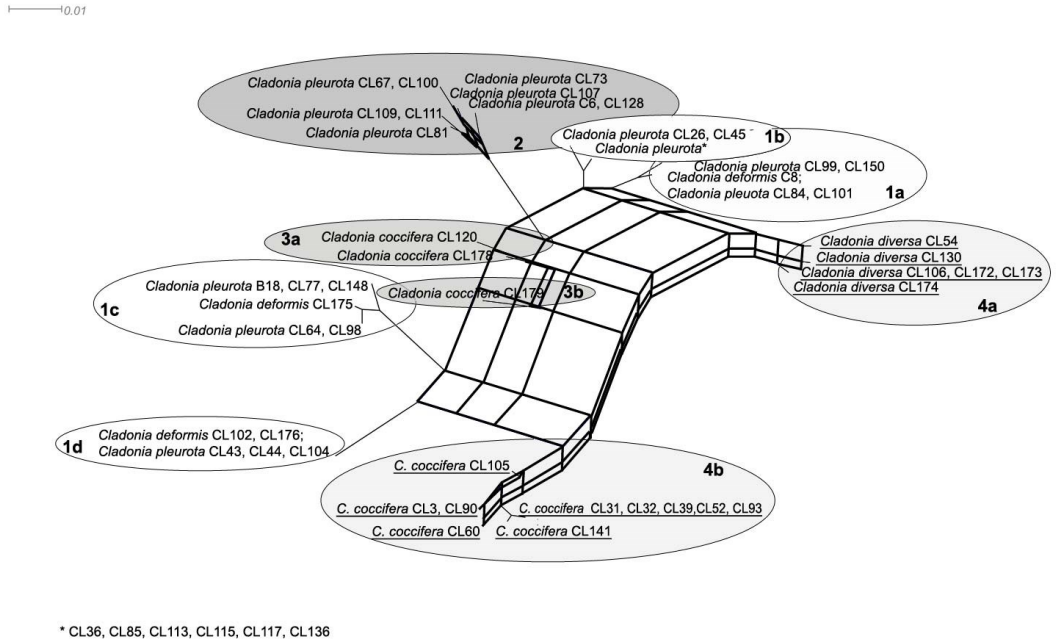


Fig. 3. Consensus network inferred from Bayesian trees from ITS and β-tubulin data. Thick lines represent hybridization events. Taxa involved in hybridization events are underlined. Scale bar shows the estimated number of substitutions per site.

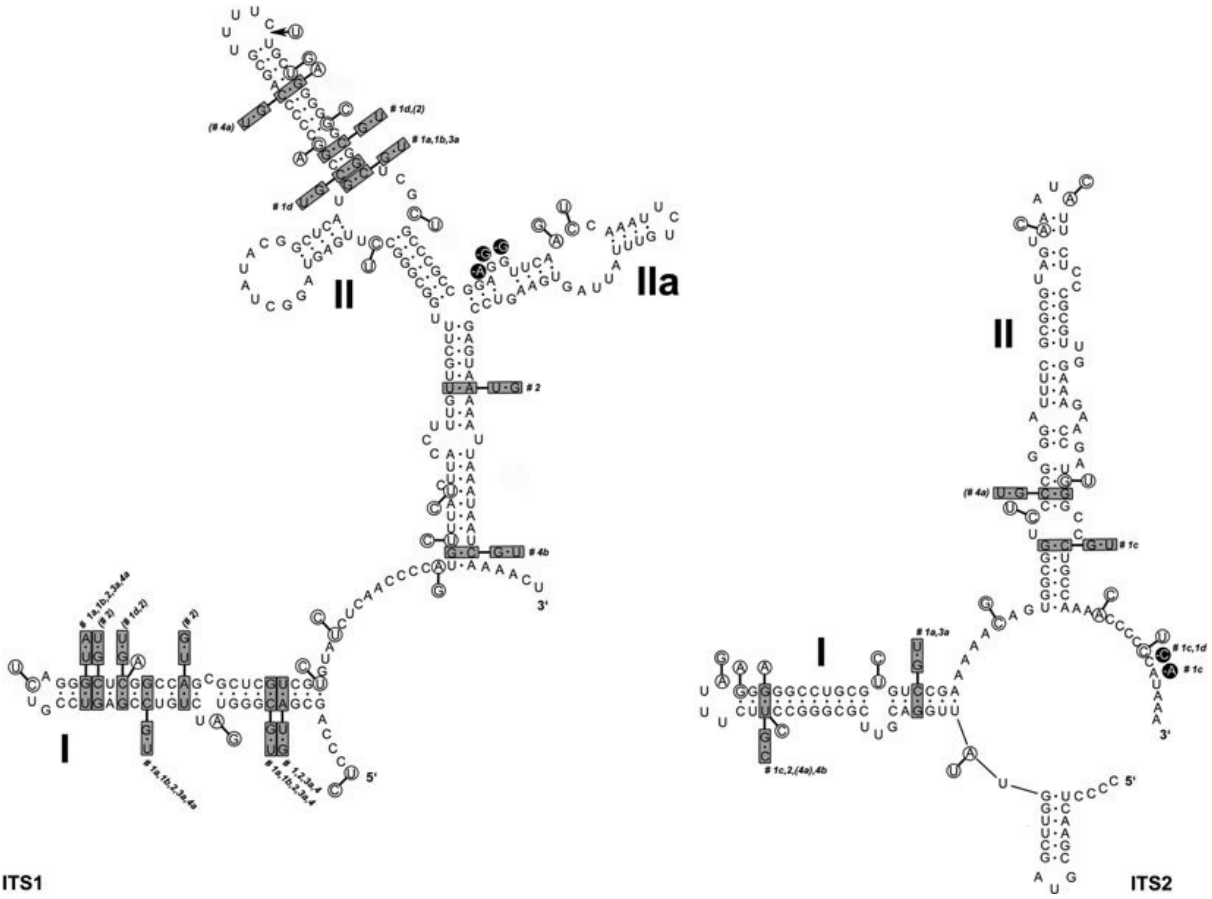
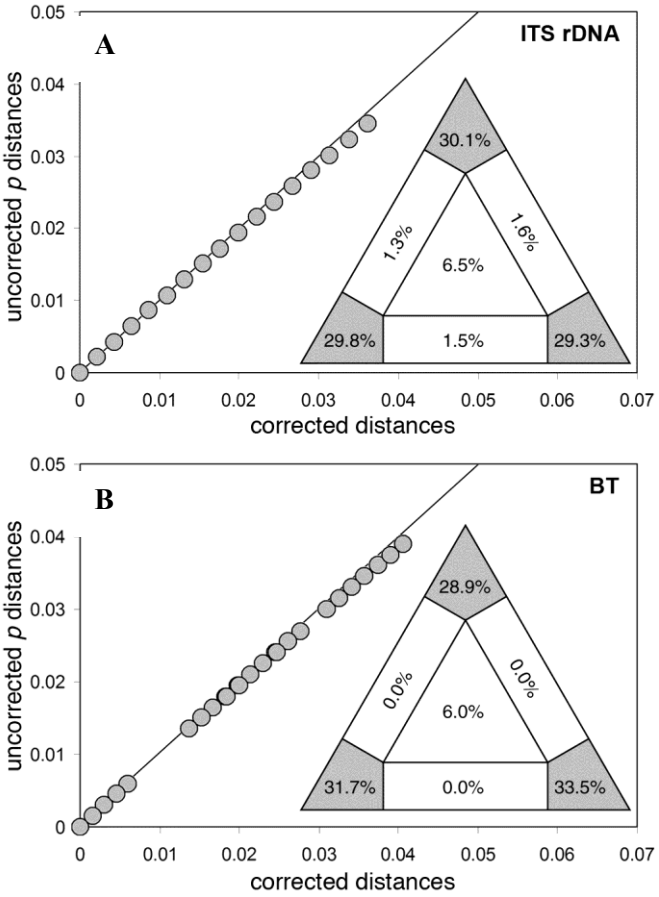


Fig. 4. Secondary structure models of ITS1 and ITS2 transcripts of *Cladonia coccifera* (DNA extraction number CL93) derived from comparison of 9 zeorin-containing red-fruited *Cladonia* lineages. Base changes between the different *Cladonia* genotypes are indicated: the base pairs marked in grey boxes indicate hemi-CBCs; single base changes are marked in circles. The numbers next to the boxes (#1a–4b) specify the *Cladonia* clades in which the base changes occurred (see Figs 2 & 3).

APPENDIX



Appendix 1. Analysis of substitution saturation. The graphs visualize the saturation of the ITS rDNA and β-tubulin datasets by plotting ML-corrected distances against uncorrected p-distances. Corrected distances are calculated using models estimated by PAUP/Modeltest for each specific data partition. A, analysis of ITS rDNA sequences; B, analysis of β-tubulin sequences. The triangles in the lower right of the graphs illustrate likelihood mapping results. The values in the panels indicate proportion of fully resolved (corners), partially resolved (along the sides), and fully unresolved quartets (in the centre).

5.3 Paper 3

Škaloud P., **Steinová J.**, Řídká T., Vančurová L. and Peksa O. (2015):

ASSEMBLING THE CHALLENGING PUZZLE OF ALGAL BIODIVERSITY: SPECIES DELIMITATION WITHIN THE GENUS *ASTEROCHLORIS* (TREBOUXIOPHYCEAE, CHLOROPHYTA)

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Assembling the challenging puzzle of algal biodiversity: species delimitation within the genus *Asterochloris* (Trebouxiophyceae, Chlorophyta)

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ABSTRACT

The genus *Asterochloris* represents one of the most common, widespread, and diverse taxa of lichen photobionts. In this report, we describe and characterize six new species (*A. echinata*, *A. friedlii*, *A. gaertneri*, *A. leprarii*, *A. lobophora*, and *A. woessiae*) that were identified during our recent investigation of photobiont diversity. We found that the species differed genetically, morphologically, ecologically, and with respect to their mycobiont partners. Statistical analyses revealed significant morphological differentiation of all six newly described species, as well as their separation from previously described *Asterochloris* species. Chloroplast morphology represented the best morphological marker for species delineation. In fact, each species can be recognized by the dominance and unique assemblage of particular chloroplast types. Although genetically well recognized by rapidly evolving internal transcribed spacer rDNA and actin intron markers, all 13 investigated *Asterochloris* species shared identical small subunit rDNA sequences. We therefore demonstrated that morphologically and ecologically diverse species can frequently be grouped into a single taxonomic unit in whole-transcriptome sequencing studies, considerably affecting the resulting estimates of species diversity. Finally, we demonstrated the presence of isogamous sexual reproduction in *Asterochloris*, disputing the current symbiotic dogma of the loss of sexual reproduction in algal symbionts.

Key index words: *Asterochloris*; cryptic species; green algae; lichens; morphology; phylogeny; speciation; symbiosis; taxonomy

Abbreviations: BI, Bayesian inference; BIC, Bayesian information criterion; CAUP, Culture Collection of Algae of the Charles University in Prague, Czech Republic; CBCs, compensatory base changes; CM, confocal microscopy; GDA, General discriminant analysis; ITS, internal transcribed spacer; MCMC, Markov chain Monte Carlo; ML, maximum likelihood; NGS, next-generation sequencing; OTUs, operational taxonomic units; PCA, principal component analysis; rbcL, ribulose-bisphosphate carboxylase; SAG, Culture Collection of Algae at the University of Gottingen, Germany; UTEX, Culture Collection of Algae at the University of Texas at Austin, USA; wMP, weighted maximum parsimony

INTRODUCTION

Species are fundamental units of biology, comparable to atoms in chemistry or theorems in mathematics. Therefore, the proper delimitation of species is essential for both biologists and the general public. Species delimitation is a fundamental requirement for our understanding of ecosystems and biodiversity, which is necessary for effective decision making about conservation efforts. In addition, taxonomy is a language used by scientists to help the public recognize the diversity, ecology, distribution, and evolutionary history of living organisms. However, evolving over time, species are not unchanging entities. We are therefore surrounded by a plethora of species that vary based on their evolutionary ages, which can make it extremely difficult to perform species identification and delimitation. In fact, the issue of species delimitation has been further complicated by the species problem, that is, the difficulty in defining the concept of species. To date, a wide range of species concepts have been proposed, many of which are associated with several definitions. Moreover, many of these concepts are incompatible in that they can lead to different conclusions concerning the boundaries and number of species (De Queiroz 2007).

In protists, the problem of species delimitation is enhanced by the near absence of morphological features that could be used to clearly distinguish one species from another. As a consequence, a high level of hidden diversity is usually present within nominal, morphologically defined species and genera. Hidden, morphologically highly similar species are frequently described in green algae (Lewis and Flechtner 2004, Fawley et al. 2011, Demchenko et al. 2012), chrysophytes (Škaloud et al. 2012, 2014, Jo et al. 2013), diatoms (Mann et al. 2004, Lundholm et al. 2012), ciliates (Quintela-Alonso et al. 2013), and heterotrophic flagellates (Hausmann et al. 2006, Harper et al. 2009). Particularly in green algae, authors often refrain to differentiate the hidden species morphologically, and they define these species according to the phylogenetic species concept (Bock et al. 2011, Krienitz et al. 2011, 2012, Fučíková et al. 2012).

In this study, we focused on species delimitation within the genus *Asterochloris* Tschermak-Woess, one of the most common lichen photobionts. This genus was described by Tschermak-Woess (1980), who differentiated it from the closely related *Trebouxia* Puymaly based on chloroplast morphology. Subsequently, molecular investigations revealed the paraphyly of the genus *Trebouxia* (Friedl and Zeltner 1994, Friedl and Rokitta 1997) and the close relationship of several *Trebouxia* species with the genus *Asterochloris* (Helms et al. 2001, Piercey-Normore and DePriest 2001, Škaloud and Peksa 2008). Splitting of the genus *Trebouxia*, as well as formal delineation of the genus *Asterochloris*, was proposed by Škaloud and Peksa (2010), who also emphasized a huge amount of hidden diversity within the latter genus. According to current knowledge, the genus *Asterochloris* represents one of the most common lichen symbionts, occurring in thalli of more than 20 lichen genera worldwide (Piercey-Normore and DePriest 2001, Yahr et al. 2004, 2006, Cordeiro et al. 2005, Nelsen and Gargas 2006, 2008, Beiggi and Piercey-Normore 2007, Bačkor et al. 2010, Škaloud and Peksa 2010, Peksa and Škaloud 2011).

During our recent investigation of *Asterochloris* photobionts, we identified a number of new lineages occurring in lichen thalli sampled across Europe (Bačkor et al. 2010, Škaloud and Peksa 2010, Peksa and Škaloud 2011). Apart from their delimitation by unique internal transcribed spacer (ITS) rDNA and actin

I sequences, the lineages were significantly differentiated by their substrate and climatic preferences (Peksa and Škaloud 2011), suggesting that, in fact, they represent hidden species. However, the virtual identity of all three published *Asterochloris* SSU rDNA sequences (i.e., *A. erici* -AB080310, *A. magna* - Z21552, and *A. phycobiontica* - GU017647) could call determining these lineages as hidden species into question.

The principal aim of this study was to assess whether genetically differentiated *Asterochloris* lineages could be considered to represent distinct, well-defined species. In particular, we aimed to morphologically differentiate among the 13 lineages, selected to include cultured photobiont strains obtained either from personal or public culture collections. Apart from the seven currently accepted *Asterochloris* species, we investigated six additional lineages representing the putative new species. We performed a detailed morphological investigation of all photobiont strains and investigated whether the lineages could be morphologically delineated. Since species of *Trebouxia* and *Asterochloris* are traditionally differentiated based on their chloroplast morphology, we investigated the chloroplast structure and development of cultivated photobiont cells using modern light and confocal microscopy (CM). Finally, we took advantage of previously published diversity surveys to trace the genetic diversity, ecology, biogeography, and mycobiont specificity of each lineage.

MATERIALS AND METHODS

Origin and cultivation of investigated strains. The majority of strains used in this study were isolated by Škaloud and Peksa (2010) from the lichen thalli sampled in central and eastern Europe (Table S1 in the Supporting Information). The remaining strains were obtained from the Culture Collection of Algae at the University of Texas at Austin, USA (UTEX), the Culture Collection of Algae at the University of Göttingen, Germany (SAG), and the Culture Collection of Algae and Protozoa at Argyll, United Kingdom (CCAP). The strains were grown on 2% agarized Bold's basal medium as modified by Bischoff and Bold (1963). All cultures were maintained at a temperature of 15°C, under constant illumination of 7–15 μmol photons · m^{-2} · s^{-1} (cooling box Helkama C5G).

Morphological observations and statistical analyses. To obtain a detailed morphological characterization of particular *Asterochloris* lineages, we investigated the cultivated strains by both conventional light and CM. Light microscopy observations were performed using an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) equipped with a differential interference contrast. For CM, a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with an argon–krypton laser was used. We applied a 488 nm excitation line and an AOBS filter-free system collecting emitted light between 498 and 700 nm. The autofluorescence of chlorophyll was exploited for visualization of the chloroplast structure. A series of optical sections through chloroplasts were captured and used for 3-dimensional reconstruction of their morphology. The

chloroplast reconstructions were produced by the ImageJ 1.34p program (Abramoff et al. 2004), using the "Volume viewer" plugin.

Individual strains were regularly observed during the 3-month period of culturing, to well characterize the overall morphological variability. Zoospore formation was induced by transferring the cultures to a 1% glucose solution (Hildreth and Ahmadjian 1981). Pyrenoid was visualized by staining with a chloriodine solution (an aqueous solution of 5 g I2 and 10 g of 2,2,2-trichlor-1,1-ethandiol in 5 mL of distilled water). Since some lineages were represented only by a single or two cultured strains, we repeated the morphological investigation of selected strains in a half-year interval, after the inoculation of cells onto the fresh agar plates. During each investigation, the following characters were observed: (i) the average cell width (calculated from at least 45 replicates); (ii) cell shape (a portion of spherical, oval, and pyriform cells); (iii) the maximum number of pyrenoids per cell; (iv) the number of aplanospores per sporangia (16, 32, 48, or 128 spores); (v) chloroplast shape (a portion of following chloroplast types as viewed in CM: shallowly lobed, deeply lobed, crenulate, parietal, echinate, flat lobed, globular); and (vi) chloroplast lobe termination (a portion of following lobe termination types as viewed in CM: elongated, simple, flat, finger like, not formed). Statistical analyses of measured data (principal component and general discriminant analyses) were performed using Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA). All graphs were created in R (R Core Team 2014), using the package ggplot2 (Wickham 2009).

DNA extraction, PCR, and sequencing. To well characterize the particular *Asterochloris* lineages, we investigated the genetic variation at four loci including the slowly evolving SSU rRNA and rbcL genes, and the rapidly evolving ITS rDNA and actin type I intron markers. Most of the analyzed ITS rDNA and actin sequences originated from our previous studies (Škaloud and Peksa 2010, Peksa and Škaloud 2011). However, to gain a better resolution of species relationships, we additionally obtained 12 ITS rDNA and 7 actin sequences from lichen thalli and cultured *Asterochloris* strains, respectively (Table S1).

Total genomic DNA was isolated following the standard cetyl trimethylammonium bromide protocol (Doyle and Doyle 1987). The amplification of SSU rRNA and rbcL genes was performed as described in Neustupa et al. (2013), using the primers 18S-F (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and 18S-R (5′-TGA TCC TTC TGC AGG TTC ACC TAC G-3′; Katana et al. 2001), and primers PRASF1 (5′-ATG GTT CCA CAA ACA GAA AC-3′) and PRASR1 (5′-TTG TCA ATA GTA TCA AAT TC-3′; Sherwood et al. 2000). The amplification of ITS rDNA and actin type I locus was performed as described in Peksa and Škaloud (2011), using the primers nr-SSU-1780 (5′-CTG CGG AAG GAT CAT TGA TTC-3′; Piercey-Normore and DePriest 2001) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; White et al. 1990), and primers ActinF2 Astero (5′-AGC GCG GGT ACA GCT TCA C-3′) and ActinR2 Astero (5′-CAG CAC TTC AGG GCA GCG GAA-3′; Škaloud and Peksa 2010). The PCR products were quantified on a 1% agarose gel stained with ethidium bromide and purified using the JetQuick PCR Purification kit (Genomed). The purified amplification products were sequenced using the PCR primers with an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730XL) in Macrogen Corp. (Seoul, Korea).

Sequence analyses. The newly determined sequences were aligned to other sequences from the GenBank database. Three different alignments were constructed for the phylogenetic analyses: (i) an SSU rDNA alignment of 22/38 unique/ total sequences of Trebouxiales, (ii) an rbcL alignment of 34/ 39 unique/total sequences of Trebouxiales, and a (iii) concatenated ITS rDNA + actin alignment of 63/79 unique/total *Asterochloris* sequences selected to encompass all known lineages characterized by both loci. The sequences were aligned using MAFFT v. 6 software (Katoh et al. 2002) under the QINS-I strategy and checked for obvious sequencing errors. The alignment of actin sequences was improved by eliminating the ambiguously aligned regions using the program Gblocks v. 0.91b (Castresana 2000). The resulting alignments had lengths of 1772 (SSU rDNA), 1158 (rbcL), and 1142 (ITS rDNA + actin) characters, respectively. All alignments were submitted to TreeBase (http://www.treebase.org/treebaseweb/home.html) and are available under No. S16886.

For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (Darriba et al. 2012). This BIC-based model selection procedure selected the following models: (i) TrNef + I + Γ for SSU rDNA, (ii) TIM2 + Γ for the first codon position of the rbcL gene, (iii) JC + I for the second codon position of the rbcL gene, (iv) TIM3 + I for the third codon position of the rbcL gene, (v) TrNef + Γ for ITS1, (vi) K80 + Γ for ITS2, actin exon, and actin intron 248, (vii) JC for 5.8S rDNA, and (viii) HKY +Γ for actin intron 206.

The phylogenetic trees were inferred by Bayesian inference (BI) using MrBayes version 3.2.1 (Ronquist et al. 2012). With the exception of SSU rDNA data, the analyses were carried out on partitioned data sets using the different substitution models selected by jModelTest 2.1.4. For those models having complicated substitution types, a mixed substitution type was selected to sample across the substitution model space in the Bayesian Markov chain Monte Carlo (MCMC) analysis itself. All parameters were unlinked among partitions. Two parallel MCMC runs were carried out for five million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF values of SSU rDNA, rbcL, and concatenated ITS rDNA + actin analyses were 0.0008, 0.0035, and 0.0053, respectively. Finally, the burn-in values were determined using the "sump" command.

Bootstrap analyses were performed by maximum-likelihood (ML) and weighted maximum parsimony (wMP) criteria using GARLI, version 2.01 (Zwickl 2006), and PAUP*, version 4.0b10 (Swofford 2002), respectively. ML analyses consisted of rapid heuristic searches (100 pseudoreplicates) using automatic termination (genthreshfortopoterm command set to 100,000). The analyses were performed on partitioned data sets using the different substitution models selected by jModelTest 2.1.4. The wMP bootstrapping (1,000 pseudoreplicates) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences, and gap characters treated as missing data. Character weights were assigned using the rescaled consistency index on a scale of 0 to 1,000. New weights were based on the mean fit values for each character over all trees in the memory.

To show the genetic diversity within the newly characterized lineages, we constructed the haplotype networks on the basis of maximum parsimony analyses of all available sequences, using the Haplotype Viewer (G. Ewing, available at www.cibiv.at/~greg/haploviewer).

RESULTS

Analyses of molecular data. BI of the SSU rDNA and rbcL data yielded similar tree topologies, resolving *Asterochloris*, *Trebouxia*, and *Myrmecia* Printz as well-defined, distinct genera (Fig. 1). In the SSU rDNA analysis, a clade of environmental sequences from soil samples (Lesaulnier et al. 2008) was additionally inferred in the affiliation of the genus *Asterochloris*. Comparison with other SSU rDNA sequences showed that six investigated *Asterochloris* strains (SAG 26.81, UTEX 911, Bayerova 3401, Peksa 183, Peksa 236, and Peksa 999) contained IB3 group I introns at position 516 relative to the *Escherichia coli* coding region. The exon SSU rDNA sequences of all investigated *Asterochloris* strains were completely identical. Resequencing of a single genetically distinct strain (*A. magna* UTEX 902, accession Z21552) confirmed that all nucleotide differences correspond to sequencing errors (Fig. 1a). The *Asterochloris* rbcL sequences were slightly different from each other, but analysis of these sequences did not reveal any highly supported clades with the exception of a lineage comprising *A. glomerata* and *A. irregularis strains* (Fig. 1b).

Bayesian analysis of the concatenated ITS rDNA and actin data set revealed the existence of more than 20 well-resolved lineages within the genus *Asterochloris* (Fig. 2). The relationships among the lineages correspond well with the phylogeny presented by Škaloud and Peksa (2010), including the presence of three moderately to well-supported major clades, A, B, and C. All seven formerly described species (*A. erici, A. excentrica, A. glomerata, A. irregularis, A. italiana, A. magna,* and *A. phycobiontica*) formed well-recognized, distinct lineages. The *Asterochloris* cultures that we isolated during our recent investigation of lichen photobionts clustered within the six distinct lineages recognized by Škaloud and Peksa (2010) as lineages 6, 7, 10, 11, 14, and 16. Lineages 6 and 7 (here referred to as *A. leprarii* sp. nov. and *A. gaertneri* sp. nov.) were inferred to belong within clade B, together with *A. excentrica* and three additional lineages. The remaining four lineages (here referred to as *A. echinata* sp. nov., *A. friedlii* sp. nov., *A. lobophora* sp. nov., and *A. woessiae* sp. nov.) were inferred to be members of clade C, including *A. italiana*, *A. phycobiontica*, and five additional lineages. Within clade C, the relationship among the lineages remained unresolved, with the exception of the close, significant relationship between *A. phycobiontica* and *A. lobophora*.

The comparison of all available ITS rDNA sequences pointed to wide differences in genetic variability within the six newly recognized species (Fig. 2b). *A. leprarii* and *A. gaertneri* showed almost no intraspecific genetic diversity. Of the 30 investigated *A. gaertneri* isolates, only one differed by a single nucleotide substitution. On the other hand, *A. lobophora* represented the most diverse species, containing a total of 18 different ITS rDNA genotypes. *A. lobophora* also stands out due to its higher occurrence in *Cladonia* lichens.

Morphological analyses. The diameter of vegetative cells varied, ranging from 4 to 29 µm. The cells were generally spherical and occasionally oval or pyriform. The cell wall was thin, and occasionally, a flat, localized thickening of the wall was detected in mature cells. In old cultures, the walls of some cells were slightly thickened along their entire surface. A single nucleus with distinct nucleolus was situated parietally in the broad chloroplast infolding. The majority of the cell volume was occupied by the chloroplast. In young cells, the chloroplast was parietal or ribbon shaped. Soon, it shifted to a central position and began to develop into a massive, lobed form. Mature vegetative cells therefore contained a central axial chloroplast with variously arranged lobes reaching the cell periphery. In the late ontogenetic stages, specifically prior to zoo- or aplanosporogenesis, the chloroplast transformed into the parietal type, with smooth, never lobed margins. After a short time, it began to divide into numerous parts in preparation for asexual reproduction. Taking advantage of laser scanning CM, we recognized seven specific chloroplast types occurring in mature *Asterochloris* cells (Fig. 3), as follows: (i) a deeply lobed type, characterized by long, branched, or unbranched lobes emerging directly from the thin chloroplast layer spreading around the pyrenoid ("Tieflappig Typ" sensu, Gärtner 1985a); (ii) a shallowly lobed type, which is similar to the previous type but differs in that the chloroplast lobes emerge from the central mass of the chloroplast layer encircling the pyrenoid ("Normaltyp" sensu, Gärtner 1985a); (iii) a crenulate type, distinguished by a central, massive chloroplast with a regularly nodulated surface ("Crenulater Typ" sensu, Gärtner 1985b); (iv) a parietal type, characterized by parietally positioned nodulated chloroplast with the margins extended into divided finger-like lobes; (v) a flat lobed type, representing an axial chloroplast with long lobes that appear flattened over their entire length; (vi) an echinate type, characterized by numerous thin radial lobes emerging uniformly from the central mass of the chloroplast layer; and (vii) a globular type, a simple spherical chloroplast without, or with very shallow, lobes.

In addition to these seven morphological chloroplast types, we distinguished four types of lobe terminations (Fig. 3), as follows: (i) an elongated type, with lobes extending longitudinally at their ends, therefore giving the chloroplast a finny appearance in surface view ("Rippenformig Typ" sensu, Gärtner 1985a); (ii) a simple type, characterized by simply terminated lobes at their ends; (iii) a flat type of chloroplast lobe, terminated by irregular plates, perpendicularly oriented with respect to the lobe axis; and (iv) a finger-like type, distinguished by lobes branched into several finger-like projections. These projections were perpendicularly oriented with respect to the lobe axis, spreading below the plasma membrane.

Pyrenoids were present in all *Asterochloris* species except *A. magna*. While *A. erici* and *A. lobophora* had single pyrenoids, the cells usually contained one to several pyrenoids lying in the chloroplast center. Often, one large centrally located pyrenoid was surrounded by several smaller satellite pyrenoids, which were created by budding. The pyrenoids were generally distinct; only in *A. erici* did the pyrenoid gradually change over to a chloroplast matrix without a distinct pyrenoid margin. Various structures could occasionally be observed in the pyrenoid matrix by both conventional light and CM. The pyrenoids were

granulated, striated, or perforated. In *A. phycobiontica*, the pyrenoid contained distinct rings of puzzling origin. The frequency and markedness of subpyrenoidal structures significantly increased with cell age. Pyrenoids were usually surrounded by a conspicuous starch sheet, which could be visualized by staining with chloriodine solution.

To investigate morphological differences between the 13 recognized *Asterochloris* species in detail, we characterized each species based on a number of features, including cell shape, dimensions, and chloroplast morphology (Table S2 in the Supporting Information). We found no significant differences in average cell dimensions among species; however, some species could be distinguished by the prevailing shape of their cells. The closely related *A. glomerata* and *A. irregularis* frequently produced oval and pyriform cells (Fig. 4a); by contrast, these cells were never observed in *A. echinata*. In addition, all investigated species were obviously heterogeneous in the overall morphological complexity of chloroplast types (Fig. 4b). Two to four chloroplast types were usually observed during cell ontogeny. The shallowly lobed chloroplast was the most common type, occurring in 11 of 13 investigated *Asterochloris* species. Four species (*A. glomerata*, *A. irregularis*, *A. woessiae*, and *A. excentrica*) were characterized by the prevailing occurrence of the deeply lobed chloroplast type, while the flat lobed type was only observed in the first three species. By contrast, deeply lobed chloroplasts were never found in *A. phycobiontica* or *A. lobophora*, which were characterized by the presence of crenulate and finger-like types. Two species, *A. magna* and *A. echinata*, could be easily recognized by their specific chloroplast morphology, the former by the presence of the simplest globular type and the latter by the combination of crenulate and echinate types. Finally, *A. italiana* was exceptional because it produced a single, shallowly lobed chloroplast type.

Morphological differences among the species were also observed in the shape of chloroplast lobe terminations (Fig. 4c). Elongated terminations were most commonly produced, which were observed in all species except *A. magna* and *A. echinata*. These two species could clearly be recognized by the common absence of any lobes (*A. magna*) and by the exclusive formation of single lobe terminations (*A. echinata*). Moreover, the prevalence of finger-like node terminations is characteristic of *A. phycobiontica*.

Principal component analysis (PCA) of the entire dataset resulted in a relatively well-defined grouping of investigated strains belonging to particular species (Fig. 4d). For example, *A. erici* and *A. magna* were plotted in two distinct clusters in the upper left corner of the PCA plot. On the other hand, several strains belonging to different species were intermixed with each other (e.g., *A. excentrica* and *A. woessiae*). Interestingly, the closely related species pairs (*A. glomerata* - *A. irregularis* and *A. phycobiontica* – *A. lobophora*) were obviously similar based on their morphology. General discriminant analysis (GDA) yielded much better grouping of species into separate clusters (Fig. 4, e and f). *A. magna* formed a strong outlying cluster with a negative value on the second GDA axis (Fig. 4e). A scatter plot based on the first and third GDA axes showed the separation of all 13 *Asterochloris* species into distinct clusters (Fig. 4f). Discriminant analysis (DA) indicated strongly significant differentiation among all investigated species (Wilk's $k < 0.00001$; $P < 0.00001$). Forward stepwise analysis selected the globular chloroplast, parietal chloroplast with finger-like lobes, the number of autospores, and the number of pyrenoids as the best

discriminating characters. The first and third GDA axes, which well discriminated all investigated species, were highly correlated with the four factors examined. Whereas the first GDA axis was correlated with parietal and flat chloroplast types with elongated lobes (correlation coefficients 0.34 and 0.38, respectively), the third axis was correlated with deeply lobed chloroplasts with elongated lobes and globular chloroplasts (correlation coefficients 0.49 and 0.41, respectively). The globular chloroplast type was also highly correlated with the second GDA axis (correlation coefficient 0.70). The average correct discrimination of individual strains based on their morphology reached 94.3%; that is, only three investigated strains (one *A. excentrica* and two *A. woessiae* strains) were classified incorrectly by the discriminant model.

Reproduction. The life cycle and reproductive processes are schematically delineated in Fig. 5. Asexual reproduction occurred by the formation of aplanospores, zoospores, and autospores. Autospore production was relatively rare; it was observed only in some species. Autospore production was initiated by slight cell enlargement and subsequent chloroplast division (Fig. 5b). In general, autospores were formed in relatively small numbers (mostly four or eight) and were liberated by either decomposition or rupturing of the mother cell wall, without producing any special openings (Fig. 5d). The formation of aplanospores and zoospores was much more frequent. Prior to the first cleavage, the chloroplast flattened, migrated to the parietal position, and started to divide (Fig. 5f). Ultimately, a large number of daughter cells (usually 64 or 128) were present within the sporangium (Fig. 5h). Once all the daughter cells formed, a distinction between aplanosporangia and zoosporangia was observable. In the case of aplanospores, the daughter cells rounded and produced a cell wall of their own. Mature aplanospores were liberated by rupturing of the mother cell wall (Fig. 5i). Mature zoosporangia were discernible by the irregular shape of their daughter cells (Fig. 5k). The zoospores were all liberated simultaneously with the rupturing of the mother cell wall, enclosed in a gelatinous vesicle that could slightly evaginate from the mother cell wall during the liberation process. The zoospores were dorsiventrally flattened, with two equal anterior flagella, a posterior chloroplast, a medianto-posterior nucleus, and indistinct stigma. Following liberation from the sporangium, the zoospores swam in a packet, the remaining joined together by their posterior extensions (Fig. 5l). Shortly thereafter, single zoospores started to detach from the packet and swam separately. Despite this, the majority of zoospores still bore posterior extensions even if observed several minutes after separation (Fig. 5n).

Sexual reproduction was very scarce and was observed only twice in *A. woessiae* (strain CAUP H1009). Biflagellate isogamous gametes were morphologically indistinguishable from zoospores. After their fusion, they gave rise to large quadriflagellate planozygotes (Fig. 5p). The germination of planozygotes was not observed.

TAXA DESCRIPTIONS

In this study, we revealed a clear morphological differentiation of the studied *Asterochloris* lineages. Given the genetic distinctiveness, previously published ecological diversification, and mycobiont

specificity of these lineages, we are proposing that they represent new species. Descriptions and characteristics of these new taxa are provided below.

Asterochloris Tschermak-Woess; Pl. Syst. Evol. 135, pp. 291, 292 emend. Škaloud et Peksa

Type species: Asterochloris phycobiontica TschermakWoess 1980; Pl. Syst. Evol. 135, p. 292

Emended description: Cells spherical, occasionally oval or pyriform. Cell wall thin, occasionally with a flat local thickening. Single nucleus situated parietally in the broad chloroplast infolding. Chloroplast single, asteroid, with variously arranged lobes reaching the cell periphery. One to several pyrenoids usually lie in the chloroplast center, surrounded by a conspicuous starch sheet. Prior to aplano- and zoosporogenesis, the chloroplast flattens and assumes a parietal position. Asexual reproduction by usually 64–128 aplanospores and zoospores, occasionally by 2–8 autospores. Zoospores naked, dorsiventrally flattened, with two apical flagella, a posterior chloroplast, a median-to-posterior nucleus, and indistinct stigma. Following liberation from the sporangium, the zoospores shortly swam in a packet, joined together by their posterior extensions. Sexual reproduction scarce, by the fusion of two isogamous gametes. Photobionts of many lichens (genera *Anzina, Cladia, Cladonia, Diploschistes, Lepraria, Pilophorus, Pycnothelia, Stereocaulon*, etc.). Widely distributed, cosmopolitan. From the morphologically similar genus *Trebouxia*, it generally differs by the presence of deeply lobed chloroplast, parietal position of chloroplast prior to zoo- or aplanosporogenesis, and the production of aplanospores as a prevailing type of asexual reproduction.

Asterochloris leprarii Škaloud et Peksa **sp. nov.** (Fig. 6, a–k)

Vegetative cells usually spherical, occasionally oval and pyriform, (5-)7.5-24(-28) µm in diameter (Fig. 6, a–c). Cell wall thin, seldom a flat local thickening of the cell wall can be distinguished. Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading toward the cell's periphery. Mature cells exhibit central chloroplasts of either shallowly lobed (Fig. 6d) or crenulate form (Fig. 6e). Rarely, the deeply lobed (Fig. 6f) and parietal chloroplast (Fig. 6g) is observed as well. The chloroplast lobes can be simply terminated (Fig. 6e), elongated at their ends (Fig. 6f), or finger like (Fig. 6g). Occasionally, the lobe ends are flat (Fig. 6h). The chloroplast contains from one to many pyrenoids. Besides the typical, centrally located pyrenoid, up to seven smaller ones may be present in its vicinity (Fig. 6i). Sometimes, an indistinct granulation or striation can be visible inside pyrenoids. Starch grains are either embedded in a layer around the pyrenoid or distributed evenly throughout the chloroplast. Asexual reproduction by 64–128 aplanospores or 64 zoospores produced in spherical or ellipsoidal sporangia (Fig. 6j). Occasionally, 2–4 autospores are also produced. Zoospores dorsiventrally flattened, drop shaped, arcuate in lateral view, 6–10 µm long and 2.8–4 µm wide, with posterior extensions (Fig. 6k).

Holotype: Cryopreserved photobiont cells isolated from the specimen Peksa 183, deposited in the Culture Collection of Algae of the Charles University in Prague (CAUP) as the item TYPE-H 1010.

Reference strains: CAUP H 1010, SAG 2280.

Type locality: Phycobiont of *Lepraria neglecta*, collected on siliceous rock, Rybárna, Šumava Mts, Czech Republic, May 23, 2005. The lichen specimen is deposited in herbarium of O. Peksa in PL (Collection of The West Bohemian Museum in Pilsen), No. 183.

Etymology: The species is named in reference to the mycobiont genus *Lepraria* Ach.

Distribution: So far known only from Europe: Czech Republic, Norway, Slovakia (Nelsen and Gargas 2008, Škaloud and Peksa 2010, Peksa and Škaloud 2011).

Ecology: In temperate Europe, it prefers altitudes of about 800–1,000 m a.s.l; associates with ombrophilic lichens growing on acidic substrates, especially siliceous rocks (Peksa and Škaloud 2011).

Specificity: found exclusively in the thalli of lichen genus *Lepraria*.

Asterochloris gaertneri Škaloud et Peksa **sp. nov.** (Fig. 6, l–t)

Vegetative cells spherical, very rarely oval or pyriform, 5.5–26(-29.5) µm in diameter (Fig. 6, k and l). Cell wall thin, seldom a flat local thickening of the cell wall can be observed. Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading toward the cell's periphery. Mature cells often possess shallowly lobed axial chloroplasts (Fig. 6, m and n). Deeply lobed (Fig. 6o), crenulate (Fig. 6p), and echinate (Fig. 6, q and r) chloroplast forms observed, as well. The chloroplast lobes are simply terminated, extended longitudinally at their ends, or terminated by finger-like extensions. Flat lobes produced very rarely. The chloroplast contains from one to many distinct pyrenoids. If many, they usually jointly occupy the chloroplast's center (Fig. 6s). Sometimes, an indistinct striation can be visible inside pyrenoids. Starch grains are embedded in a layer around the pyrenoid. Asexual reproduction by 64–128–256 aplanospores or 128 zoospores produced in large spherical or ellipsoidal sporangia (Fig. 6t). Occasionally, 4–8 autospores are also produced. Zoospores dorsiventrally flattened, 6–7.5 µm long and 2.5–4 µm wide.

Holotype: Cryopreserved photobiont cells isolated from the specimen Peksa 236, deposited in the CAUP as the item TYPE-H 1013.

Reference strains: CAUP H 1013, SAG 2283.

Type locality: Phycobiont of *Lepraria rigidula*, collected on bark of *Acer pseudoplatanus*, near Stříbrnická Mt., Králický Sněžník Mts, Czech Republic, February 10, 2005. The lichen specimen is deposited in herbarium of O. Peksa in PL, No. 236.

Etymology: The species epithet is in honor of the work of Dr. Georg Gärtner, who published several reports on *Trebouxia* s.l.

Distribution: So far known only from Europe: Czech Republic, Germany, Slovakia (Škaloud and Peksa 2010).

Ecology: Associates with lichens growing on tree bark and siliceous rocks, in rain-sheltered situations (ombrophobic); in temperate Europe, it was found at altitudes of about 500–900 m a.s.l (Peksa and Škaloud 2011).

Specificity: found exclusively in the thalli of lichen genus *Lepraria*.

Asterochloris woessiae Škaloud et Peksa **sp. nov.** (Fig. 7, a–k)

Vegetative cells usually spherical, rarely oval and pyriform, (5-)6.5–19(-25.5) µm in diameter (Fig. 7a). Cell wall thin, seldom a flat local thickening of the cell wall can be made out (Fig. 7b). Very rarely, the cell wall is slightly thickened along its entire surface. Chloroplast in young cells assumes the central position with several lobes spreading toward the cell's periphery (Fig. 7c). Mature cells display a structurally complicated, central, deeply lobed chloroplast with branched lobes that emerge directly from the thin layer spreading around the pyrenoid (Fig. 7d). Mature cell chloroplasts can further exhibit several other ontogenetic stages, alternating during the cell's ontogeny. The chloroplast can be shallowly lobed (Fig. 7c), or appearing flattened over their entire length (Fig. 7e). Rarely, the crenulate (Fig. 7f) and parietal chloroplasts can be formed as well. The chloroplast lobes can be terminated by all four known types, usually by elongated, flat (Fig. 7g), or finger-like extensions. The chloroplast contains 1–3 distinctively delimited pyrenoids (Fig. 7h). Sometimes, an indistinct granulation can be visible inside the pyrenoids. Starch grains are embedded in a layer around the pyrenoid. Asexual reproduction by 128 aplanospores or zoospores produced in large spherical or ellipsoidal sporangia (Fig. 7i). Occasionally, 2–4 autospores are also produced. Zoospores dorsiventrally flattened, 4.5–7.5 µm long and 2.5–4.5 µm wide, with posterior extensions (Fig. 7j). Sexual reproduction by fusion of biflagellate isogamous gametes; planozygotes with four longitudinal flagella (Fig. 7k).

Holotype: Cryopreserved photobiont cells isolated from the specimen Bayerová 3401, deposited in the CAUP as the item TYPE-H 1009.

Reference strains: CAUP H 1009, SAG 2279.

Type locality: Phycobiont of *Lepraria borealis*, collected on sunlit slate rock, Stara planina Mts, Central Balkan National Park, Bulgaria, July 1, 2004. The lichen specimen is deposited in collection of S. Bayerová-Slavíková in PRA (Herbarium of Institute of Botany of the ASCR, Czech Republic), No. 3401.

Etymology: The species epithet is in honor of the work of Dr. Elisabeth Tschermak-Woess, who described the genus *Asterochloris*.

Distribution: Cosmopolitan, widely distributed. Europe: Bulgaria, Czech Republic, Great Britain, Slovakia, Spain, Sweden; America: Costa Rica, USA; Africa: Canary Islands (Nelsen and Gargas 2006, Bačkor et al. 2010, Škaloud and Peksa 2010, Peksa and Škaloud 2011, Pino-Bodas et al. 2010).

Ecology: Prefers lichens growing on moderately basic substrates (shale, basalt, serpentine rocks etc.) and low altitudes of about 300–600 m a.s.l of temperate Europe (Peksa and Škaloud 2011).

Specificity: Found in a number of lichen species belonging to genera *Lepraria*, *Cladonia*, and *Stereocaulon*.

Asterochloris friedlii Škaloud et Peksa **sp. nov.** (Fig. 7, l–t)

Vegetative cells spherical or slightly oval, (4.5-)6– 18(-21) µm in diameter (Fig. 7, l and m). Cell wall thin, seldom a flat local thickening of the cell wall can be detected. Chloroplast in young cells assumes the central position with several simple lobes spreading toward the cell's periphery (Fig. 7n). Mature cells generally display a structurally complicated, central, deeply or shallowly lobed chloroplast with branched lobes (Fig. 7, o and p). Sometimes, the crenulate (Fig. 7q) or parietal chloroplast (Fig. 7r) can be formed

as well. In mature cells, the chloroplast can be slightly asymmetrically positioned. The chloroplast lobes are mostly extended longitudinally at their ends, but they can be terminated by additional three types, as well. The chloroplast generally contains a single distinct, granulated pyrenoid. Especially in older cells, the pyrenoid buds at its surface and gives rise to several smaller ones in its vicinity (Fig. 7s). Starch grains are embedded in a layer around the pyrenoid in the form of large granules. Asexual reproduction by 64–128 aplanospores or zoospores produced in large spherical, ellipsoidal, or irregular sporangia (Fig. 7t). Zoospores dorsiventrally flattened, 4.5–7 μm long and 3–3.5 μm wide, with posterior extensions.

Holotype: Cryopreserved photobiont cells isolated from the specimen Peksa 235, deposited in the CAUP as the item TYPE-H 1011.

Reference strains: CAUP H 1011, SAG 2281.

Type locality: Phycobiont of *Lepraria caesioalba*, collected on bryophytes on siliceous rock, Klenovský Vepor Mt., Slovenské Rudohorie Mts, Slovakia, July 12, 2004. The lichen thallus is deposited in herbarium of O. Peksa in PL, No. 235.

Etymology: The species epithet is in honor of the work of Dr. Thomas Friedl, who published several reports on *Trebouxia* s.l.

Distribution: Cosmopolitan, widely distributed. Europe: Czech Republic, Romania, Slovakia; America: Canada, USA; Asia: China (Nelsen and Gargas 2006, 2008, Škaloud and Peksa 2010, Peksa and Škaloud 2011).

Ecology: Mainly in ombrophobic lichens growing on acidic as well as basic substrates (Peksa and Škaloud 2011).

Specificity: With a single exception (*Cladonia*) found in thalli of lichen genus *Lepraria*.

Asterochloris echinata Škaloud et Peksa **sp. nov.** (Fig. 8, a–i)

Vegetative cells always spherical, (5-)7–18(-21) μm in diameter (Fig. 8a). Cell wall thin, without any local thickenings. Young and mature cells exhibit central crenulate chloroplasts (Fig. 8b). In mature cells, the chloroplast often transform into the echinate form characterized by many thin radial lobes giving it a bristly appearance (Fig. 8, c and d). The crenulate chloroplast of old cells frequently transform into a highly specific, simple form without any lobes (Fig. 8, e and f). This form has a distinctive chloroplast ultrastructure in its central and marginal regions. In the center, the starch accumulation causes the decrease in thylakoid numbers, and subsequent modification of the chloroplast's texture (Fig. 8g). The chloroplast contains from one to many distinct or indistinct pyrenoids. In the latter case, up to eight smaller pyrenoids are present in the vicinity of the central one (Fig. 8h). Sometimes, an indistinct granulation can be visible inside pyrenoids. Starch grains are embedded evenly throughout the chloroplast. Asexual reproduction by 64-128 aplanospores produced in spherical or ellipsoidal sporangia (Fig. 8i). Zoospores very rare, dorsiventrally flattened, 6 μm long and 4 μm wide.

Holotype: Cryopreserved photobiont cells isolated from the specimen Peksa 186, deposited in the CAUP as the item TYPE-H 1012.

Reference strains: CAUP H 1012, SAG 2282.

Type locality: Phycobiont of *Lepraria rigidula*, collected on bryophytes on basalt rock, Klíč Mt., Lužické hory Mts, Czech Republic, September 18, 2004. The lichen specimen is deposited in herbarium of O. Peksa in PL, No. 186.

Etymology: The species epithet is named in reference to the echinate shape of chloroplast, which appeared in certain ontogenetic stages.

Distribution: So far known only from Europe: Bulgaria, Czech Republic, Portugal, Slovakia, Spain (Škaloud and Peksa 2010, Peksa and Škaloud 2011).

Ecology: Associates with ombrophilic lichens growing on acidic substrates (Peksa and Škaloud 2011).

Specificity: Found in the thalli of lichen genera *Lepraria* and *Cladonia*.

Asterochloris lobophora Škaloud et Peksa **sp. nov.** (Fig. 8, j–u)

Vegetative cells usually spherical, occasionally oval and pyriform, 6–23(-25.5) µm in diameter (Fig. 8, j and k). Cell wall thin, seldom a flat local thickening of the cell wall can be distinguished (Fig. 8l). Very rarely, the cell wall is slightly thickened along its entire surface (Fig. 8m). Chloroplast in young cells assumes the central position with several lobes spreading toward the cell's periphery. Mature cells exhibit shallowly lobed chloroplast (Fig. 8n), sometimes transformed into the crenulate (Fig. 8o) or parietal form (Fig. 8, p and q). Even though the chloroplast can sometimes appear to be deeply lobed, the lobes never emerge directly from the pyrenoid surroundings. The chloroplast lobes can be terminated by all four known types, usually by finger-like or elongated extensions. The chloroplast contains single distinct granulated pyrenoid (Fig. 8r). Particularly prior to cell division, the pyrenoid sometimes divides into two parts. Starch grains are embedded either in a layer around the pyrenoid or evenly throughout the chloroplast. Asexual reproduction by 64–128 aplanospores or 128–256 zoospores produced in large spherical or slightly ellipsoidal sporangia (Fig. 8, s and t). Zoospores dorsiventrally flattened, 4–7 µm long and 2.5–3.5 µm wide, with posterior extensions (Fig. 8u).

Holotype: Cryopreserved photobiont cells isolated from the specimen Peksa 166, deposited in the CAUP as the item TYPE-H 1014.

Reference strain: CAUP H 1014.

Type locality: Phycobiont of *Lepraria caesioalba*, collected on siliceous rock, Kašperk Mt., Šumava Mts, Czech Republic, March 31, 2005. The lichen specimen is deposited in herbarium of O. Peksa in PL, No. 166.

Etymology: The species epithet is named in reference to the lobed chloroplast shape.

Distribution: Cosmopolitan, widely distributed. Europe: Czech Republic, Slovakia; America: Canada, USA; Asia: India (Piercey-Normore and DePriest 2001, Yahr et al. 2004, 2006, Cordeiro et al. 2005, Nelsen and Gargas 2006, Beiggi and Piercey-Normore 2007, Bačkor et al. 2010, Kotelko and Piercey-Normore 2010, Škaloud and Peksa 2010, Peksa and Škaloud 2011, Řídká et al. 2014).

Ecology: In temperate Europe, it prefers altitudes of about 500–1,000 m a.s.l; associates with ombrophilic lichens growing mainly on acidic substrates (Peksa and Škaloud 2011).

Specificity: Found in a number of lichen species belonging to genera *Cladonia*, *Lepraria*, *Diploschistes*, and *Stereocaulo*n.

DISCUSSION

***Species delineation in* Asterochloris.** To date, a total of seven species are recognized within the genus *Asterochloris*. These species, established during the second half of the 20th century, were exclusively delimited based on morphological features such as cell size, cell shape, chloroplast morphology, cell wall thickness, and dissociation of aplanospores (Ahmadjian 1960, Archibald 1975, Tschermak-Woess 1980, Hildreth and Ahmadjian 1981). Each species formed a distinct, well-supported lineage in the concatenated ITS rDNA + actin phylogenetic tree (Fig. 2); however, our phylogenetic reconstruction points out the existence of several additional, well supported lineages within the genus. Six of these new lineages were investigated in detail in this study. In general, we can apply two alternative taxonomic approaches to assess the observed genetic diversity. First, we can consider each genetic lineage to be a separate, distinct species and based on this approach, the genus *Asterochloris* would encompass tens, if not hundreds, of undescribed species. Second, genetic diversity can be considered to be a manifestation of substantial infraspecific variability. Applying this "large species" concept would involve merging all described species into a single species, namely *A. phycobiontica*.

The existence of a single, genetically divergent species is supported by the presence of identical SSU rDNA sequences in all of the investigated strains and by the observation that the relatively low genetic variation in the ITS rDNA region is correlated with the absence of compensatory base changes (CBCs) among a number of *Asterochloris* lineages (Škaloud and Peksa 2010). In prokaryotes, it was proposed that species' boundaries should be defined by a fixed threshold of genetic divergence in SSU rDNA (Rossello-Mora and Amann 2001). This concept considers that all strains showing a similarity higher than 97% belong to the same species. Applying this concept to *Asterochloris* would result in recognizing only a single species; however, this species concept was never applied to eukaryotic organisms, and its applicability to prokaryotes has been seriously criticized (Pedrós-Alió 2006, Stackebrandt and Ebers 2006). Similarly, the presence of CBCs in the conserved regions of the ITS2 molecule has been proposed to represent a threshold for defining species boundaries in eukaryotes (Coleman 2000); however, this concept has been subjected to mounting criticism (Caisová et al. 2011, 2013, Assuncao et al. 2012). In particular, it was demonstrated that the CBCs are not diagnostic at the species level and that even genera, families, and orders of green algae can lack CBCs in such regions (Caisová et al. 2011, Škaloud and Rindi 2013). Indeed, a causal link between ITS2 secondary structure and speciation mechanisms in eukaryotes simply does not exist. Therefore, the presence and number of CBCs are most probably direct consequences of the accumulation of mutations during the evolutionary process, simply reflecting the genetic distance among organisms.

In this study and during our previous investigation of *Asterochloris* algae, we detected substantial genetic, morphological, and ecological differences among particular lineages. First, although the overall mean distance among the lineages was rather low in ITS rDNA (P-distance: 0.022), a considerable genetic differentiation was observed in the actin locus (P-distance: 0.168). Contrary to their broad utilization in fungal research (e.g., Grube and Kroken 2000, Daniel and Meyer 2003), actin intron sequences are still

rarely used for species identification in protists. The potential of this marker for identifying and delimiting protist species has been demonstrated in photobiont genera *Asterochloris* (Nelsen and Gargas 2006, Škaloud and Peksa 2010) and *Trebouxia* (Kroken and Taylor 2000, Muggia et al. 2010) and in the heterotrophic chrysophycean genus *Spumella* Cienkowsky (Stoeck et al. 2008).

Second, our statistical analyses revealed a significant morphological differentiation of all investigated *Asterochloris* lineages. Although cell size was determined to be highly plastic under culture conditions, all other morphological features appear to well discriminate among species. Chloroplast morphology, in particular, could be considered to be the best morphological marker for species delineation. Škaloud and Peksa (2008) pointed to the existence of several specific chloroplast types occurring during species ontogeny. In the current study, we demonstrated that although these types are frequently shared by more than one species, particular species could be well recognized by the dominance and unique assemblage of particular types of chloroplasts (Fig. 4, b and c). For example, the parietal lobed chloroplast type prevails in *A. phycobiontica*, frequently occurs in *A. magna*, only occasionally appears in *A. lobophora*, and very rarely develops in *A. leprarii*, *A. woessiae*, *A. friedlii*, and *A. irregularis*. Moreover, some chloroplast types occur in a small minority of species and could be used to easily define these species. For example, the combination of crenulate and echinate chloroplast types occurs only in *A. echinata*, while the presence of a globular type defines *A. magna*. Interestingly, closely related lineages were generally similar in terms of morphology (i.e., *A. gaertneri–A. irregularis* and *A. phycobiontica–A. lobophora*).

Finally, previous molecular investigations detected clear ecological differentiation of *Asterochloris* lineages. Initially, Piercey-Normore and DePriest (2001) explained the photobiont switching among the symbiotic lichen associations as an analogy to human agriculture, where locally best adapted crops are selected and subsequently distributed. Later on, Piercey-Normore (2006) hypothesized that algal genotypes coexisting in the same lichen thalli may be adapted to different forest light levels. Yahr et al. (2006) then suggested the ecological specialization of photobiont lineages, depending on the local environment. Quite recently, specific ecological factors that drive the specialization of *Asterochloris* lineages were detected by Peksa and Škaloud (2011). Besides substrate and climatic preferences, exposure to rain and sun was the most significant environmental factor, clearly distinguishing particular lineages. The photobionts from ombrophobic and ombrophilic lichens were clustered into completely distinct clades.

In light of the above-mentioned genetic, morphological, and ecological inferences, we believe that each well-resolved *Asterochloris* lineage should be considered to be a distinct species. In fact, by applying an alternative, broader species concept, obvious differences among the lineages would be ignored. This would ultimately prevent us from understanding the true diversity, distribution, and specificity of *Asterochloris* species, as well investigating evolutionary processes that occur at the species level. Therefore, we described all lineages investigated in this study as new species.

Biogeography and specificity of new Asterochloris species. A comparison with previously published diversity surveys allows us to trace the biogeography and specificity of newly proposed *Asterochloris*

species. Although the biogeography of microorganisms has become a highly discussed topic (Caron 2009), investigations dealing with the biogeography of symbiotic protists are very scarce. Geographic separation of particular lineages has been reported for reef-coral dinoflagellate endosymbionts (e.g., Finney et al. 2010, LaJeunesse and Thornhill 2011), as well as for endosymbiotic green algae of the ciliate *Paramecium bursaria* (Hoshina et al. 2005). By contrast, population studies on lichenized *Trebouxia* species indicated that the distribution of particular genotypes is particularly shaped by either climatic factors (Fernández-Mendoza et al. 2011) or distribution patterns of mycobiont partners (Buckley et al. 2014).

The single study dealing with the biogeography of *Asterochloris* photobionts indicated generally wide (eurychoric) distribution of species (Řídká et al. 2014). Nevertheless, the habitat area of common lineages seems to be more or less restricted based on climatic preferences (e.g., warm-temperate to (sub)arctic distribution of *A. glomerata*). Though the real diversity of *Asterochloris* algae is still greatly undersampled, it seems that at least some lineages exhibit restricted geographic distribution independent of climatic factors. According to the actually available genetic data, three of the six newly proposed species (*A. echinata*, *A. gaertneri*, and *A. leprarii*) occur only in Europe (Fig. 2b). These species have never been reported from climatically analogous regions in eastern USA, though the investigations were performed on identical or closely related lichen species (Nelsen and Gargas 2008).

In lichen associations, the term "specificity" is used to refer the range of compatible partners for a given symbiont (Yahr et al. 2006). Lichen specificity is usually perceived from the perspective of a fungal partner, that is, as the range of possible photobionts for a given fungal species. In general, both fungal specialists (having a high specificity) and generalists (having a low specificity) have been distinguished in many lichen genera (see Muggia et al. 2014). However, the specificity could be conceived from the algal perspective, as well. Using our present data, we can compare the algal specificity toward the fungal genera of the newly described *Asterochloris* species. All species seem to be highly specific toward the genera *Cladonia* and *Lepraria* (Fig. 2b). Two species (*A. gaertneri* and *A. leprarii*) even form the symbiotic associations exclusively with the fungal genus *Lepraria*. Interestingly, though a number of *Stereocaulon* thalli have been investigated for the diversity of their algal partners (Piercey-Normore and DePriest 2001, Nelsen and Gargas 2006, Bačkor et al. 2010, Škaloud and Peksa 2010, own unpublished data), the species herein described as new are obviously not preferred by this fungal genus. In fact, *Stereocaulon* is much widely preferred by two closely related species *A. glomerata* and *A. irregularis* (Fig. 2a).

Assessing species diversity in protists. Estimation of the total species diversity in protists remains a highly controversial topic (Caron 2009). Global protist diversity has been proposed to be extraordinarily high by some (Foissner 1999) and generally much lower and fundamentally different from the biodiversity of macroorganisms by others (Fenchel and Finlay 2003). Accordingly, while Finlay and Fenchel (1999) estimated that there are approximately 20,000 protist species, others estimate that there are several million undescribed protist species (Pawlowski et al. 2012). Such substantial differences are primarily caused by

differences in methodology (e.g., morphological vs. molecular approaches), species concept (see Boenigk et al. 2012), and theoretical framework (e.g., dispersal-gene flow paradox; De Meester et al. 2002); however, the vast majority of recent investigations have provided undeniable evidence that the overall species diversity of protists is greatly underestimated (e.g., Caron et al. 2012, Pawlowski et al. 2012).

During the past decade, analysis of SSU ribosomal RNA genes has become the most commonly used approach to investigate the diversity of protists. A number of studies have revealed an extremely high proportion of SSU rDNA sequences that could not be assigned to any described species (e.g., López-García et al. 2001, Behnke et al. 2011). Exploration of SSU rDNA sequences has often revealed the existence of several novel, highly diverse lineages (Dolven et al. 2007, Howe et al. 2009). More recently, technological progress in sequencing has enabled researchers to investigate protist diversity at previously unattainable scales. Using next-generation sequencing (NGS) technologies, thousands of SSU rDNA amplicon sequences can be produced from a single sample (Edgcomb et al. 2011); however, the sequence length obtained by NGS sequencing is (at the time of writing) insufficient to characterize complete SSU rDNA genes. Therefore, only short, hypervariable SSU rDNA regions are usually targeted to assess protist diversity (Dunthorn et al. 2012).

In NGS studies, sequence data are typically converted into operational taxonomic units (OTUs) based on sequence similarity; these units are often treated as being synonymous to species (Schmidt et al. 2014). Several methods have been developed to cluster SSU rDNA sequences into OTUs; however, they often partition sequence data differently (Sun et al. 2011). In addition, the taxonomic power of OTUs generated based on sequence similarities has been questioned (Boenigk et al. 2012). In the current study, we demonstrated that morphologically and ecologically diverse species can share identical SSU rDNA sequences. As a consequence, such species would be grouped into a single OTU by NGS data processing. Considering that closely related, mostly cryptic species of protists are commonly identical in their SSU rDNA sequences, the true diversity of eukaryotes can be much greater than that estimated by NGS data. In addition, organisms sharing high SSU rDNA sequence similarity can significantly differ in their ecology and distribution. Therefore, we suggest that the rapidly evolving ITS region should be sequenced, in addition to the broadly used SSU rDNA gene, in NGSbased protist diversity investigations (e.g., Bachy et al. 2013).

Sexuality of lichen photobionts. Focusing on the main objective of this study, we performed a detailed investigation of the morphology and life cycle of many isolated photobionts, which involved hundreds of hours of microscopic observations. On two occasions, we had a brief opportunity to observe sexual reproduction in *Asterochloris woessiae* cultures, as evidenced by the fusing of biflagellate gametes. According to contemporary symbiotic dogma, lichen symbiosis should lead to the loss of sexual reproduction in the algal symbiont as a result of highly evolved and integrated symbiotic association (Law and Lewis 1983). The absence of sexual reproduction in lichen photobionts (except for the genus *Trentepohlia*) is, in fact, frequently mentioned in the literature (e.g., Ahmadjian 1987, Gärtner 1992, Friedl and Büdel 1996) and is interpreted as preventing the production of novel genotypes that would be less

suited to the mycobiont (Ahmadjian 1993). However, records confirming sexual reproduction in photobionts do exist. The first exhaustive description of sexual reproduction in *Trebouxia* was published by Waren (1920), who observed frequent production of gametes and their subsequent fusion in photobionts of *Anaptychia ciliaris*, *Physconia distorta*, and *Xanthoria parietina*. Nine years later, Jaag (1929) reported sexual reproduction by iso- and anisogamy in a "*Cystococcus parmeliae*" photobiont isolated from *Flavoparmelia caperata*. Another observation was made by Ahmadjian (1959, 1960), who described frequent sexual reproduction in *Trebouxia impressa* isolated from *Physcia stellaris*. In both cases, isogamous sexual reproduction resulted in the formation of spherical, smooth-walled zygotes. Finally, indirect evidence of sexual reproduction in *Trebouxia* was presented by Kroken and Taylor (2000), who found a recombining population structure in photobionts of *Letharia* spp. by comparing ITS and actin sequences.

Although the existence of sexual reproduction has been disregarded, for example, by Gärtner (1985b), we consider that all of the abovementioned data, as well as our direct observation, clearly establish the presence of sexual reproduction in photobiont genera Trebouxia and *Asterochloris*. As sexual reproduction was previously reported only for *Trebouxia* s. str., gamete fusion in *A. woessiae* represents the first record of sexual reproduction in *Asterochloris*.

In conclusion, we demonstrated the obvious existence of a great number of distinct species within the genus *Asterochloris*, which differ genetically, morphologically, and ecologically. In general, the existence of an extraordinarily high number of cryptic, functionally differentiated species is probably the rule rather than the exception in protists. As a consequence, real species entities are poorly defined, providing little use in evaluating their distribution and diversity patterns or clarifying their ecological roles within ecosystems (e.g., Vyverman et al. 1996, Lilly et al. 2007). In addition, the existence of cryptic species with narrow ecological optima would significantly affect our strategies for conservation management (van Oppen and Gates 2006, Cotterill et al. 2008).

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FIGURES

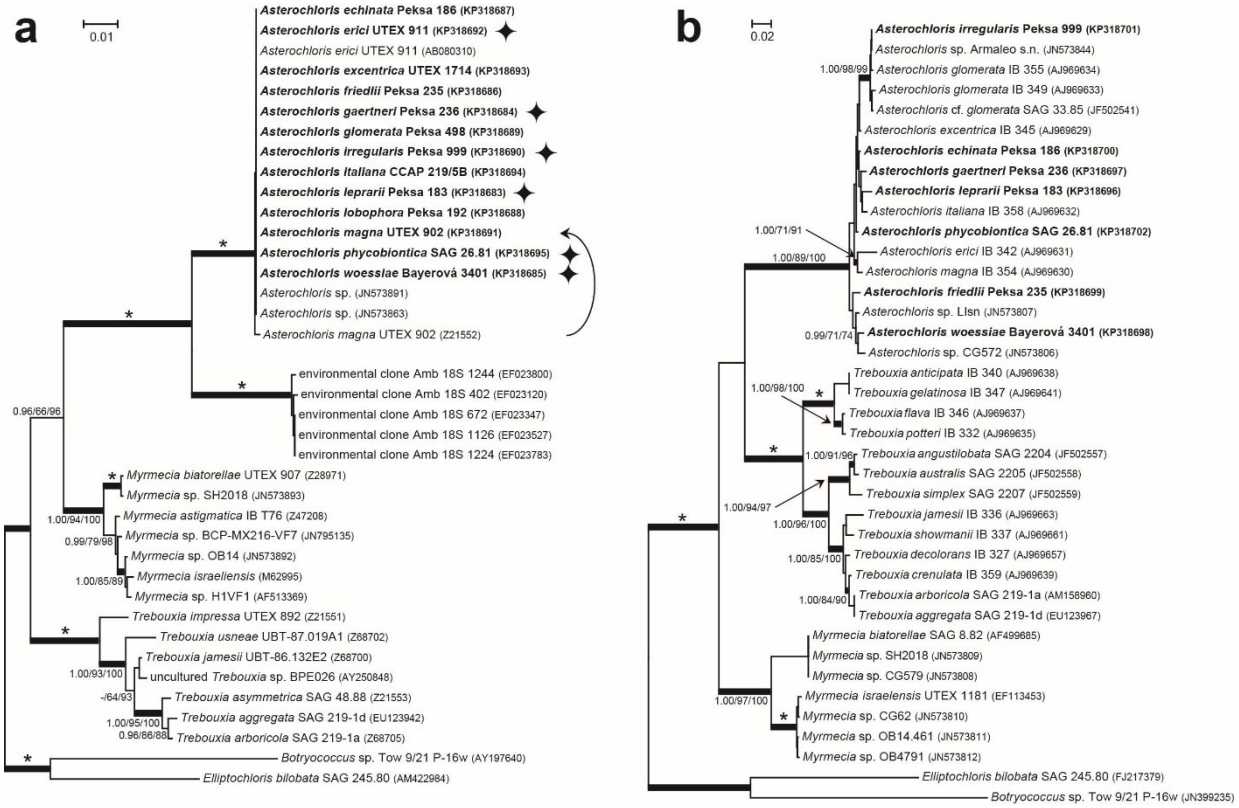

Fig. 1. Phylogeny of the Trebouxiales obtained by Bayesian inference of the SSU rDNA (a) and rbcL (b) data sets. Values at the nodes indicate statistical support estimated by three methods: MrBayes posterior-node probability (left), maximum-likelihood bootstrap (middle), and weighted maximum parsimony bootstrap (right). Full statistical support (1.00/100/100) is marked with an asterisk. Thick branches represent nodes receiving the highest posterior probability support (1.00). Newly sequenced strains are marked in bold. Those sequences containing the IB3 group I introns are marked by stars. An arrow indicated the corrected phylogenetic position of the strain UTEX 902 (*Asterochloris magna*), which SSU rDNA sequence was deposited in GenBank with putative sequencing errors. Scale bar represents the expected number of substitutions per site.

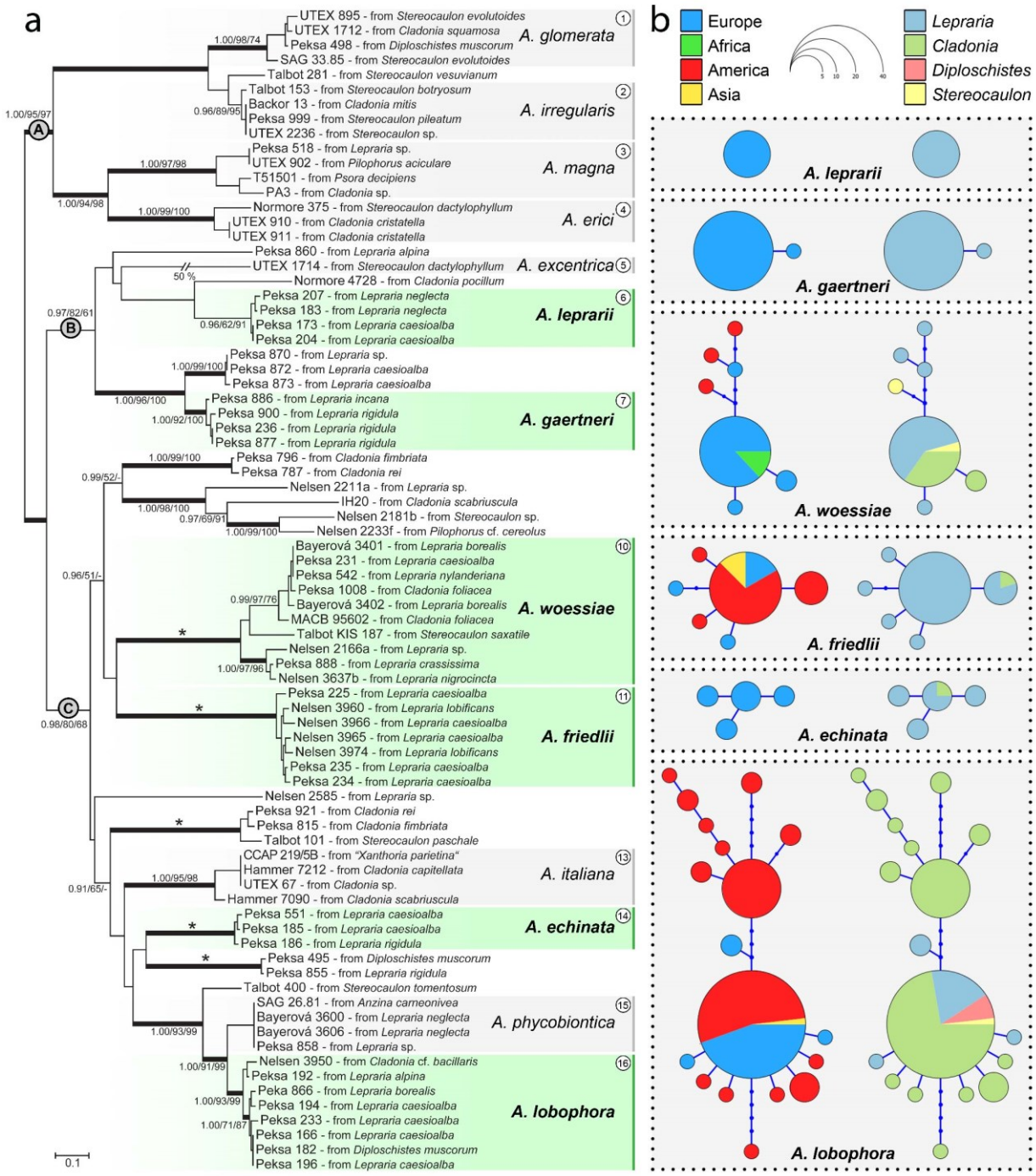

Fig. 2. Genetic diversity within the genus *Asterochloris*. (a) The Bayesian majority rule tree based on the concatenated ITS rDNA + actin alignment. See Fig. 1 for the explanation of node values. Clade numbering and affiliation into the three major clades (A-C) follows Škaloud and Peksa (2010). Scale bar represents the expected number of substitutions per site. (b) Statistical parsimony haplotype networks of all available ITS rDNA sequences, showing the intraspecific diversity within the newly proposed species. Genotypes are colored according to the sampling continent (on the left) and respective mycobiont symbiotic partner (on the right). The sizes of circles representing genotypes reflect the number of sequences that share a genotype. Inferred intermediate haplotypes that were either not sampled or are extinct are represented by small noncolored circles.

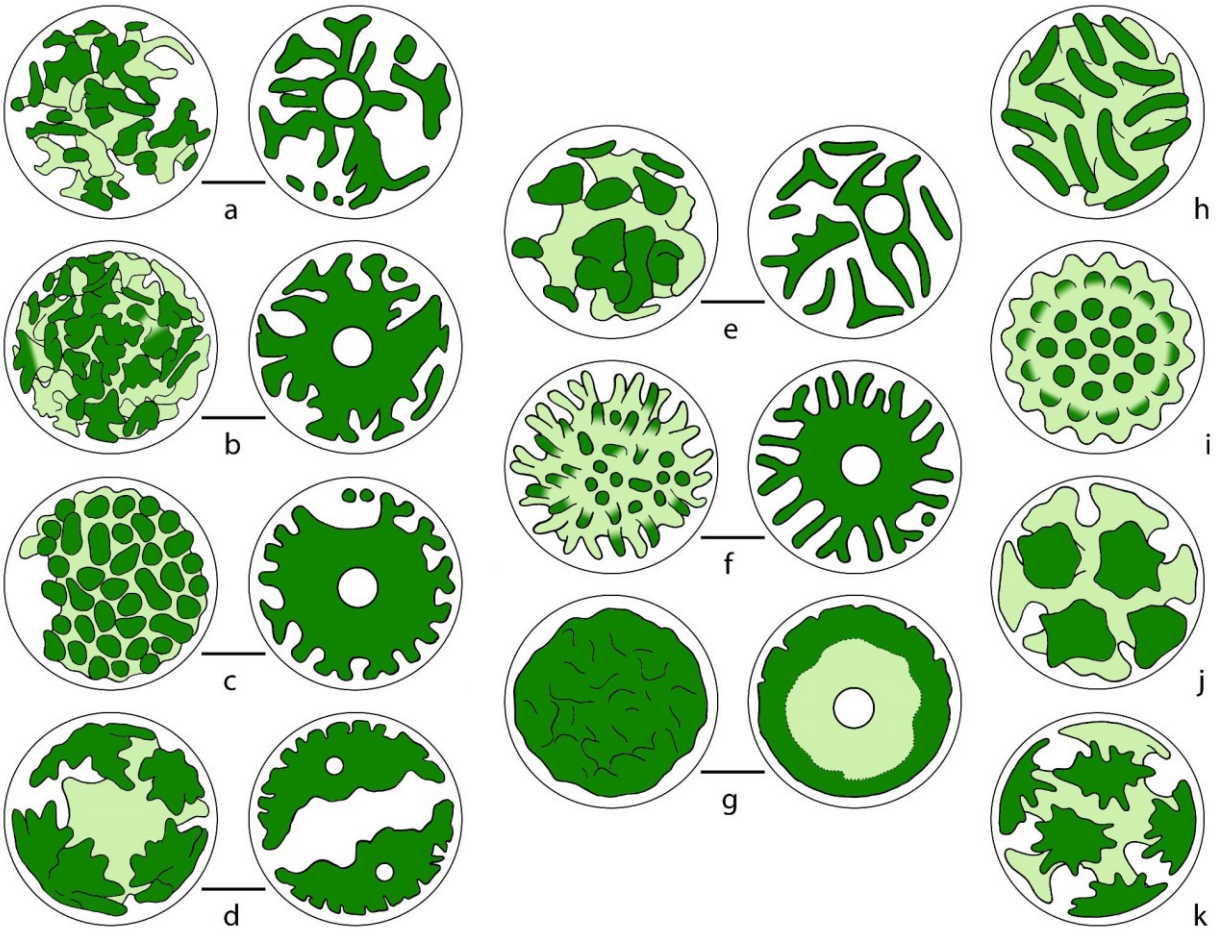

Fig. 3. Schematic drawings of particular chloroplast and lobe termination types in *Asterochloris*. (a–g) Chloroplast types (left: surface view; right: view in optical section): (a) deeply lobed, (b) shallowly lobed, (c) crenulate, (d) parietal, (e) flat lobed, (f) echinate, (g) globular. (h–k) Lobe termination types: (h) elongated, (i) simple, (j) flat, (k) finger-like.

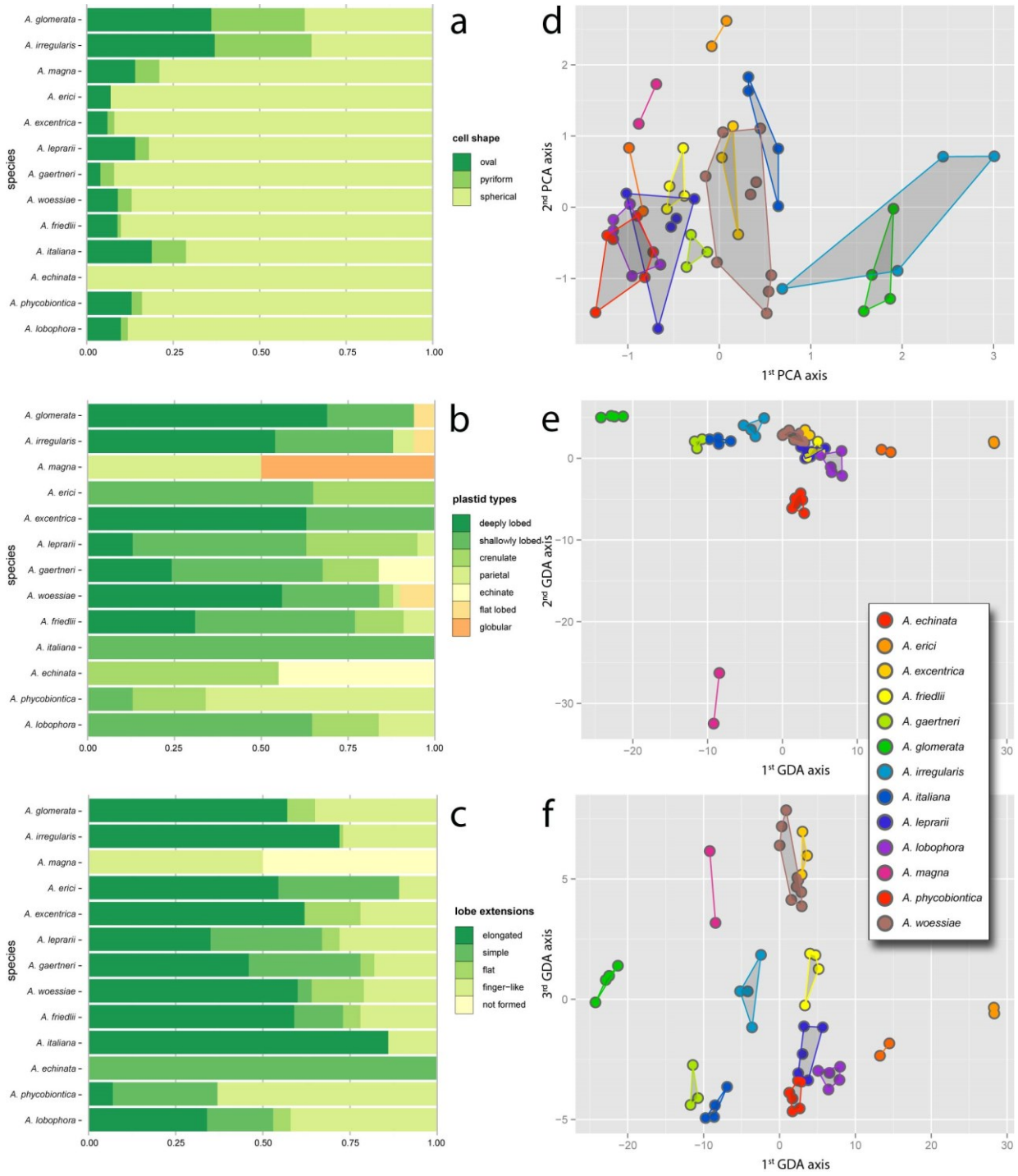

Fig. 4. Morphological comparisons of 13 investigated *Asterochloris* species. (a) Proportion of three different cell shapes. (b) Proportion of seven recognized plastid types. (c) Proportion of five distinguished lobe termination types. (d) Principal component analysis (PCA) of the entire measured morphological features data set. (e, f) General discriminant analysis (GDA) the same data set: (e) the two-dimensional plot of the 1st and 2nd GDA axes, (f) the two-dimensional plot of the 1st and 3rd GDA axes.

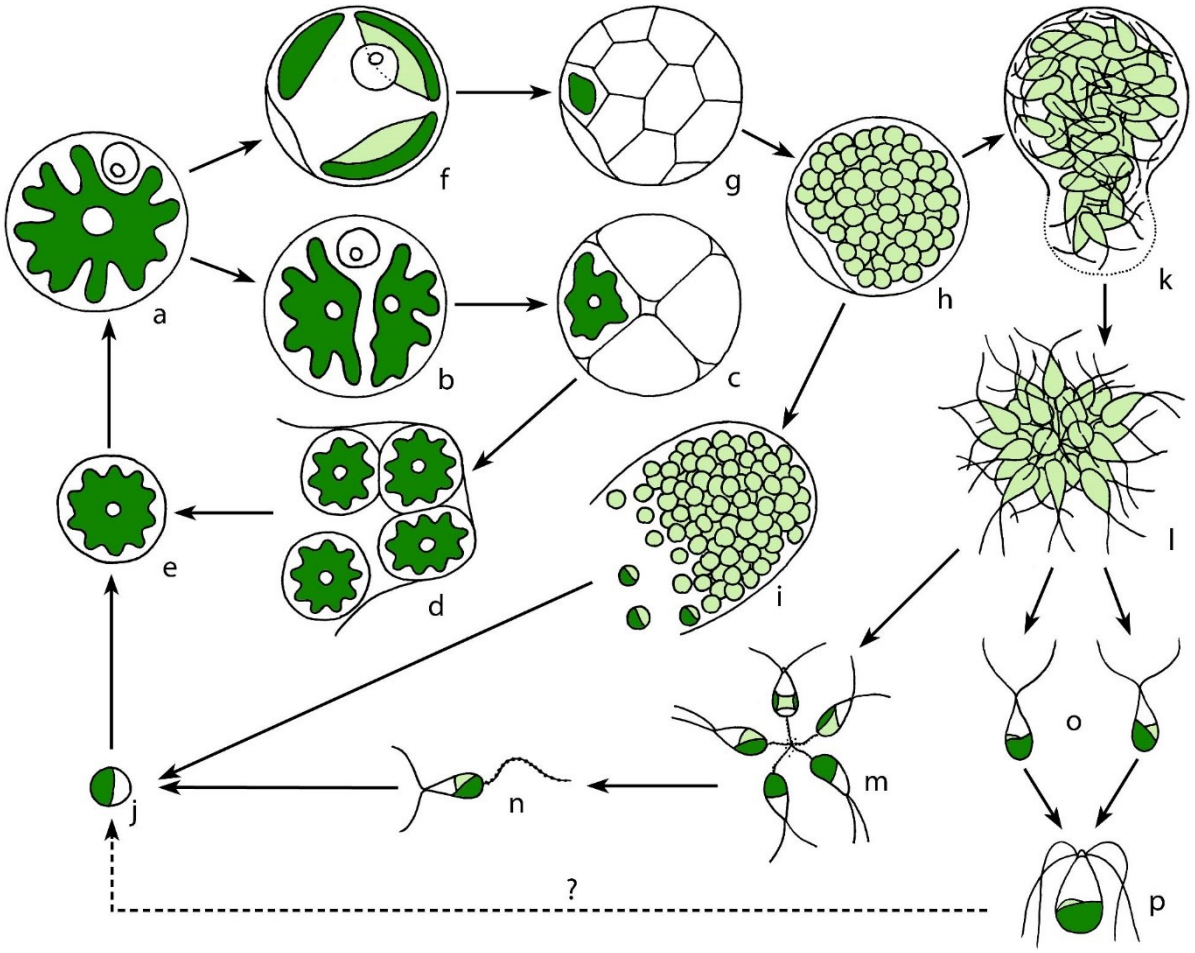

Fig. 5. Schematic representation of the life cycle and reproduction processes in *Asterochloris*. (a) Vegetative cell. (b–e). Autosporogenesis: (b) chloroplast division, (c) young autosporangium, (d) release of autospores, (e) mature autospore. (f–n). Aplano- and zoosporogenesis: (f) chloroplast flattening, note a local thickening of the cell wall, (g) young aplano/zoosporangium, (h) mature aplano/ zoosporangium, (i) release of aplanospores, (j) young aplanospore, (k) release of zoospores, note evagination of gelatinous vesicle, (l) zoospore packet, (m) releasing of zoospores from the packet, (n) single zoospore with a posterior extension. (o, p). Sexual reproduction: (o) gametes, (p) planozygote.

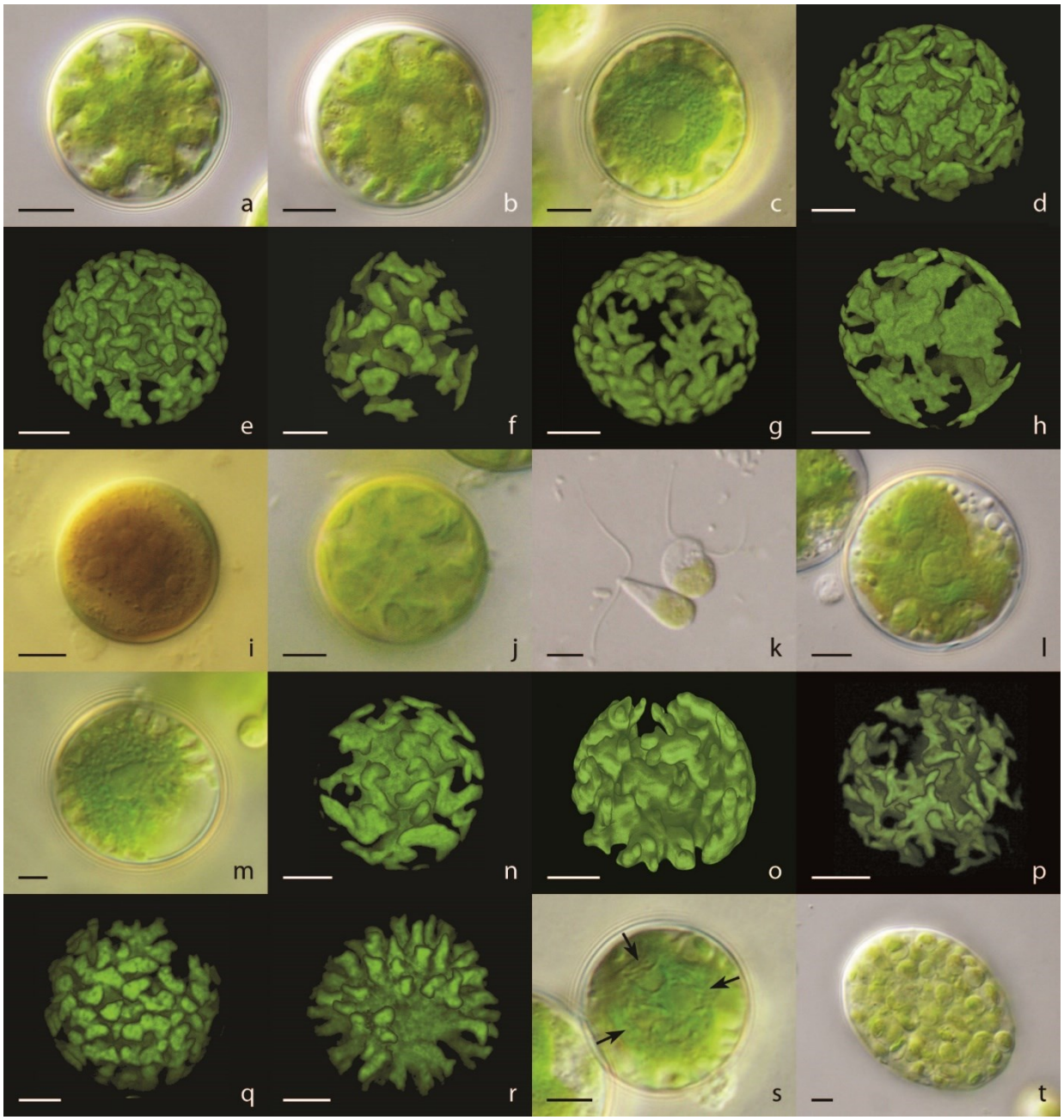

Fig. 6. Light micrographs and confocal reconstructions of chloroplast structures in *Asterochloris leprarii* and *A. gaertneri*. (a–k) *A. leprarii*. Light micrographs of mature vegetative cells possessing deeply lobed (a), shallowly lobed (b), and crenulate (c) chloroplasts. Confocal reconstructions of shallowly lobed (d), crenulate (e), deeply lobed (f), and parietal (g) chloroplast types. (h) Deeply lobed chloroplast with flat lobe ends. Several pyrenoids occur around the large central pyrenoid (i); cells stained by chloriodine solution. (j) Young aplanosporangium. (k) Zoospores. (l–t) *A. gaertneri*. Light microscopy of shallowly lobed (l) and crenulate (m) chloroplast. Confocal reconstructions of shallowly lobed (n, o), deeply lobed (p), crenulate (q), and echinate (r) chloroplasts. Several pyrenoids of equal size (arrows) occur in the chloroplast's center (s). (t) Mature aplanosporangium; scale bar - 5 µm.

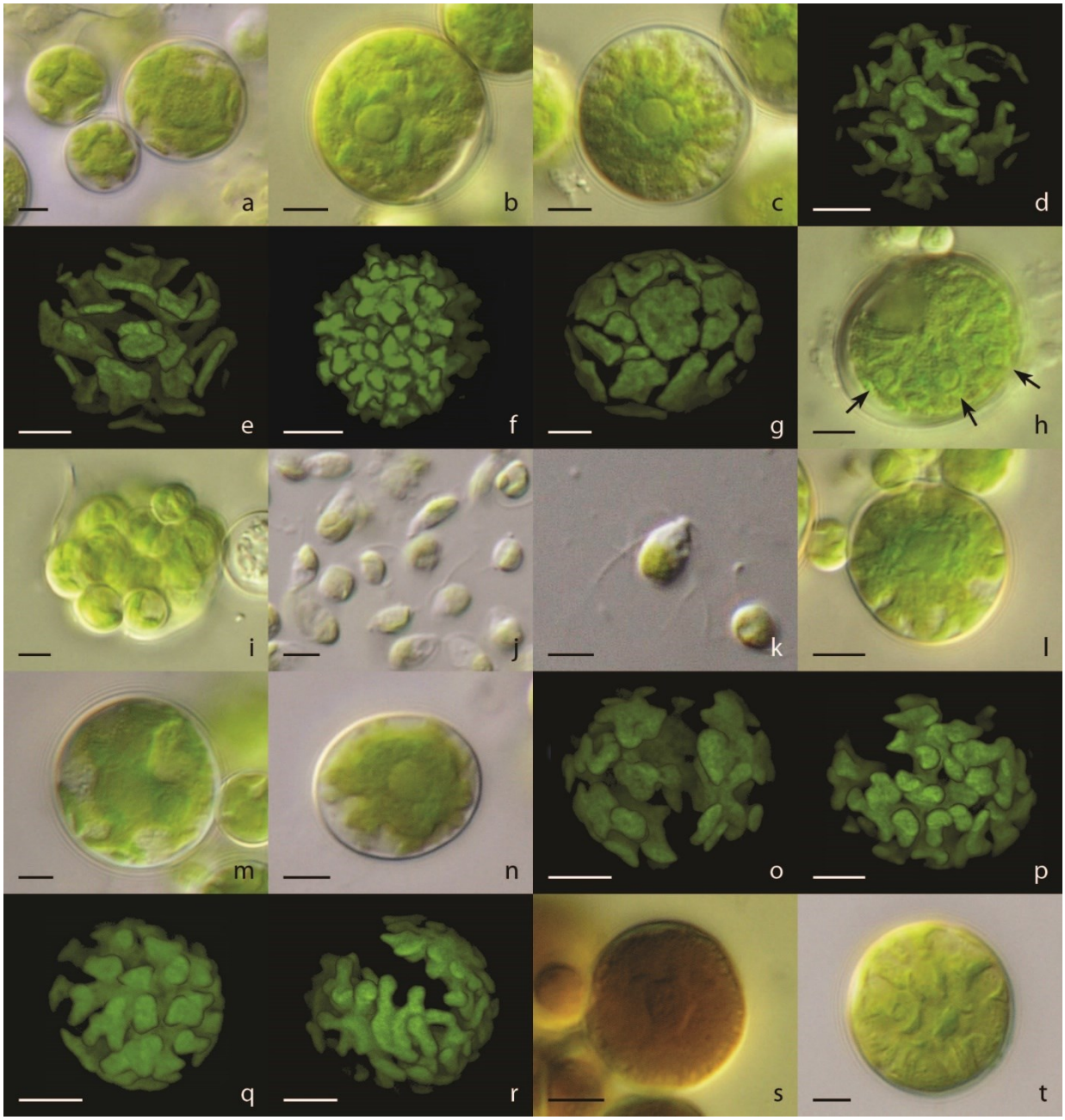

Fig. 7. Light micrographs and confocal reconstructions of chloroplast structures in *Asterochloris woessiae* and *A. friedlii*. (a–k) *A. woessiae*. Light micrographs of young (a) and mature vegetative cells (b, c). Confocal reconstructions of deeply lobed (d), flat lobed (e), and crenulate (f) chloroplasts. (g) Flat terminations of chloroplast lobes. (h) Several pyrenoids (arrows) are formed within the chloroplast. (i) Aplanosporangium. (j) Zoospores. (k) Planozygote with four flagella. (l–t) *A. friedlii*. Light microscopy of shallowly lobed (l) and deeply lobed (m) vegetative cells. (n) Young vegetative cell with simple asteroid chloroplast. Confocal reconstructions of deeply lobed (o), shallowly lobed (p), crenulate (q), and parietal (r) chloroplasts. (s) Single budding pyrenoid. (t) Young aplanosporangium. Cells in Figure(s) stained by chloriodine solution; scale bar - 5 µm.

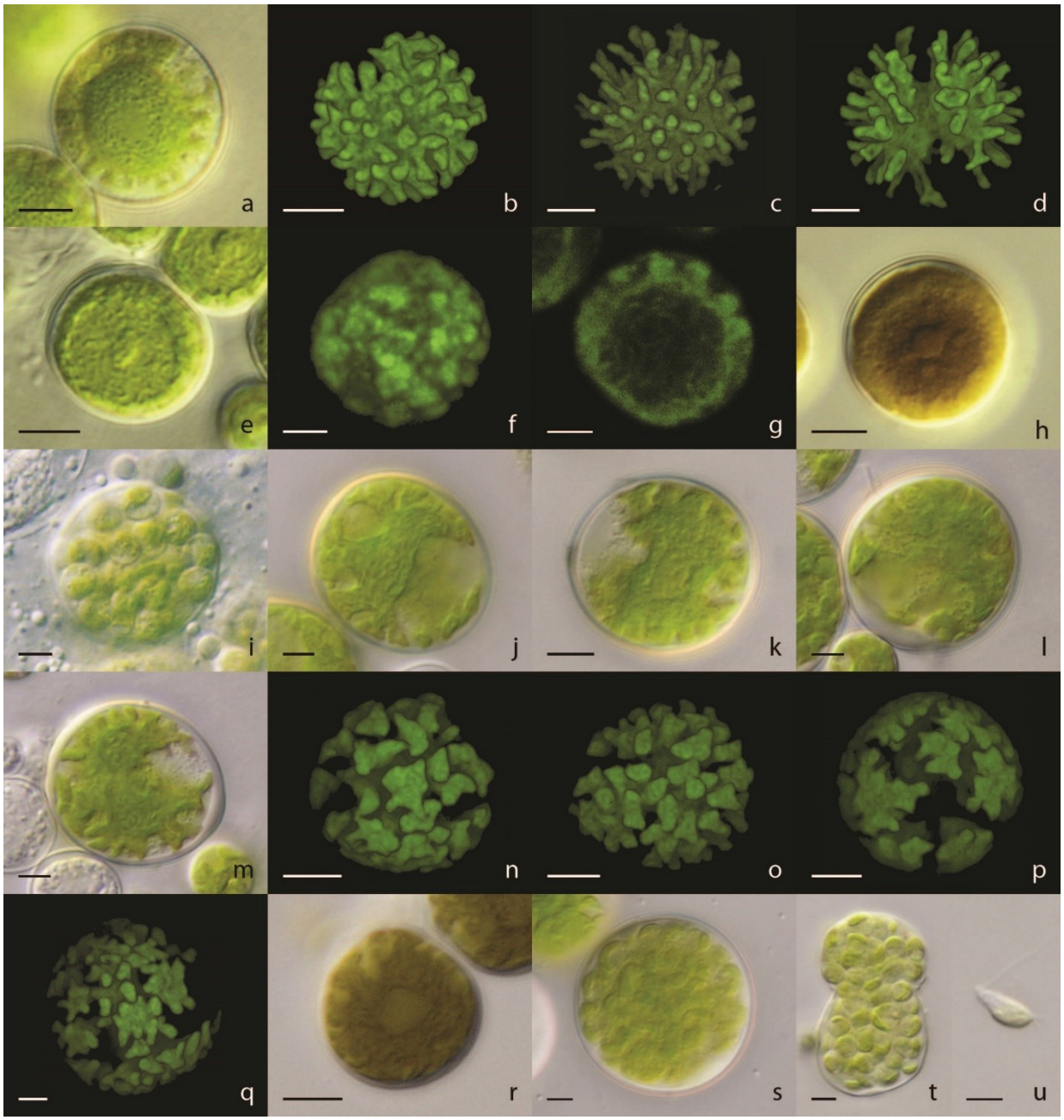

Fig. 8. Light micrographs and confocal reconstructions of chloroplast structures in *Asterochloris echinata* and *A. lobophora*. (a–i) *A. echinata*. (a) Light micrograph of mature vegetative cell. Confocal reconstructions of crenulate (b) and echinate (c, d) chloroplast. Light micrograph (e) and confocal reconstruction (f) of globular chloroplast. (g) Confocal section through the globular chloroplast. (h) Several pyrenoids occur around the large central pyrenoid. (i) Aplanosporangium. (j–u) *A. lobophora*. Light micrographs of deeply lobed (j) and shallowly lobed (k) chloroplasts. (l) Vegetative cell with a flat local thickening of the cell wall. (m) Mature vegetative cell with the thickened cell wall. Confocal reconstructions of shallowly lobed (n), crenulate (o), and parietal (p, q) chloroplasts. A single pyrenoid is situated in the chloroplast lumen (r). (s) Mature aplanosporangium. (t) Liberated aplanospores. (u) Zoospore. Cells in Figure (h, r) stained by chloriodine solution; scale bar - 5 µm.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. *Asterochloris* sequences used in phylogenetic reconstruction an haplotype networks, including strain and sample numbers, mycobiont species, geographic origin, and GenBank accession numbers for the ITS rDNA, actin, SSU rDNA, and rbcL loci.

Table S2. Morphological characteristics of investigated *Asterochloris* strains.

5.4 Paper 4

Steinová J., Škaloud P., Yahr R., Bestová H. and Muggia L.:

REPRODUCTIVE AND DISPERSAL STRATEGIES SHAPE THE DIVERSITY OF MYCOBIONT-PHOTOBIONT ASSOCIATION IN LICHEN SYMBIOSES

Submitted manuscript.

Reproductive and dispersal strategies shape the diversity of mycobiont-photobiont association in lichen symbioses

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ABSTRACT

Ecological preferences, partner compatibility, or partner availability are known to be important factors shaping obligate and intimate lichen symbioses. We considered a complex of four *Cladonia* species, differentiating by the extent of sexual reproduction and the type of vegetative propagules, to assess if the reproductive and dispersal strategies affect mycobiont-photobiont association patterns. In total 85 lichen thalli from 72 European localities were studied, two genetic markers for both *Cladonia* mycobionts and *Asterochloris* photobionts were analyzed. Variance partitioning analysis by multiple regressions on distance matrices was performed to describe and partition variance in photobiont genetic diversity. The asexual *Cladonia* species were found to be strongly selective towards their photobionts two closely related *Asterochloris* species. In contrast, sexually reproducing lichens associated with seven unrelated *Asterochloris* lineages, thus being photobiont generalists. The reproduction mode had the largest explanatory power, explaining 44% of the total photobiont variability. Reproductive and dispersal strategies are the key factors shaping photobiont diversity in this group of lichens. A strict photobiont specialisation observed in two studied species may steer both evolutionary flexibility and responses to ecological changes of these organisms, and considerably limit their distribution ranges.

Key words: *Asterochloris*; *Cladonia*; lichen distribution; reproductive strategy; specialist pattern; symbiosis.

INTRODUCTION

Lichens, as one of the most spectacular examples of mutualistic symbiotic associations, result from interdependent relationships between heterotrophic fungi, the mycobionts, and one or more population(s) of photosynthetic partners, the photobionts, these being either green or blue-green algae or both (Hawksworth and Honegger, 1994). The obligate and intimate associations between mycobionts and photobionts can lead to co-evolution of both partners and to concerted diversification (del Campo et al., 2013; Rambold et al., 1998). These processes are in many cases conditioned by the ecological

preferences for one or both partners and by the degree of partner specificity, with possibilities spanning from generalist associations for both partners, to strong reciprocal specificity, or any of a range of intermediate outcomes including local ecological specialization (Belinchón et al., 2015; Otálora et al., 2010; Yahr et al., 2004). In lichens, species distributions and ecological adaptations to a certain niche depend on abiotic conditions, such as the substrate, the availability and the different requirements of light, habitat quality and climate (Bannister et al., 2004; Giordani and Incerti, 2007). Lichen distributions have been hypothesized to strongly correlate, however, with the ecological specialization and the physiological responses of the photobionts (e.g., Casano et al., 2011; Peksa and Škaloud, 2011; Yahr et al., 2006).

The degree of partner specificity (defined as the range of compatible partners for a given symbiont; (Yahr et al., 2004, 2006), is usually considered as that of the mycobiont towards the photobiont, and it has been correlated with the distributional range of the mycobionts (e.g., Blaha et al., 2006; Fernández-Mendoza et al., 2011; Muggia et al., 2014). In cosmopolitan lichen species-complexes with wide ecological amplitude, low photobiont selectivity apparently allows the mycobiont to establish successful symbioses with locally adapted photobionts in a multitude of habitats (Muggia et al., 2014). Alternatively, a widely distributed, but ecologically more restricted mycobiont species was reported to have a narrower photobiont range, likely explained by habitat-scale factors (Domaschke et al., 2012; Fernández-Mendoza et al., 2011).

Diverse reproductive strategies, interaction regimes between lichen species and interactions of mycobionts with the available photobionts have been associated with different patterns of fungal-algal specificity (Belinchón et al., 2015; Cao et al., 2015; Fedrowitz et al., 2012, 2011). While algal cells reproduce clonally by cell division inside the thallus, the different reproductive and dispersal modes which the mycobionts develop account for evolutionary advantages and drawbacks of the symbioses. Asexual propagules (in some species even thallus fragments), represent clonal diaspores in which the mycobiont and its compatible algal partner are co-dispersed. Soredia are tiny, abundant, powdery propagules of fungal hyphae wrapped around photobiont cells that detach easily from the thallus. Isidia, granules, plates and microsquamules are outgrowths of the thallus in which photobiont cells are enclosed by a cortex of fungal hyphae. Soredia are lighter and smaller in size than the corticate propagules (see Fig. 1), and potentially allow greater dispersal distances from the parent thallus (Büdel and Scheidegger, 2008). Asexual reproduction circumvents problems of low symbiont availability (Wornik and Grube, 2009), but it reduces the opportunities for adaptive evolution (Eckert, 2002). It is hypothesized that clonal dispersion can lead to high co-evolutionary rate of the two symbionts and their specialization to certain niches but it might decrease the genetic diversity of both partners (Otálora et al., 2012; Wornik and Grube, 2009). Alternatively, a sexually reproducing lichen mycobiont disperses independently by spores and must re-synthesize the thallus with a suitable photobiont. The thallus re-synthesis requires the presence of compatible algae in the environment where the spore germinates and is triggered by the degree of preference by the fungi towards the available photobionts (e.g., Beck et al., 1998; Honegger,

2008, 1993; Ott, 1987; Yahr et al., 2004). Though sexually reproducing lichen fungi have to re-establish the symbiosis *de novo* every time, this type of reproduction increases the genotypic diversity and the successful dissemination by long range dispersal (Bailey, 1976; Belinchón et al., 2015; Pyatt, 1973; Werth et al., 2006). In addition, many closely related lichen species present either sexual or asexual reproductive structures, or both, and they proved to be, therefore, ideal subjects to investigate the dispersal patterns and the genetic diversity of the symbionts (Bannister et al., 2004; Cao et al., 2015; Otálora et al., 2012; Wornik and Grube, 2009).

The genus *Cladonia* is a group of lichenized fungi for which asexually and sexually reproducing taxa are known, and in some species both vegetative propagules and apothecia producing ascospores are present. *Cladonia* is distributed world-wide and is one of the most species-rich and morphologically most distinctive genera of lichenized fungi (over 400 described species, Ahti, 2000; Stenroos et al., 2002). *Cladonia* species are also known for their high specificity towards the green photobiont genus *Asterochloris* (Trebouxiophyceae; Bačkor et al., 2010; Beiggi and Piercey-Normore, 2007; Nelsen and Gargas, 2006; Piercey-Normore et al., 2010; Škaloud et al., 2015; Yahr et al., 2004, 2006). In the same way, the genus *Asterochloris* has been found to associate only with a limited number of lichenized fungal genera which share similar ecological conditions, and these are correlated with the environmental factors preferred by *Asterochloris* photobionts (Peksa and Škaloud, 2011).

Recent phylogenetic analyses coupled with microscopy observations disentangled 20 phylogenetic lineages within genus *Asterochloris* and seven new species were described and characterized by genetic diversity, morphological and anatomical traits (Moya et al., 2015; Škaloud et al., 2015, 2010). The species diversity of *Asterochloris* was recorded across multiple, ecologically diverse lichen species (Škaloud et al., 2015; Škaloud and Peksa, 2010) but has never been investigated within a group of closely related lichens so far.

In the *Cladonia-Asterochloris* symbioses different patterns of selectivity and specificity of the mycobionts towards the photobiont have already been documented (Piercey-Normore and DePriest, 2001; Yahr et al., 2004, 2006). The role of different factors possibly shaping the algal-fungal association in *Cladonia* symbiosis was evaluated on *Cladonia subtenuis* by Yahr et al. (2006). The authors demonstrated that geographic position and habitat are the best predictors of algal genotype distribution. However, the relation of the photobiont diversity and the dispersal mode(s) of the mycobionts has not been studied so far in this group of lichens.

Here we considered a complex of four red-fruited *Cladonia* species which are characterized by sharing chemical patterns but are differentiated by the type of vegetative propagules (soredia, granules, plates or microsquamules) and by the incidence of producing sexually reproductive structures (apothecia with ascospores; Fig. 1). Because esorediate species present heavy, corticated, vegetative propagules (Fig. 1) (plates, granules or microsquamules) which are produced in small amounts and are firmly attached to the podetial surface, and regularly build sexual reproductive structures (apothecia, producing spores in

large amounts), they have been considered here to reproduce mainly sexually. In contrast, sorediate species are seldom recovered with apothecia (Ahti et al., 2013) and therefore their primary dispersal mode has been hypothesized to be asexual and to depend on the small, light and ecorticate soredia produced in large amounts. Indeed, Molina et al., (2013) demonstrated that the viability of spores of the mixed lichen species *Physconia grisea* is much lower compared to its related sexual species (*P. distorta*).

In this context, we set out to test whether the type of reproductive strategy is the key factor shaping photobiont diversity in a group of four *Cladonia* lichen species across a broad geographical scale. In particular, we tested two main hypotheses: i) in sorediate species, in which mycobiont and photobiont are assumed strictly to co-disperse by soredia, the mycobionts show higher specificity towards their photobiont than the esorediate species; ii) the photobiont diversity of esorediate species is determined by their sexual reproduction and not by the vegetative dispersal of their propagules (plates, granules or microsquamules). To strengthen these hypotheses, the photobiont diversity recovered was tested against the genetic distance of the mycobionts, as well as the geographic, climatic and reproductive variances.

MATERIAL AND METHODS

Taxon sampling

The four *Cladonia* taxa here treated are: *C. deformis* (L.) Hoffm., *C. pleurota* (Flörke) Schaer, *C. coccifera* (L.) Willd. and *C. diversa* Asperges ex S. Stenroos. These lichens are characterized by the production of rhodocladonic acid causing the red coloured hymenium, and by forming scyphi (cups). They share a similar secondary metabolite pattern (presence of usnic acid derivates and zeorin occasionally accompanied by porphyrilic acid) but differ by the type of vegetative propagules and by the incidence of producing sexually reproductive structures (Ahti et al., 2013; Stenroos, 1989). *C. deformis* and *C. pleurota* present mainly soredia of different size and seldom produce apothecia (Stenroos, 1989); *C. coccifera* and *C. diversa* build heavier corticate propagules, such as granules, microsquamules or plates, and often bear apothecia (Stenroos, 1989) (Fig. 1). The four species occur in habitats with low rate of competition by vascular plants, e.g., on sandy and rocky acidic soils, on soil in rock crevices; they are seldom found on bark or rotten wood on siliceous bedrock. In Europe the sorediate species *C. deformis* and *C. pleurota* are common in the Northern Scandinavian countries and in Central Europe. In British Isles, Western and Southern Europe they are usually restricted to mountains. On the other hand the esorediate taxa *C. coccifera* and *C. diversa* have broader distributions, and they dominate in areas where sorediate species are very rare (e.g., British Isles, Western Europe). *C. coccifera* is widespread in Europe growing from arctic to warm temperate areas (Ahti et al., 2013). C. *diversa* shows oceanic tendencies, is rather rare in the area of Fennoscandia and avoids high altitudes (Ahti and Steinová, personal observation).

A total of 85 lichen thalli from 72 localities in Europe were included in this study (see Table 1, Fig. 2). The lichen material was freshly collected or retrieved from herbarium collections (BG, C, CBFS, GZU,

H, MACB, NMW, PL, PRA). The starting dataset for the mycobionts was the one published by (Steinová et al., 2013) and it was here complemented with additional 44 new samples. A total of 43 specimens of the two sorediate species, *C. deformis* and *C. pleurota*, and 42 specimens of the esorediate species, *C. coccifera* and *C. diversa*, were used for molecular analyses. At five localities in the Czech Republic and in Germany we collected both sorediate and esorediate species growing up to 10 m from each other (Table 2). The specimens were determined using morphological and chemical characters. The presence of zeorin, the key trait of this lichen species complex, was confirmed by thin-layer chromatography (TLC) according to (Orange et al., 2001).

DNA extraction, PCR and sequencing

Dry lichen material was ground to powder and was used for DNA extraction following either the CTAB protocol (Cubero et al., 1999) or the DNA extraction kit InstaGene Matrix (Bio-Rad). Genetic loci were analyzed for both the mycobionts and the photobionts. The fungal ITS region and an intron-containing portion of the β-tubulin gene were amplified as described in Steinová et al. (2013). The algal ITS rRNA gene was amplified using the algal-specific amplification primers ITS1T and ITS4T (Kroken and Taylor, 2000). The actin type I locus was amplified with primers actin_F and actin_R (Cocquyt et al., 2010). PCR conditions were applied as in Steinová et al. (2013) and Muggia et al. (2014). The PCR products were visualized on a 1% agarose gel stained with ethidium bromide and subsequently cleaned using the QIAquick PCR Purification Kit (Genomed) according to manufacturer's instructions. PCR products were sequenced with the same forward and reverse primers used for the PCR amplifications at Macrogen Corp. (Amsterdam, The Netherlands).

Sequence alignment and phylogenetic analyses

The new obtained sequences were assembled using the software SeqAssem (Hepperle, 2004) and checked for their identity in the GenBank database by blast similarity search (Altschul et al., 1990). The sequences were aligned using MAFFT v.6 software (Katoh et al., 2002) under the QINS-I strategy. Ambiguous SNPs and aligned regions were estimated using the program Gblocks v.0.91b (Castresana, 2000) and were excluded from the alignment. Beginning and ending parts of the sequences containing missing data were also removed from the alignment. For a number of specimens we were unable to generate sequences for all of the selected loci. Additional mycobiont and photobiont sequences were retrieved from the previous study by Steinová et al. (2013) and from GenBank and included in the dataset (Table 1). Identical sequences were removed to speed-up the analyses.

Two different multilocus alignments were prepared for the phylogenetic analyses: *(i)* the fungal ITS rRNA concatenated with β-tubulin genes alignment, *(ii)* the algal ITS rRNA gene concatenated with actin alignment. Photobiont sequences were selected to encompass all known lineages of *Asterochloris* (Bačkor et al., 2010; Škaloud et al., 2015) for which data of both loci were available.

The phylogenetic network analyses of *Cladonia* mycobionts was conducted with the program SplitsTree 4 (Huson and Bryant, 2006) as in our previous study (Steinová et al., 2013). The consensus network

based on the combined dataset of ITS rRNA and β-tubulin genes sequences was reconstructed using NeighborNet analysis option.

We used single locus trees analyses to detect possible phylogenetic conflicts between the *Asterochloris* photobiont ITS rRNA and the actin genes. As both phylogenies resulted in congruent topologies we used the concatenated dataset for the final analysis.

The phylogenetic analyses were performed with Bayesian inference (BI), Maximum Likelihood (ML) and weighted Maximum Parsimony (wMP) approaches. Models of molecular evolution were selected independently for the two photobiont loci, ITS rRNA and actin genes, according to the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (Darriba et al., 2012). The models applied were the TIM2ef+G for the photobiont ITS rRNA gene partition, and the TrNef+G for the actin partition. A Bayesian analysis was implemented using MrBayes version 3.2.1 (Ronquist et al., 2012). Two parallel MCMC runs were carried out for five million generations, each with four chains. Trees and parameters were sampled every 100 generations. The convergence of the chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). Further, the log-likelihood scores were plotted against generation time using Tracer 1.4 (Rambaut and Drummond, 2007) to determine when the stationarity of likelihood values have been reached (e.g., the burn-in stage; Ronquist et al., 2012). Burn-in was set at one million generations and the majority rule consensus trees were calculated from the posterior samples of 40000 trees. The SDSF value between simultaneous runs was 0,006174 in the concatenated dataset. ML and MP phylograms were obtained using Garli version 2.0, and PAUP version 4.0b10 (Swofford, 2002), respectively. The same programs were used for the bootstrap analyses. ML analyses consisted of rapid heuristic searches (100 pseudo-replicates) by using automatic termination (the genthreshfortopoterm command set to 100000). The weighted parsimony (wMP) bootstrapping (1000 replications) was performed using heuristic searches with 100 random sequence addition replicates, tree bisection reconnection swapping, random addition of sequences (the number limited to 10000 for each replicate), and gap characters treated as a fifth character state. The weight to the characters was assigned using the rescaled consistency index on a scale of 0 to 1000. New weights were based on the mean of the fit values or each character over all of the trees in memory. The phylogenetic trees were visualized in TreeView (Page, 1996).

Environmental data

Climatic variation was modeled using climatic database WorldClim (Hijmans et al., 2005) at resolution of 2.5 arc minutes. Principal component analysis of 19 bioclimatic variables was used to reduce dimensionality (for list of bioclimatic variables and PCA biplot see Supplementary material Fig. S1 and Table S1). The first PC axis explained 40.39% of variability and was mainly following a precipitation gradient. The second axis corresponded to temperature range and seasonality gradient and explained 31.52% of variability in the climatic data set. We used scores of sites for PC1 and PC2 as climatic scores for further analyses.

Variance partitioning

We performed variance partitioning by multiple regression on distance matrices (MRM; Legendre et al., 1994; Lichstein, 2006; Manly, 1986; Smouse et al., 1986) to describe and to partition variance in photobiont genetic diversity. This method computes adjusted R^2 for the complete model, estimating how much of the total variability is defined by explanatory variables. It also allows estimating R^2 for each of the explanatory variables as well as their shared components. The photobiont genetic distance matrix was used as response matrix, whereas genetic distance of the mycobionts as well as geographic, climatic and reproductive distances were used as explanatory matrices. Pairwise genetic distances of photobionts was obtained from the alignment of ITS rDNA using JC69 model of evolution (Jukes and Cantor, 1969). The JC69 model was also used for the concatenated fungal ITS rRNA and β-tubulin genes alignment. Geographic distances were calculated from longitudinal and latitudinal data of the sampling localities. A climatic similarity matrix was calculated as Euclidean distance between scores of PC1 and PC2. Similarity in reproductive strategy (asexually vs. sexually) was defined as Jaccard distance (Jaccard, 1912). All the analyses of the variance partitioning were performed using R (ver. 3.0.3; R Development Core Team).

RESULTS

The final dataset contained 271 sequences, 187 of which were newly obtained in this study: 80 algal ITS rRNA sequences, 28 algal actin sequences, 38 fungal ITS rRNA sequences and 41 fungal β-tubulin sequences (Table 1). Additional 84 sequences (both fungal and algal) were retrieved from our previous datasets (Bačkor et al., 2010; Škaloud et al., 2015; Steinová et al., 2013) and from GenBank. Fungal ITS rRNA and β-tubulin sequences were 547 and 673 base pairs in length, respectively, with 69 and 79 variable and 52 and 47 informative characters, respectively. For algal loci, ITS rRNA sequences were 509 base pairs long, with 76 variable characters of which 58 were parsimony-informative. Algal actin sequences were 576 base pairs long with 392 variable sites and 342 parsimony-informative sites.

Phylogenetic network analyses of *Cladonia* mycobionts

The consensus network of ITS rRNA and β-tubulin sequences (see Fig. 3) resulted in conflicting relationships among species. The broad edges at the core of the network and the absence of long branches (except one subgroup of *Cladonia pleurota* containing specimens C6, CL67, CL73, CL100 and CL128) suggested incomplete lineage sorting or ongoing speciation among the four studied taxa, and corroborate the results previously shown by Steinová *et al.* (2013). Four samples of *C. coccifera* (CL178, CL179, CL379 and CL396) were inferred on isolated splits and remained separated from both the sorediate and esorediate groups.

Phylogenetic analyses of the *Asterochloris* photobionts

The Bayesian, MP and ML phylogenies resulted in similar topologies and were in congruence with the phylogenetic inference of Škaloud et al. (2015). Bayesian analysis of the concatenated ITS rDNA and actin dataset resulted in 19 well-resolved *Asterochloris* lineages (see Fig. 4). Thirteen clades represented already described *Asterochloris* species, the remaining six have not been assigned a name yet (two of which were preliminary identified by the names "clade 8" and "clade A9"). The species *A. excentrica* was represented by a single sequence and was on its own single branch. The clades were fully resolved and the majority of them were highly supported. A total of eight *Asterochloris* lineages plus two *Asterochloris* sequences, which were recovered on individual branches and did not correspond to any currently recognized lineage, were found to associate with the four *Cladonia* taxa. *Asterochloris glomerata*, *A. italiana* and *A. irregularis* were the most common photobionts and were recovered in 31, 24 and 18 samples, respectively.

All samples of the sorediate *Cladonia* species associated only with *A. glomerata* and *A. irregularis*. However, *A. irregularis* was also the photobiont of the esorediate mycobiont species, whereas *A. glomerata* associated exclusively with the sorediate mycobionts. The esorediate *Cladonia* species, on the other hand, exhibited a much lower level of specificity towards the associated photobionts, as seven *Asterochloris* lineages and those two *Asterochloris* sequences recovered on individual branches were found to associate with these. *C. coccifera* was found to associate with six *Asterochloris* lineages (*A. irregularis*, *A. woessiae*, *A. italiana*, *A. lobophora*, *Asterochloris* clade 8 and clade A9) and the two unique *Asterochloris* sequences. *C. diversa* was found to associate with four *Asterochloris* species (*A. irregularis*, *A. echinata*, *A. italiana* and *A. lobophora*).

Asterochloris italiana was the most frequent photobiont associated with the esorediate *Cladonia* taxa (24 samples), and two subclades were recognized: the first containing samples from Belgium, Denmark, Netherlands, Spain, Wales and from lower altitudes from Czech Republic, the second comprising samples from higher elevation in Czech Republic, Austria, Germany and Spain. Specimens of sorediate (CL85, CL99, CL101, CL128, CL355, CL356, and CL393) and esorediate (CL86, CL142, CL364, CL392, and CL394) samples which were collected in the same localities were always found to associate with different photobionts respectively.

Clear differences of photobiont distribution across Europe and in *Cladonia* thalli could be recognized (Fig. 2b). *A. italiana* was mostly recovered from localities spread in the North-Western oceanic part of Europe, including Great Britain, Denmark, Belgium, The Netherlands and the Norwegian coast, but also from Central Europe (Austria, Czech Republic and Germany), Portugal and Spain (Table 1). In the North-Eastern Fennoscandia we detected only *A. glomerata* and *A. irregularis* (associated both with sorediate and esorediate fungal species). *Cladonia* samples collected in Central and Southern Europe associated with a considerably higher number of *Asterochloris* lineages. Although we included only six *Cladonia* samples from the Iberian Peninsula, we found four *Asterochloris* lineages associated with the mycobionts in this region (*A. echinata*, *A. irregularis*, *A. italiana* and *A. woessiae*). Samples collected

in Central Europe contained seven *Asterochloris* lineages and the two unique *Asterochloris* sequences. The Austrian Alps and the Krkonoše Mts. in the Czech Republic were the regions with the richest *Asterochloris* diversity detected in Central Europe.

Variance partitioning

The PCA of bioclimatic variables revealed two main gradients. The first PC axis explained 40.39% of variability and was mainly following a precipitation gradient. The second axis corresponded to the temperature range and seasonality gradient and explained 31.52% of variability in the climatic data set (see PCA biplot in Supplementary material Fig. S1). In downstream analyses, we use scores of sites for PC1 and PC2 to represent climatic conditions.

Our linear regression model including reproductive strategies, geographical distance, climatic similarity and genetic distance of mycobiont significantly explained altogether 46.16% of variability in the genetic distance of photobionts (Fig. 5). The reproduction mode had the largest explanatory power. It explained 43.90% of variability, though a large portion was also represented by the covariance with the mycobiont genetic distance. The isolated effect of reproduction mode, when accounted for covariance with all other explanatory matrices, was associated with 16.7% of variability in genetic distance of photobiont (Fig. 5). The other explanatory variables accounted only for minor percentages of variability, although they were selected as significant for the complete linear model. A substantial percentage of variability remained unexplained by our model (53.8%) and might account for some unmeasured environmental heterogeneity, or alternatively, a degree of stochasticity in associations.

DISCUSSION

Symbiotic diversity is shaped by host reproductive strategies

Mutualistic interactions offer suitable examples to study co-evolution, partner selectivity, evolutionary responses and ecological adaptations to symbiotic lifestyles (Bronstein et al., 2004). So far only few studies have evaluated the influence of reproductive strategies of mycobionts on the specificity and selectivity of photobiont associations (Cao et al., 2015; Fedrowitz et al., 2011; Otálora et al., 2010; Wornik and Grube, 2009). The selected complex of the four *Cladonia* taxa is well suited to test multiple hypotheses in this context. Because the mycobionts of sorediate species rarely build sexual reproductive structures, they have been hypothesized to reproduce and disperse asexually. The prevailing asexual dispersal mode would logically justify specific mycobiont-photobiont associations, because fungi and algae are co-dispersed. Alternatively the esorediate taxa, in which mycobionts abundantly produce apothecia, are hypothesized to reproduce mainly sexually by ascospores. By reproducing sexually, these mycobionts could show different levels of specificity towards their photobiont. Within the broad spectrum of their geographic distribution in Europe, our results show that the two sexually reproducing *Cladonia* species adopted a generalist strategy (by associating with numerous *Asterochloris* lineages).

In contrast, the asexually reproducing species were associated exclusively with *A. glomerata* or *A. irregularis* even in localities where other *Asterochloris* species were detected in the sexually reproducing species (Table 2). The strict maintenance of these two *Asterochloris* lineages over large geographic distances indicates high selectivity towards its photobionts (Fedrowitz et al., 2012). A similar pattern of high mycobiont specificity towards its symbionts in asexually reproducing lichens, coupled with a low level of specificity in sexually reproducing lichens, was observed also previously in other *Cladonia* species (Yahr et al., 2004, 2006) and in *Nostoc*-associating *Nephroma* and *Degelia* species by (Fedrowitz et al., 2011; Otálora et al., 2012).

All *Cladonia* species studied here produce some kind of vegetative propagules. We hypothesize that the different specificity of the mycobiont toward its photobiont can be attributed to the differences in size and amounts of the vegetative propagules built on the podetium, and the ability to produce viable ascospores. The role in the lichen dispersal of the relatively big vegetative propagules of esorediate *Cladonia* species is likely very limited, and the mycobiont dispersion is ensured by the ascospores produced in the always abundant ascomata. Therefore, the low selectivity and specificity towards the algal partner is advantageous for the esorediate *Cladonia* fungi, which have to find a suitable algal partner shortly after their germination. On the contrary, soredia represent abundant and light vegetative propagules that detach easily and can replace ascospores as the main dispersal propagules with all pros and cons of this strategy. Interestingly, it has been reported that ascospores of sorediate lichens can have a strongly reduced reproductive function (Molina et al., 2013). The authors compared the spore viability between the mixed species *Physconia grisea* and the related sexual species *P. distorta* and showed that mature apothecia from both species discharged meiospores capable of germination, but spores from *P. grisea* rarely (0.43%) developed, whereas those from *P. distorta* developed and germinated successfully.

The algal genetic diversity in populations of lichenized fungi having distinct propagation strategies is, however, not always necessarily different. This can be explained by the switch of algal partners, denoted as “algal switching” (Piercey-Normore and DePriest, 2001). Successful horizontal photobiont transmission is common in sexually reproducing lichen fungi, but can take place also in asexual species. *Physconia* species, though reproducing vegetatively by soredia, presented an unexpected high photobiont diversity (Wornik and Grube, 2009). It was suggested that depending on the viability of the soredial algae, the soredial fungi could choose between establishing the new thallus with the co-propagated alga or with another photobiont, likely better adapted to the local conditions. The main role of the original photobiont would be to prolong the survival of the co-propagated fungal hyphae (Wornik and Grube, 2009).

Photobiont switching is now understood as a rather common phenomenon in lichen symbiosis (e.g., Nelsen and Gargas, 2009; Piercey-Normore, 2006) allowing the mycobiont to adapt to local environmental conditions (Werth and Sork, 2010) or to extend its geographical range or ecological niche (Fernández-Mendoza et al., 2011). The asexual *Cladonia* fungi, by having the possibility to switch algae, would be guaranteed the option of either maintaining their algal partner, or replacing it if a more

suitable/better-adapted one is available. Other symbiotic systems, such as corals, are well known for their *in situ* adaptation and capacity to regulate their fitness according changing ecological conditions. Studies on *Anthopleura-Symbiodinium*/*Elliptochloris* symbioses have shown how the presence and the identity of the photobionts in different environmental conditions balance the life and the reproductive strategies of the anemone host (Bingham *et al.*, 2014). In lichens the maintenance of the symbiotic association would, therefore, be an option but not a strict consequence of the joint, vegetative symbiont propagation (Wornik and Grube, 2009).

Our results show that the studied asexual *Cladonia* species do not switch photobionts, and we suggest that this may result in severe consequences for their survival in a changing environment. The ability to switch the photobionts allows the fine-tuned symbiosis to be flexible and resilient over geographic and environmental gradients in space and time, while a very selective mutualism may lead to its termination (Nelsen and Gargas, 2009). This is particularly true for asexually reproducing lichens in which substantial proportion of the evolutionary flexibility has already been lost by the absence of sexual reproduction, thought to be beneficial to the longevity of a species (Muller, 1932). Clonal reproduction via vegetative propagules helps to overcome the problem of limited availability of symbiotic partners. However, such a tight and rigid relationship between symbiotic partners together with the loss of adaptability by strictly asexual reproduction may become an evolutionary trap in the long term.

In corals, the adaptive bleaching hypothesis (Buddemeier and Fautin, 1993) explains that process in which the animal hosts reshuffle their photosynthetic symbiont to overcome environmental changes and survive (Baker, 2003; Parkinson and Baums, 2014). The studied *Cladonia* species are found in diverse ecological conditions, and we demonstrate that the photobionts are significantly structured by both climate and geography, although the explanatory power of these is smaller compared to the mode of reproduction. In the future, however, they might face severe environmental changes. If they will not evolve the ability to switch to locally adapted photobionts, they may become rare or even go extinct (Domaschke et al., 2012), as has already been shown in other systems (LaJeunesse et al., 2003).

Can the distribution of hosts be substantially controlled by the symbionts?

We observed two main patterns of *Asterochloris* diversity across Europe: i) high *Asterochloris* diversity within relatively small geographic regions, and ii) wide geographic areas dominated by only one or two *Asterochloris* species. The first group was represented in Czech Krkonoše Mts. and the Austrian Alps, where we found five *Asterochloris* lineages and a single *Asterochloris* isolate, belonging to a still unnamed taxon: these were associated with seven and ten *Cladonia* specimens respectively. The Central European mountains seem therefore to represent hotspots of *Asterochloris* species richness. In contrast, the North-Western oceanic parts of Europe were dominated by *A. italiana*. In Nordic countries (Norway and Finland), *Cladonia* species were found to associate mostly with *A. glomerata* and *A. irregularis*. This can be explained either by the preference of the *Cladonia* species to associate with those *Asterochloris* lineages adapted to the local environmental conditions or by a very low *Asterochloris*

diversity in these areas. The low *Asterochloris* diversity in the area of Fennoscandia may be caused by environmental filtering (Dal Grande et al., 2017) or, alternatively, can possibly be the consequence of the recolonization history after the last glacial maximum (e.g., Hoarau et al., 2007). This finding would contradict the hypothesis of ubiquitous distributions of microorganisms caused by their high dispersal rates (Fenchel and Finlay, 2004; Finlay, 2002; Lowe et al., 2012; Ryšánek et al., 2015). However, patterns similar to those observed here have already been reported for other symbiotic protists (Domaschke et al., 2012; LaJeunesse et al., 2010) and might well be explained by the co-propagation of both symbiotic partners constraining their distributional ranges. Further study of other potential *Asterochloris* hosts in Fennoscandia could help to test this hypothesis.

There is strong evidence that symbiotic organisms associate preferentially with locally adapted partners, both in lichens (e.g., Dal Grande et al., 2017, 2012; Muggia et al., 2014) and other mutualistic associations (e.g., Finney et al., 2010; Pánková et al., 2014; Sampayo et al., 2007; Ulstrup and Van Oppen, 2003). This implies that low selectivity of the host towards its symbiotic partner(s) helps the host to take advantage of the locally adapted symbiotic partners and colonize larger geographic areas. In contrast, hosts which strictly associate with a limited number of symbiotic partners are expected to show narrower ecological width, resulting in more restricted geographical and/or ecological distribution. In symbiotic associations the generalist pattern is far more common and has been reported from coral-algae symbioses (Pochon and Pawlowski, 2006; Rowan et al., 1997), fungus-farming insects (Schlick-Steiner et al., 2008), mycorrhizae (Porras-Alfaro and Bayman, 2007), as well as lichens (Rikkinen et al., 2002). In contrast the specialist pattern is much rarer and has been reported for rare orchid species associating with a limited number of fungal species (Graham and Dearnaley, 2011; Swarts et al., 2010) or for *Nostoc*-associating lichen fungi (Otálora et al., 2010). In our study, the sexually reproducing *Cladonia* lichens were shown to be generalists, whereas asexual *Cladonia* can be considered specialists. Also, there are clear differentiations in the geographical distributions in Europe which correlate with the degree of mycobiont selectivity of the studied *Cladonia* species. The asexually reproducing *Cladonia deformis* and *C. pleurota* are common in boreal zone and in mountain regions all over Europe but are rare in North-Western oceanic part of Europe (Belgium, Denmark, Great Britain, Ireland, Netherlands). This is, at the same time, an area in which the photobiont *A. italiana* has been shown to dominate in this lichen group and where the less selective sexually reproducing lichens *C. coccifera* and *C. diversa* are common (Fig. 2). It is possible that the distribution of sorediate *Cladonia* species in this part of Europe may be limited by the local environmental conditions not suitable for the physiological optimum of their preferred photobionts *A. glomerata* and *A. irregularis*. Another possible explanation is that the performance of both interacting partners as one unit (holobiont) can be negatively affected by the local conditions (Dal Grande et al., 2017), although *A. glomerata* and *A. irregularis* may be present in the same geographical area associated with other mycobionts. This could be confirmed or ruled out by a more extensive sampling of other *Cladonia* species potentially harboring *A. glomerata* and *A. irregularis* in W Europe. These observations would then support the hypothesis that photobiont availability and its ability to cope with local environmental conditions may play key roles in shaping the distribution of

lichens which present high specificity towards their algal partner.

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TABLES

Table 1. List of the environmental samples used in the molecular analyses. DNA extraction numbers, voucher numbers, geographic origin and NCBI accessions for the new sequences (bold) obtained for both the mycobionts and the photobionts are reported.

Taxon name	DNA extraction No.	Collection No. (herbarium)	Locality	GenBank No.			
				photobiont		mycobiont	
				ITS	actin	ITS	β-tubulin
Cladonia coccifera	CL31	Haffelner 66608 (GZU)	Austria, Stubalpe, Größenberg	**KT989915**	-	HE611155	HE611207
	CL32	Haffelner 66785 (GZU)	Austria, Stubalpe, Ofnerkogel	**KT989916**	**xxxxxx**	HE611156	HE611208
	CL39	Haffelner 66214 (GZU)	Austria, Stubalpe, Lichtengraben	**KT989919**	**xxxxxx**	HE611157	HE611209
	CL52	Bouda 778 (PRC)	Czech Rep., Novohradské hory, Kraví hora	**KT989908**	**xxxxxx**	HE611158	HE611210
	CL60	Peksa 359 (PL)	Czech Rep., Lužické hory, Studenec	**KT989888**	**xxxxxx**	HE611159	HE611211
	CL68	Vondrák 4800 (CBFS)	Czech Rep., Kašperské hory, Obří hrad	**KT989909**	-	**KU053034**	**xxxxxx**
	CL86[1]	Steinová 97 (PRC)	Czech Rep., Brdy, Žďár	**KT989907**	-	**KU053046**	**xxxxxx**
	CL90	Steinová 43 (PRC)	Czech Rep., Krkonoše, Velká Kotelní jáma	**KT989920**	**xxxxxx**	HE611160	HE611212
	CL93	Steinová 81 (PRC)	Czech Rep., Českosaské Švýcarsko, Křepelčí důl	**KT989910**	-	HE611161	HE611213
	CL105	Steinová 401 (PRC)	Spain, Somosierra, arroyo de la Peña del Chorro	KP318669	**xxxxxx**	HE611162	HE611214
	CL124[2]	Steinová 160 (PRC)	Czech Rep., Sedlčansko, Drbákov-Albertovy skály	**KT989890**	**-**	**KU053015**	**xxxxxx**
	CL141	Steinová 242 (PRC)	Austria, Nockberge, Erlacher Bockhütte	**KT989921**	-	HE611163	HE611215
	CL143	Steinová 125 (PRC)	Czech Rep., Krkonoše, Obří důl	**KT989922**	**xxxxxx**	-	**xxxxxx**
	CL178	Steinová 332 (PRC)	Norway, Rondane, Eisenthøe	**KT989936**	**xxxxxx**	HE611171	HE611223
	CL179	Steinová 334 (PRC)	Finland, Heinola, Pirttijärvi lake	**KT989929**	**xxxxxx**	HE611172	HE611224
	CL347	Steinová 537 (PRC)	Austria, Steierische Randgebirge	**KT989911**	-	**KU053042**	**xxxxxx**
	CL374	Steinová 464 (PRC)	Norway, Hordaland, Bergen	**KT989904**	-	**KU053021**	**xxxxxx**
	CL375	Steinová 529 (PRC)	Wales, Ty Canol	**KT989896**	-	**KU053037**	**xxxxxx**
	CL376	Steinová 586 (PRC)	Czech Rep., Jizerské hory, Věžní skály	**KT989914**	-	**KU053038**	**xxxxxx**
	CL377	Steinová 528 (PRC)	Wales, St. Davids Head	**KT989897**	-	**KU053022**	**xxxxxx**
	CL379	Steinová 624 (PRC)	Finland, Sondby	**KT989930**	-	**KU053017**	**xxxxxx**
	CL381	Orange 20406 (NMW)	Wales, Anglesey, Holyhead Mountain	**KT989898**	-	**KU053011**	**xxxxxx**
	CL383	Steinová 639 (PRC)	Czech Rep., Krkonoše, Sněžka	**KT989905**	**xxxxxx**	**KU053048**	**xxxxxx**
	CL394[3]	Steinová 642 (PRC)	Czech Rep., Ještěd	**KT989917**	-	**KU053041**	**xxxxxx**
	CL395	Steinová 650 (PRC)	Czech Rep., Krkonoše, Obří sedlo	**KT989918**	-	**KU053043**	**xxxxxx**
	CL396	Steinová 649 (PRC)	Czech Rep., Krkonoše, Sněžka	**KT989933**	-	**KU053016**	**xxxxxx**
	CL398	Søchting 12153 (C)	Denmark, Zealand, Melby Ovedrev	**KT989901**	-	**KU053023**	**xxxxxx**
C. diversa	CL54	Bouda 777 (PRC)	Czech Rep., Českosaské Švýcarsko, Babylon	**KT989889**	-	HE611164	HE611216
	CL106	Steinová 400 (PRC)	Portugal, Beira Alta, Parque Natural de Serra de Estrela	KP318671	**xxxxxx**	HE611165	HE611217
	CL130	Vondrák 6242 (CBFS)	Denmark, Bornholm	**KT989891**	**xxxxxx**	HE611166	HE611218
	CL172	Steinová 351 (PRC)	Belgium, Kalmthout, Van Ganzenven	**KT989892**	-	HE611167	HE611219
	CL173	Steinová 352 (PRC)	Belgium, Kalmthout, Van Ganzenven	**KT989927**	-	HE611168	HE611220

	CL363	Ahti 72006 (H)	Netherlands, Gelderland, Garderen	**KT989893**	-	**KU053047**	**xxxxxx**
	CL364[4]	Steinová 596 (PRC)	Germany, Saxony, Oberlausitzer Heide	**KT989912**	-	**KU053013**	**xxxxxx**
	CL367	Steinová 635 (PRC)	Spain, Asturias, Parque Natural de Redes	**KT989913**	-	**KU053035**	**xxxxxx**
	CL368	Steinová 634 (PRC)	Spain, Asturias, Parque Natural de Redes	**KT989894**	-	**KU053036**	**xxxxxx**
	CL370	Steinová 637 (PRC)	Czech Rep., Hradiště	**KT989906**	**xxxxxx**	**KU053044**	**xxxxxx**
	CL372	Ahti 68670 (H)	Norway, Hordaland, Bergen	**KT989895**	-	**KU053039**	**xxxxxx**
	CL392[5]	Steinová 616 (PRC)	Czech Rep., district Tábor, Mlýny	**KT989899**	-	**KU053014**	**xxxxxx**
	CL397	Søchting 28. X. 2013 (C)	Denmark, Jutland, Bredevandsbakker	**KT989900**	-	**KU053040**	**xxxxxx**
	CL400	Søchting 12154 (C)	Denmark, Zealand, Tisvilde Hegn	**KT989902**	-	**KU053024**	**xxxxxx**
	CL404	MACB 97615	Spain, Riofrío de Riaza, sierra de Ayllón	**KT989903**	-	-	-
C. deformis	CLAD 08	Peksa 918 (PL)	Czech Rep., Chvaletice	FM945357	-	HE611205	HE611257
	CL175	Steinová 330 (PRC)	Finland, Suomossalmi	**KT989946**	**xxxxxx**	HE611190	HE611242
	CL176	Steinová 336 (PRC)	Finland, Varkaus	**KT989928**	**xxxxxx**	HE611186	HE611238
	CL354	Pentti Alanko 150786 (H)	Finland, Suomenlinna	**KT989947**	-	**KU053019**	**xxxxxx**
	CL355[5]	Steinová 617 (PRC)	Czech Rep., district Tábor, Mlýny	**KT989961**	-	**KU053029**	**xxxxxx**
	CL356[4]	Steinová 603 (PRC)	Germany, Saxony, Oberlausitzer Heide	**KT989962**	-	**KU053026**	**xxxxxx**
	CL357	Steinová 627 (PRC)	Finland, Sondby	**KT989963**	-	**KU053027**	**xxxxxx**
	CL359	Steinová 587 (PRC)	Czech Rep., Krkonoše, Výrovka	**KT989964**	-	**KU053020**	**xxxxxx**
	CL360	Palice 16632 (PRA)	Czech Rep., Šumava	**KT989948**	-	**KU053030**	**xxxxxx**
	CL393[3]	Steinová 644 (PRC)	Czech Rep., Ještěd	**KT989932**	-	**KU053028**	**xxxxxx**
	CL401	Søchting 10. IX. 2013 (C)	Denmark, Harrild Hede	**KT989966**	-	**KU053031**	**xxxxxx**
	CL405	MACB 97100	Spain	**KT989940**	-	-	-
C. pleurota	Backor 18	Peksa 820 (PL)	Slovakia, Veľká Fatra, Harmanec	FM945370	-	HE611191	HE611243
	CLAD 06	Peksa 588 (PL)	Czech Rep., Chvaletice	FM945351	-	HE611181	HE611233
	CL26	Palice 11305 (PRA)	Czech Rep., Dolní Loučky	**KT989951**	**xxxxxx**	HE611193	HE611245
	CL36	Haffelner 65635 (GZU)	Austria, Stubalpe, Lahnhofen	**KT989941**	**xxxxxx**	HE611194	HE611246
	CL37	Haffelner 65828 (GZU)	Austria, Stubalpe, Lahnhofen	**KT989952**	-	**KU163444**	**xxxxxx**
	CL43	Peksa 562 (PL)	Czech Rep., Brdy, Hřebenec	**KT989942**	**xxxxxx**	HE611182	HE611234
	CL44	Peksa 564 (PL)	Czech Rep., Brdy, Hřebenec	**KT989953**	**xxxxxx**	HE611183	HE611235
	CL45	Peksa 563 (PL)	Czech Rep., Brdy, Hřebenec	**KT989943**	**xxxxxx**	HE611195	HE611247
	CL64	Vondrák 3631 (CBFS)	Romania, Retezat, Cheile Butii	**KT989954**	**xxxxxx**	HE611187	HE611239
	CL67	Vondrák 2868 (CBFS)	Czech Rep., Křivoklátksko, Na Andělu	**KT989955**	-	HE611173	HE611225
	CL73	Peksa 574 (PL)	Czech Rep., Chvaletice	**KT989956**	-	HE611174	HE61226
	CL74	Peksa 575 (PL)	Czech Rep., Radvanice	**KT989944**	-	**KU053025**	**xxxxxx**
	CL85	Steinová 103 (PRC)	Czech Rep., Brdy, Žďár	**KT989935**	-	HE611196	HE611248
	CL98	Steinová 45 (PRC)	Czech Rep., Krkonoše, Kotel	**KT989957**	-	HE611188	HE611240
	CL99	Steinová 99 (PRC)	Czech Rep., Brdy, Žďár	**KT989958**	-	HE611202	HE611254
	CL100	Steinová 65 (PRC)	Czech Rep., Slavkovský Les, Křížky	**KT989967**	**xxxxxx**	HE611176	HE611228
	CL101	Steinová 108 (PRC)	Czech Rep., Brdy, Žďár	**KT989923**	**xxxxxx**	HE611203	HE611255
	CL104	Steinová 126 (PRC)	Czech Rep., Brdy, Hřebenec	**KT989924**	**xxxxxx**	HE611185	HE611237
	CL125	Steinová 161 (PRC)	Czech Rep., Sedlčansko, Husova kazatelna	**KT989959**	-	**KU053032**	**xxxxxx**

	CL128[2]	Steinová 164 (PRC)	Czech Rep., Sedlčansko, Drbákov-Albertovy skály	**KT989960**	**xxxxxx**	HE611180	HE611232
	CL136	Steinová 215 (PRC)	Finland, Helsinki, Rastila	**KT989925**	**xxxxxx**	HE611200	HE611252
	CL148	Steinová 241 (PRC)	Austria, Gurktaler Alpen, Nassbodensee	**KT989945**	**xxxxxx**	HE611189	HE611241
	CL150	Steinová 187 (PRC)	Finland, Vantaa, Fagersta	**KT989926**	**xxxxxx**	HE611204	HE611256
	CL350	GZU 000303377	Monte Negro, Prokletije Mountain Range, Krš Bogićevica	**KT989937**	-	**KU053018**	**xxxxxx**
	CL385	Peksa 1722 (PL)	Czech Rep., Ledce, Krkavec	**KT989965**	-	**-**	-
	CL386	Steinová 551 (PRC)	Austria, Gurktaler Alpen, Hochrindl	**KT989938**	-	**KU053033**	**xxxxxx**
	CL388	Steinová 176 (PRC)	Austria, Koralpe, Weinebene	**KT989949**	-	**KU053045**	**xxxxxx**
	CL389	Steinová 312 (PRC)	Czech Rep., Slavkovský les, Dominova skalka	**KT989950**	-	-	**xxxxxx**
	CL390	Steinová 339 (PRC)	Norway, Rondane, Einsethøe	**KT989939**	-	-	-
	CL391	Steinová 341 (PRC)	Norway, Rondane	**KT989931**	-	-	-
	CL403	Tønsberg 42460 (BG)	Norway, Oppland, Lom, Breidsæterdalen	**KT989934**	-	-	**xxxxxx**

Table 2. Comparison of *Asterochloris* diversity recovered in five localities in which multiple samples have been collected. Samples in grey represent sorediate collections.

Locality ID	**Geographic origin**	***Cladonia* lichen species**	**sample ID**	***Asterochloris* photobiont**
1	Czech Rep., Brdy, Žďár	*C. coccifera*	CL86	*A. lobophora*
	Czech Rep., Brdy, Žďár	*C. pleurota*	CL85	*A. irregularis*
	Czech Rep., Brdy, Žďár	*C. pleurota*	CL99	*A. glomerata*
	Czech Rep., Brdy, Žďár	*C. pleurota*	CL101	*A. irregularis*
2	Czech Rep., district Tábor, Mlýny	*C. diversa*	CL392	*A. italiana*
	Czech Rep., district Tábor, Mlýny	*C. deformis*	CL355	*A. glomerata*
3	Czech Rep., Ještěd	*C. coccifera*	CL394	*clade 8*
	Czech Rep., Ještěd	*C. deformis*	CL393	*A. irregularis*
4	Czech Rep., Sedlčansko, Drbákov-Albertovy skály	*C. coccifera*	CL124	*A. italiana*
	Czech Rep., Sedlčansko, Drbákov-Albertovy skály	*C. pleurota*	CL128	*A. glomerata*
5	Germany, Saxony, Oberlausitzer Heide	*C. diversa*	CL364	*A. italiana*
	Germany, Saxony, Oberlausitzer Heide	*C. deformis*	CL356	*A. glomerata*

FIGURES

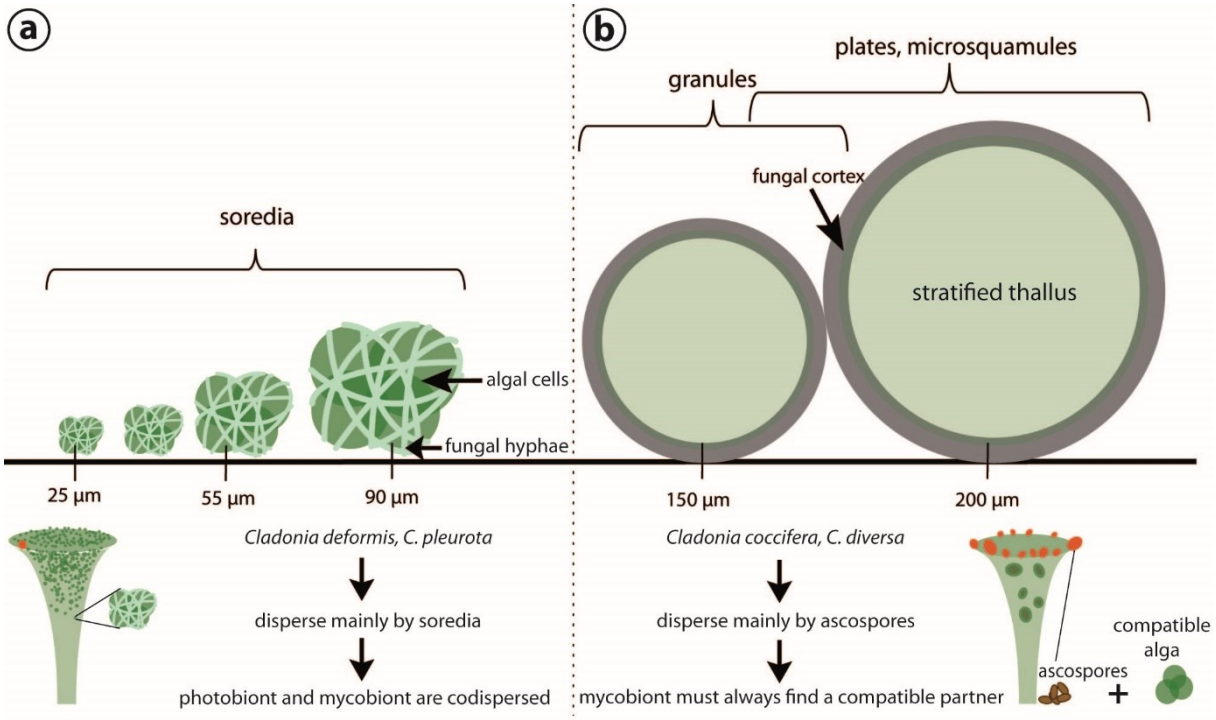

Fig. 1. Schematic representation of *Cladonia* reproductive and dispersal modes. a) The mycobionts *C. deformis* and *C. pleurota* reproduces mainly asexually and co-disperse together with the photobiont vegetative by soredia, which size is up to 90 µm. b) The mycobionts *C. coccifera* and *C. diversa* reproduce and disperse predominantly sexually by ascospores, therefore they need to find *de novo* the compatible photobiont; granules, plates and microsquamules are corticated thallus structures of 150-200 µm size.

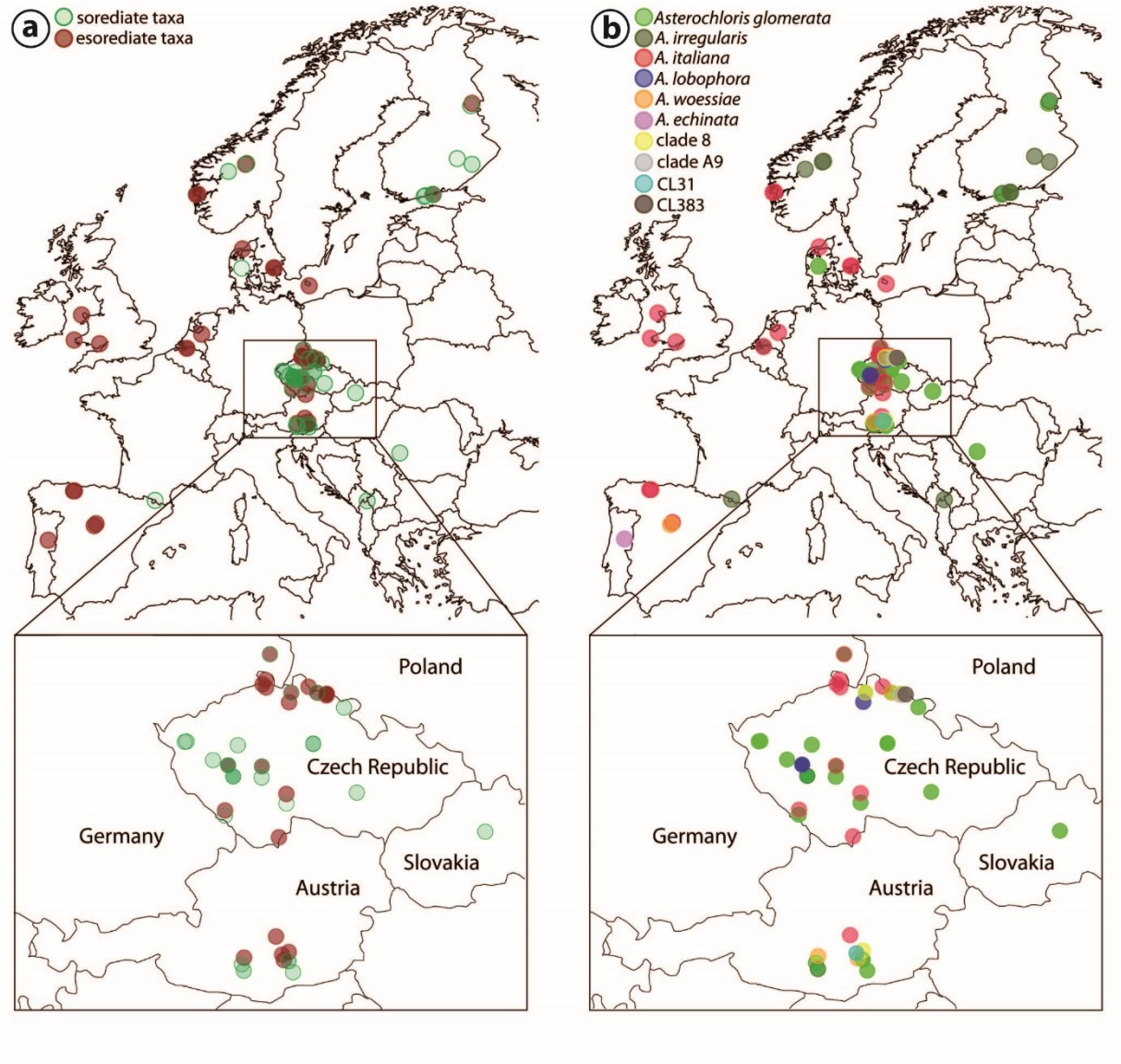

Fig. 2. Geographic maps of the collection sites across Europe with expanded maps of the Central Europe. The distribution of the *Cladonia* mycobionts (a) and the *Asterochloris* photobionts species (b) is colour coded. a) Sorediate species with asexual reproduction and vegetative dispersion (green), esorediate species with sexual reproduction and dispersion (red). b) Distribution of the *Asterochloris* photobionts: the eight lineages and the single *Asterochloris* sequences correspond to those recognized in the phylogenetic analysis of Fig. 4; the two *Asterochloris* lineages associated with the asexually reproducing *Cladonia* species are labeled in green.

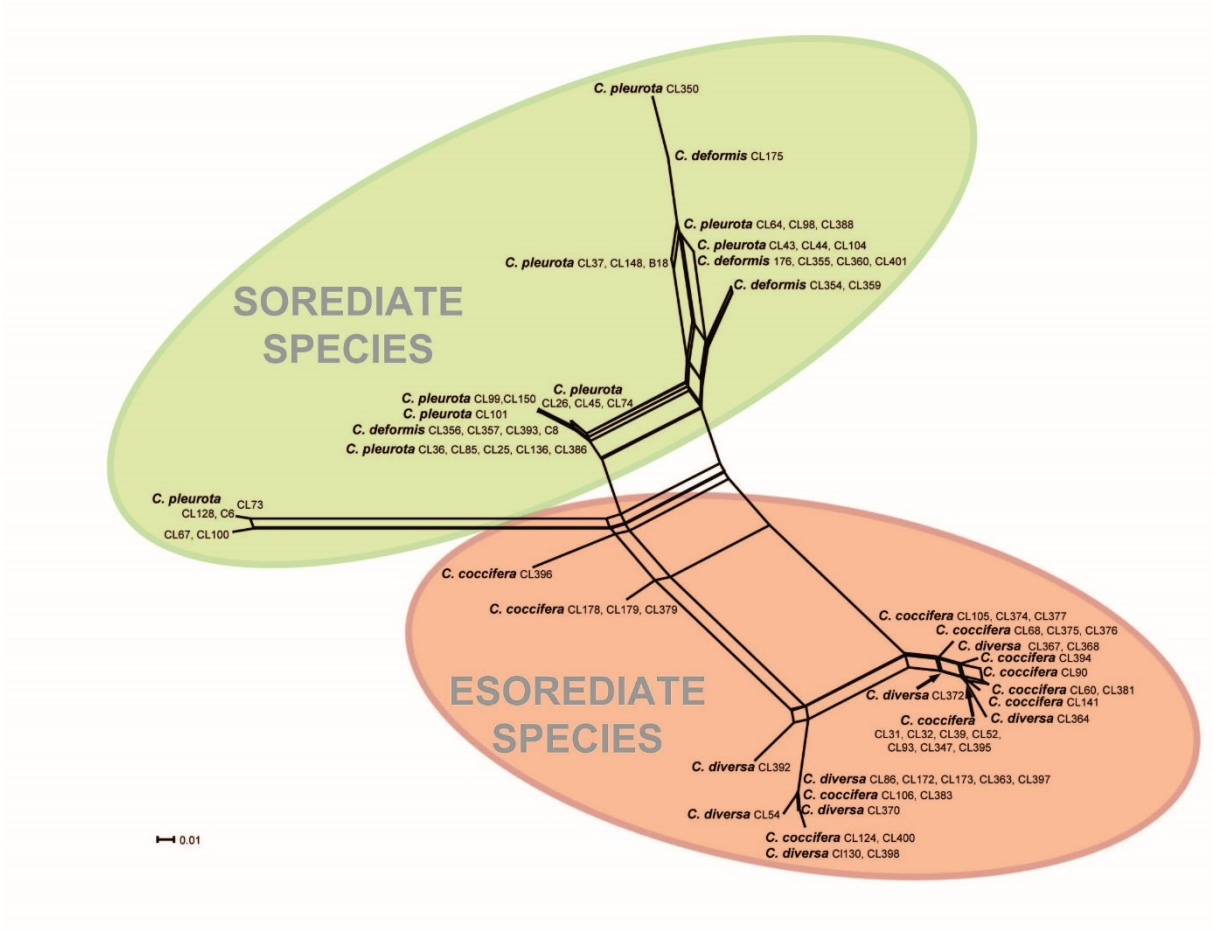

Fig. 3. Neighbor-net analysis of *Cladonia* mycobionts based on the combined fungal loci ITS and β-tubulin. Sorediate and esorediate species segregate in two defined groups joined by broad splits.

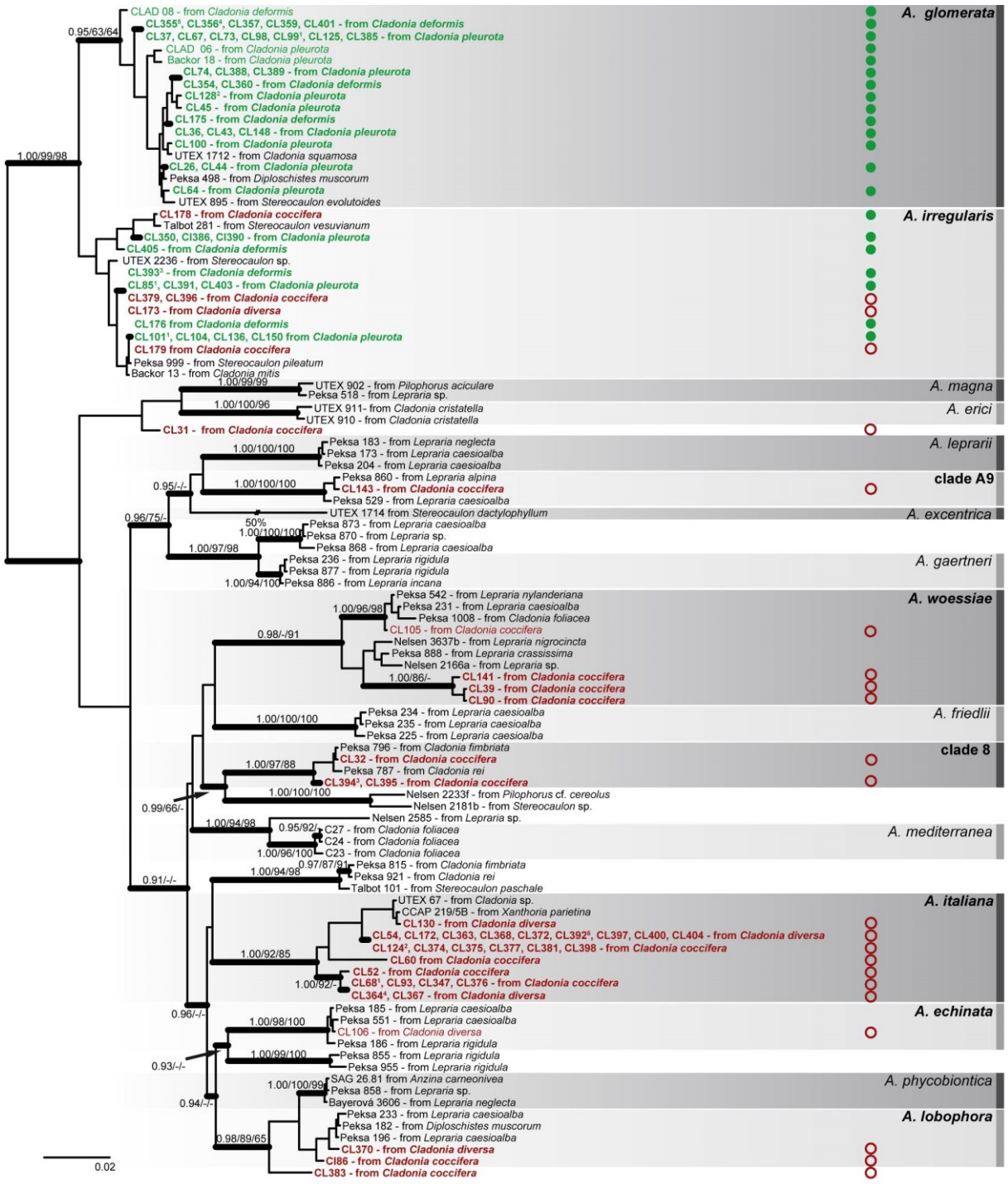

Fig. 4. Multigene phylogenetic hypothesis of *Asterochloris* photobionts: Bayesian hypothesis based on the combined dataset of ITS and actin I loci. Bootstrap support for the ML and MP analyses and the Bayesian posterior probability are reported at the corresponding branches. Branches have been collapsed to report multiple samples represented by the same sequence data. Upper case numbers (1-5) correspond to specimens of sorediate and esorediate species collected at the same locality, as reported in Table 2. Samples are colour coded according the reproductive and dispersal mode of the lichens: green, for sorediate asexual species and red for esorediate sexual species. *Asterochloris* lineages recovered in the studied *Cladonia* are in bold.

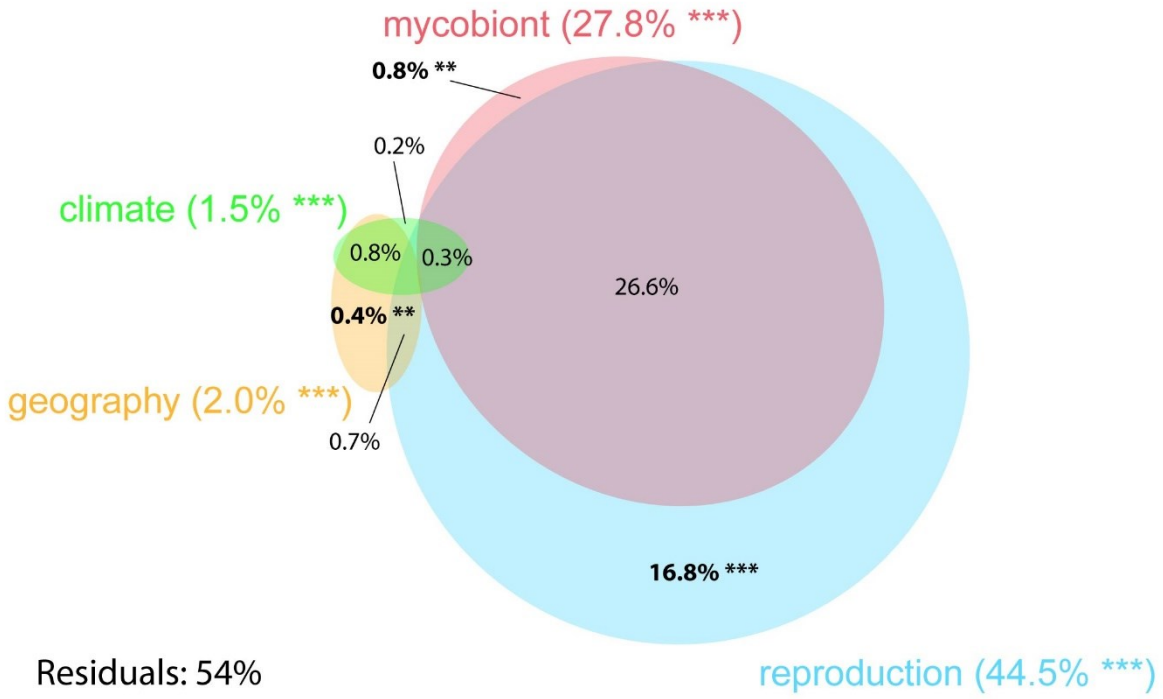

Figure 5. Variance partitioning analysis showing the percentage of explained photobiont diversity based on the four explanatory variables of *i)* the mycobiont genetic diversity, *ii)* the geographic, *iii)* the climatic and *iv)* the reproductive distances. Values in bold show pure effects of the explanatory variables.

6 CURRICULUM VITAE

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Since 2009:	Faculty of Science, Charles University, Prague – botany (lichenology) – Ph.D. studies
2006 – 2009:	Faculty of Science, Charles University, Prague – master studies in botany – (master thesis: Revision of the *Cladonia coccifera* group in Central Europe with emphasis on the Czech Republic)
2003 – 2006:	Faculty of Science, Charles University, Prague – bachelor studies in biology (bachelor thesis: Study on *Cladonia coccifera* group)

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Molecular microbiology and geomicrobiology.

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09/2013 – 06/2014	Slovak Academy of Science Bratislava, Slovakia – National Scholarship Programme of the Slovak Republic
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09/ 2012 – 12/ 2012:	Finnish Museum of Natural History, Helsinki, Finland – CIMO scholarship
09/ 2008 – 12/ 2008:	Karl-Franzens Universität Graz, Austria – AKTION
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Publications in SCI journals:

Němeček J., **Steinová J.**, Špánek R., Pluhař T., Pokorný P., Najmanová P., Knytl V., Černík M. (2018): Thermally enhanced in situ bioremediation of groundwater contaminated with chlorinated solvents – a field test. *Science of the total environment* 622-623: 743-755.

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Steinová J., Bobčíková K., Hristov D. R., Ševců A. (2016): Evaluation of two different methods for endotoxin detection in nanoparticle suspensions. *NANOCON 2016 - Conference Proceedings (8th International Conference on Nanomaterials - Research and Application)*: 627-631.

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