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EDITORIAL

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Lichens: from genome to ecosystems in a changing world

Kansri Boonpragob[1], Peter D. Crittenden[2], H. Thorsten Lumbsch[3]

1 *Department of Biology, Faculty of Science, Ramkhamhaeng University, Huamak, Bangkok 10240, Thailand*
2 *School of Biology, The University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom*
3 *Department of Botany, Field Museum of Natural History, 1400 S. Lake Shore Drive, Chicago, IL 60605, USA*

Corresponding author: *H. Thorsten Lumbsch* (tlumbsch@fieldmuseum.org)

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Fungi that form stable associations with algae and/or cyanobacteria, so-called lichens, are among the evolutionary most successful symbiotic systems with about 28,000 estimated species. In addition, certain groups of bacteria have recently been found to be present in lichen thalli. This symbiotic system consists of partners from at least two domains of life and major clades of eukaryotes and prokaryotes. Thus, it is an excellent model to study the evolution of cooperation. The International Association for Lichenology (IAL) is the society for all scientists working on different aspects of the lichen symbiosis, from diversity and evolution over ecology and physiology to conservation and bioprospecting. Between January 9-13, 2012 the 7th International Symposium of the IAL7 attracted about 300 lichenologists from all over the world (47 countries) to come to Bangkok, Thailand. At the beginning of most days during the meeting, a plenary talk was given on recent progress in different areas of lichenology. Two speakers of plenary talks agreed to prepare a review paper on their presentations. In addition, three of the other contributors to the general symposia agreed to write review papers on recent progress in their fields of lichen research. Four of these five contributions are published following the editorial in this issue of MycoKeys, while one manuscript has been published in the previous issue (Triebel et al. 2012). The scope of the contributions spans from using information technology to handle

data in megascience platforms, over molecular approaches to understand the ecology, distribution and phylogeny of lichens, to modern approaches to understand metabolism and ecosystem change.

The paper by Triebel et al. (2012) discussed processing and handling of large amounts of data available in megascience platforms and models in order to put the maintenance of those databases on a sustainable basis; it included specific references to databases with a focus on mycological and lichenological data. The paper by Joukko Rikkinen (Helsinki) summarizes recent progress in our understanding of the diversity of cyanobacteria in the lichen symbiosis and previously overlooked aspects that potentially have great importance for the fitness of cyanobacteria, including the production of toxins by those symbionts in the lichenized state. The second paper in this issue by Christian Printzen (Frankfurt) and his co-workers illustrates the enormous increase in our knowledge of the biogeography and ecology of lichens by the combination of using molecular approaches and ecophysiological measurements using *Cetraria aculeata* as a model species. In the following paper Eimy Rivas Plata (Durham, NC) and twelve co-authors present a molecular phylogeny of the tropical lichen family Graphidaceae. It includes 437 species and represents one of the largest molecular phylogenies of lichenized fungi undertaken to date and clearly demonstrates the potential of collaborative approaches in evolutionary biology. The last review paper in this series is written by Cristina Máguas (Lisbon) and her collaborators and summarizes our understanding of the role of the atmosphere in carbon, nitrogen and water relationships in lichens. These studies allow us to now understand much better than previously the impact of ecosystem change on lichens and to better understand the metabolism in these symbiotic organisms.

We thank each contributor for taking the time to prepare these review papers that give an overview of current developments in lichenology. We also thank the members of the local and scientific organizing committees for their hard work in planning and running this most successful and enjoyable meeting. We also thank all contributors to the different symposia for their efforts in making this meeting extremely successful.

Reference

Triebel D, Hagedorn G, Rambold G (2012) An appraisal of megascience platforms for biodiversity information. MycoKeys 5: 45–63. doi: 10.3897/mycokeys.5.4302

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REVIEW ARTICLE

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Molecular studies on cyanobacterial diversity in lichen symbioses

Jouko Rikkinen[1]

1 *University of Helsinki, Department of Biosciences, P.O.Box 65, Helsinki, Finland*

Corresponding author: *Jouko Rikkinen* (jouko.rikkinen@helsinki.fi)

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Abstract

Symbioses between cyanobacteria and lichen-forming fungi occur worldwide in a wide range of terrestrial environments, ranging from tropical rainforests to hot and cold deserts. The evolutionary success of these symbioses is evident from the wide range of fungal groups that have established associations with cyanobacteria. The diversity of symbiotic cyanobacteria is also high, and it is obvious that symbioses between different cyanobacteria and different lichen-forming fungi have evolved on multiple occasions. From the late 1990s cyanobacterial lichens have been the subject of a steadily increasing number of molecular investigations. This chronological review examines how these studies have contributed to present knowledge and highlights some conceptual developments that have been instrumental in the process.

Key words

Cyanolichens, symbiosis, specificity, coevolution, phylogeny, trnL, $tRNA^{Leu}UAA$ intron, 16S rDNA, rbcLX, *Nostoc*, *Collema*, *Nephroma*, *Pannaria*, *Peltigera*, *Pseudocyphellaria*

Introduction

Lichens are symbiotic associations between a fungus and a photosynthetic partner that may be a eukaryotic alga or cyanobacterium. While a clear majority of lichen-forming fungi, mainly Ascomycota, associate with green algae, over 1500 species of lichen-forming fungi have cyanobacteria as primary or accessory photosynthetic partners and are therefore collectively referred to as "cyanolichens". Cyanobacterial symbioses have evolved repeatedly in different lineages of lichen-forming Fungi, and often con-

vergent evolution has led to superficially similar thallus structures in distantly related cyanolichens (Rikkinen 2002).

Within lichen thalli the symbiotic cyanobacteria contribute to the host fitness by provisioning sugar and/or fixed atmospheric nitrogen. In return, the fungal host provides the cyanobacteria with a relatively stable environment, host-derived water and carbon dioxide, and a special niche that is relatively well protected from environmental extremes and predation. In many cases the cyanobacterial symbionts are vertically transmitted within specialized asexual propagules and maintained through host generations, insuring a close and long-term symbiosis. Most lichen symbioses are thought to be obligate as the fungal hosts cannot survive without their photosynthetic partners and the cyanobacterial symbionts do not seem to commonly establish aposymbiotic populations outside lichen thalli.

Lichen symbioses are often perceived as pair wise interactions between a single fungal host and a single photosynthetic symbiont. However, many lichen-forming fungi are in fact associated with two or more species of photobionts. In most bipartite cyanolichens the cyanobacterial symbiont forms a more or less continuous layer immediately below the upper cortex of the thallus (Figs 1A–B). Tripartite cyanolichens, on the other hand, house both green algal and cyanobacterial symbionts. In these lichens the cyanobacteria, which usually represent a minor proportion of total photobiont biomass, are restricted to special structures called cephalodia (Figs 1C–D). Hundreds of lichen species are known to have external or internal cephalodia. In addition, some green algal lichens commonly establish ephemeral associations with free-living cyanobacteria, most probably in order to access a supply of fixed nitrogen.

The fungal hosts of certain tripartite lichens (some species of *Lobaria*, *Nephroma*, *Peltigera*, *Pseudocyphellaria*, and *Sticta*) can occasionally produce different thallus morphologies in symbiosis with compatible green algae and cyanobacteria. Chimeroid lichens with green algae and cyanobacteria as primary photobionts in different parts of the same thallus are called photosymbiodemes. The contrasting morphotypes may either combine into a compound thallus or, in some cases, live separate lives.

Lichen-symbiotic cyanobacteria can deliver photosynthate and/or fixed nitrogen to their fungal partners. The relative importance of these activities is known to vary between bi- and tripartite lichens. The cyanobionts of bipartite lichens often show lower heterocyst frequencies and lower rates of nitrogen fixation than those of tripartite species. In tripartite cyanolichens, the cyanobionts usually show relatively high rates of nitrogen fixation, while the green algal photobiont typically delivers most of the photosynthate.

Nostoc is by far the most common genus of cyanobacteria in lichens, especially in the temperate and cool regions of the world. The lichen symbiontic *Nostoc* genotypes are closely related to plant symbiotic and free-living forms of the same genus (Fig. 2). All *Nostoc* species are filamentous and have complex life cycles. Their non-branching filaments consist of cylindrical or spherical vegetative cells with intercalary heterocysts (large nitrogen-fixing cells) developing in mature trichomes (Figs 2D–E). The filaments are usually covered in mucilage and many free-living *Nostoc* genotypes can form large gelatinous colonies (Figs 2A–B).

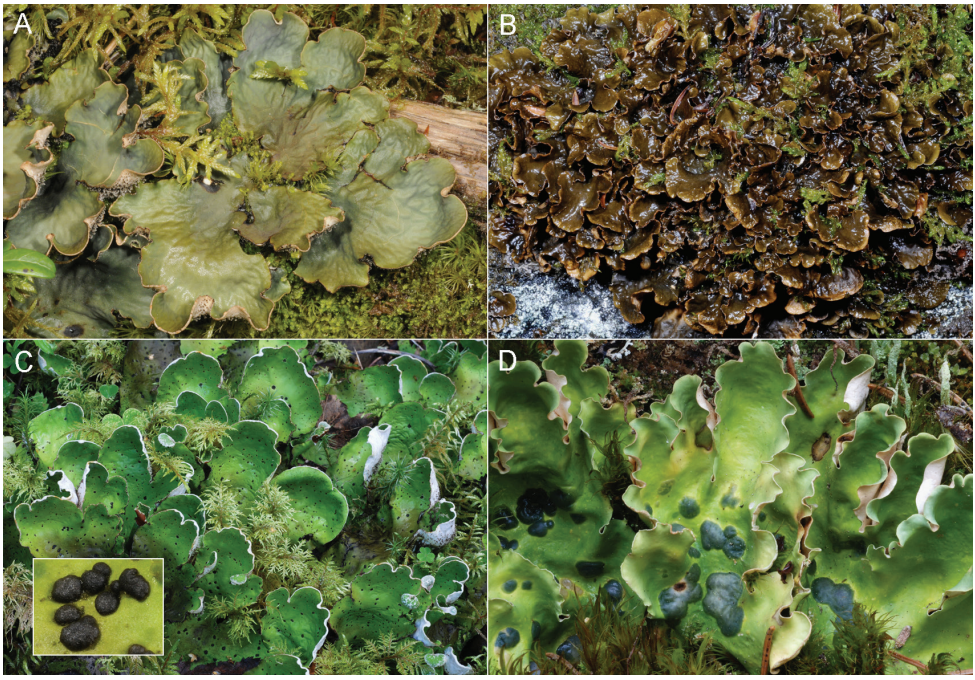

Figure 1. Bipartite and tripartite cyanolichens. **A** In the bipartite cyanolichen *Peltigera scabrosa* the cyanobacterial symbiont (*Nostoc*) forms a continuous layer just below the upper cortex of the lichen thallus **B** *Nephroma bellum* is another example of bipartite cyanolichens **C** In the tripartite cyanolichen *Peltigera aphthosa* the *Nostoc* symbiont is restricted to wart-like cephalodia (shown magnified) on the upper surface of the thallus, while the green algal symbiont (*Coccomyxa*) forms the photobiont layer **D** *Nephroma arcticum* is another example of tripartite cyanolichens. The large cephalodia of this species are internal, but clearly visible through the upper cortex of the hydrated thallus.

At present, symbiotic *Nostoc* genotypes and other lichen symbiotic cyanobacteria cannot be reliably identified to species. This relates to a general confusion in the species level taxonomy of cyanobacteria. From early in the 19th century to the mid 20th century numerous genera and species of blue-green algae were described on the basis of morphological characteristics and life-history traits (e.g. Geitler 1932). While DNA data have since confirmed that cyanobacteria represent a lineage among the Eubacteria, cyanobacterial taxa can still be described following two sets of rules, i.e., those of the Botanical and the Bacteriological Code of Nomenclature, respectively (e.g. Oren 2004). While the taxonomic criteria of many botanical cyanobacterial groups were established more than a century ago, adequate type material is available for relatively few species. Most early species descriptions were based on insufficient observations, and many taxa were defined using unstable features. Furthermore, many morphological characteristics of free-living cyanobacteria are not apparent within lichen thalli, as the morphology and development of the organisms tend to be modified in symbiosis.

The *Nostoc* symbionts of many cyanolichens, including both bi- and tripartite species of *Peltigera*, have usually been called *Nostoc punctiforme*, but also cyanobacterial

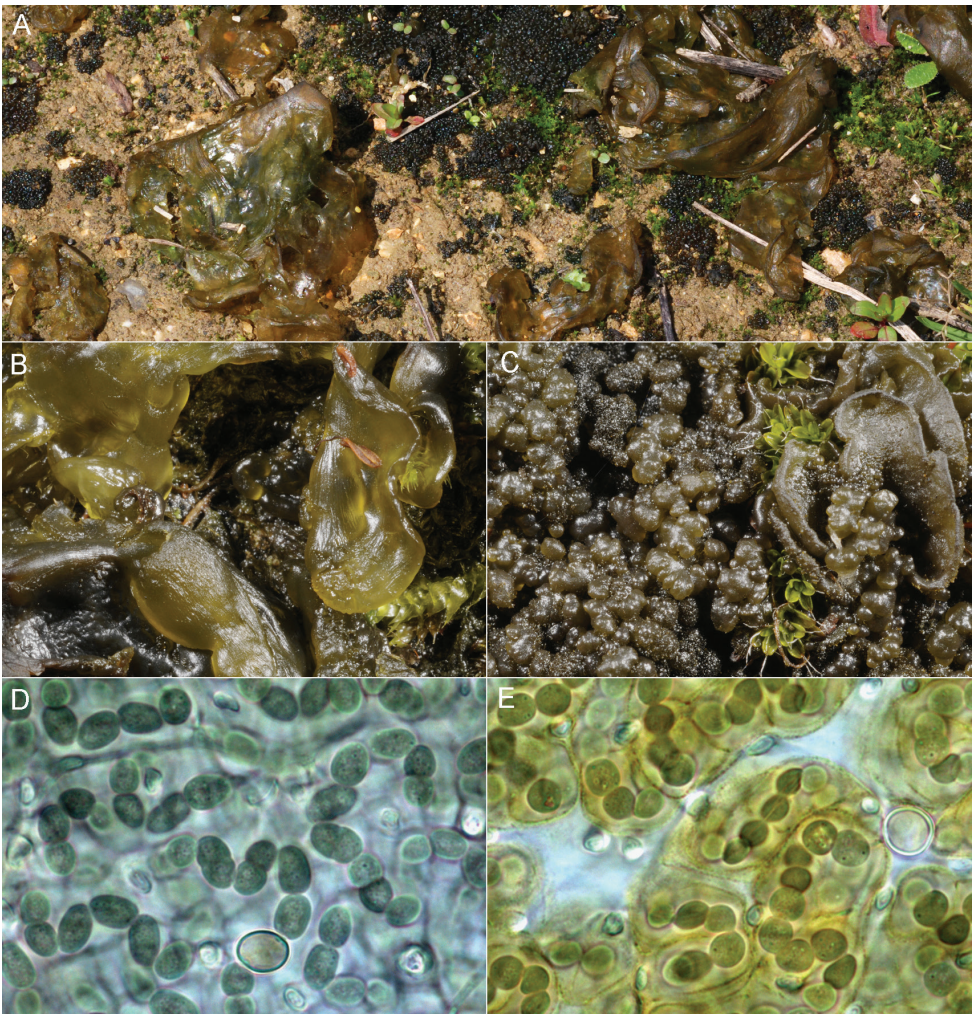

Figure 2. Free-living and lichen-symbiotic *Nostoc* strains. **A** Free-living *Nostoc* colonies and gelatinous cyanolichens (*Collema* sp.) growing on mineral soil in northern Spain **B** Free-living *Nostoc* colony on limestone in northern Italy **C** Lichenized *Collema* thallus (containing *Nostoc* symbionts) on limestone in northern Italy **D–F** Morphological variation of *Nostoc* symbionts inside two *Leptogium* thalli in south-western Kenya. The large clear cells are nitrogen-fixing heterocysts, the smaller translucent structures are fungal hyphae in optical cross-section.

strains resembling *N. muscorum*, *N. sphaericum* and *N. linckia* have been cultured from some *Peltigera* species (e.g. Forssel 1883, Bergman and Hällbom 1982, Tschermak-Woess 1988). Degelius (1954) pointed out that *N. punctiforme* may not represent any one taxon, but rather include developmental stages (e.g. Mollenhauer 1988) of several different *Nostoc* species. Feige (1978) found both intra- and infraspecific differences in the lipid patterns of cyanobacterial heterocysts from *Peltigera* species indicating that several different cyanobacterial species would be involved in these lichens. Further-

more, tripartite *Peltigera* and *Nephroma* species have sometimes been found to possess several different cyanobacterial morphotypes within single thalli and occasionally even in the same cephalodium (Jordan and Rickson 1971). The cephalodia of some *Stereocaulon* species may even contain cyanobacteria of several different genera (Tschermak-Woess 1988).

As a whole, in symbiosis the few structural features of cyanobacteria tend to be strongly modified, making the accurate identification of morphospecies impossible. Thus, molecular techniques offer the only practical methods for studying the genetic diversity of these organisms, and for making comparisons between lichen symbiotic and other symbiotic or free-living cyanobacteria. During the past fifteen years the cyanobacterial symbionts of lichens have been the subject of many molecular investigations which have greatly increased our understanding of symbiont diversity in these interesting systems. The following chronological review describes how such studies have gradually built up our present knowledge and examines some conceptual developments that have been important in the process.

From single thalli to global distribution patterns

The first DNA-based studies on lichen-symbiotic cyanobacteria were those of Kardish et al. (1989, 1990) who by using Southern hybridizations got different patterns from laboratory cultures and fresh *Nostoc* isolates from the bipartite cyanolichen *Nephroma laevigatum*. Leizerovich et al. (1990) used the same method to compare *Nostoc* cultures from several bipartite cyanolichen species, two thalloid bryophytes, *Cycas*, *Gunnera*, and a free-living specimen. The hybridization patterns were diverse and did not appear to correlate with the phylogenic position of the host organisms. Several years later, using cyanobacterial 16S rDNA sequences, Miao et al. (1997) detected different *Nostoc* genotypes in two *Peltigera membranacea* thalli from western North America. However, only one *Nostoc* genotype could be cultured from the lichen specimens. The two thalli analyzed represented different color forms of the bipartite cyanolichen species.

These early studies indicated that while individual lichen thalli seemed to contain a rather limited variety of cyanobacteria, isolating and culturing the correct symbiont could be difficult. Similar experiences had previously been gained from studies on plant-cyanobacterial symbioses, especially those concerning the water fern *Azolla*. The findings emphasized the general need to first identify symbiotic cyanobacteria directly from lichen thalli, before trusting the identification of symbionts in culture.

Paulsrud and Lindblad (1998) pioneered the use of cyanobacterial trnL sequences in lichen studies by identifying *Nostoc* genotypes from several specimens of the tripartite cyanolichen species *Nephroma arcticum* (Fig. 1D) and *Peltigera aphthosa* (Fig. 1C), and the bipartite species *Peltigera canina* and *P. membranacea* from boreal forests in Sweden. The results demonstrated that the symbionts of the studied lichens did not consist of a community of different strains but, rather, of one *Nostoc* genotype in each lichen thallus. Although several different *Nostoc* trnL genotypes were identified in the

study, even from lichens growing close to each other, no variation was observed within a sample or within a single lichen thallus. Similar conclusions have since been reached concerning all bipartite cyanolichens analyzed in subsequent studies. The authors discussed possible reasons for the apparent lack of variation within one thallus, and particularly that in tripartite lichens species, where the symbiont population is confined to small cephalodia separated by wide cyanobacterium-free areas of only fungus and green alga, and concluded that each lichen species was probably restricted in their choice of cyanobiont to a relatively small number of *Nostoc* strains. In any case, the sequence patterns of *Nostoc* trnL genotypes in the studied lichens were clearly more restricted by the species identities of fungal hosts than by the spatial segregation of lichen thalli.

Paulsrud et al. (1998) expanded the earlier sampling by identifying *Nostoc* symbionts from additional lichen specimens from central Finland. The study revealed that the same *Nostoc* trnL genotypes that had been identified from Sweden were also present in Finnish specimens of *Peltigera aphthosa*, *Peltigera canina,* and *Nephroma arcticum*. The cephalodia of the two tripartite lichen species analyzed always contained different *Nostoc* trnL genotypes, with two different genotypes occurring in different thalli of both species in both countries. They also identified *Nostoc* symbionts in the cephalodia and bipartite thallus sections of a *Peltigera aphthosa* photosymbiodeme collected from Finland and found that the same *Nostoc* genotype was present in both parts of the chimeroid lichen thallus. This indicated that a single *Nostoc* genotype was able to participate in the two structurally different symbioses of bipartite and tripartite lichens, respectively. Two *Nostoc* trnL intron genotypes were identified from different *Peltigera neopolydactyla* specimens from Finland. One of these was very similar to those found from *P. canina* and *P. membranacea*, and since from numerous other bipartite *Peltigera* species (Fig. 1A), while the second genotype was identical to that identified from several specimens of *P. aphthosa* from both Finland and Sweden. This confirmed that some cephalodial *Nostoc* genotypes also occurred in bipartite cyanolichens. Finally, a new trnL intron type, characterized by a peculiar inserted sequence in the P6b region, was found from an epiphytic *Nephroma resupinatum* thallus from Finland.

Paulsrud et al. (2000) continued to widen the sampling by identifying *Nostoc* symbionts from new cyanolichen specimens from different localities in Oregon and Washington in western North America. A single *Nostoc* trnL genotype was again found in each lichen thallus in all except one lichen species. The exception was *Peltigera venosa* (Fig. 3C), in which different *Nostoc* genotypes were identified from different cephalodia in several thalli. However, in a photosymbiodeme of this species, the same *Nostoc* genotype was found both in the cephalodia and a free-living cyanosymbiodeme collected from one locality. In *Peltigera britannica*, two different *Nostoc* genotypes were identified from cephalodia of different thalli, and the more common genotype was also found in one cephalodium of *P. venosa*. In the bipartite cyanolichen species, two different *Nostoc* genotypes were found in different thalli of the local *Peltigera neopolydactyla*. Both of these were different than those previously identified from Finnish specimens of the same species complex. However, one of the new *Nostoc* genotypes, that was also found from two *Peltigera membranacea* specimens from Oregon, was sequence

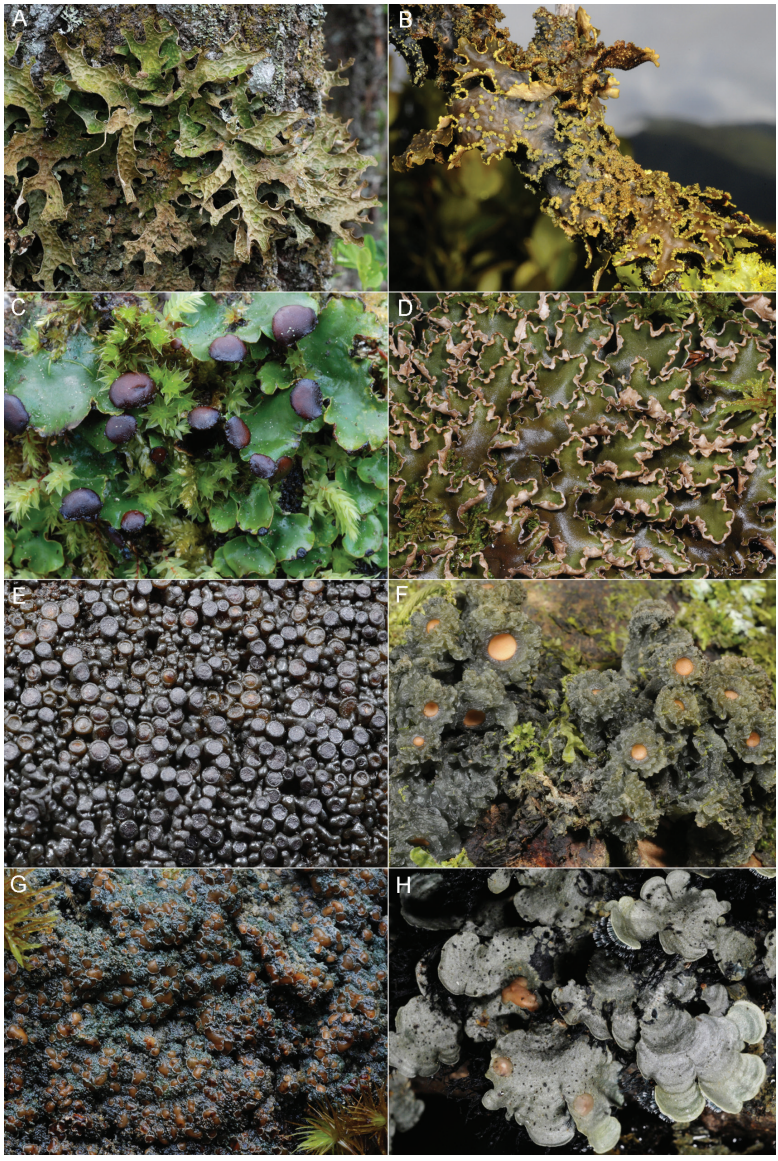

Figure 3. Examples of cyanolichens examined in molecular studies of cyanobacterial diversity. **A** Cephalodial symbionts of the tripartite cyanolichen *Lobaria pulmonaria* remain poorly known (e.g. Rikkinen et al. 2002, Myllys et al. 2007) **B** *Nostoc* symbionts of *Pseudocyphellaria* species have been analyzed in several studies (e.g. Summefield et al. 2002, 2006, Rikkinen et al. 2002, Stenroos et al. 2006) **C** *Peltigera venosa* may have different *Nostoc* genotypes in different cephalodia (Paulsrud et al. 2000) **D** *Nostoc* symbionts of bipartite *Peltigera* species have been identified in many studies (e.g. Paulsrud and Lindblad 1998, O'Brien et al. 2005, Kaasalainen et al. 2012) **E** Otalora et al. (2010) analyzed genetic diversity of *Nostoc* in *Collema* and related cyanolichens **F** Many tropical *Leptogium* specimens were screened by Kaasalainen et al. (2012) **G** A *Nostoc* strain isolated from *Pannaria pezizoides* produces potent hepatotoxins in culture (Oksanen et al. 2004, Kaasalainen et al. 2009) **H** The cyanobacterial symbionts of *Coccocarpia* species are only distantly related to *Nostoc* (Lücking et al. 2009).

identical to that previously identified from *P. membranacea* in Sweden. Also the *Nostoc* genotype now found from *Nephroma resupinatum* in Oregon was quite similar to that previously found from the same lichen species in Finland. However, it did not have the peculiar insertion in the P6b region of the trnL intron.

The new findings indicated that some patterns of symbiont specificity could hold true over vast geographical distances. Many of the cyanolichen specimens analyzed came from mixed collections of different cyanolichen species growing in close physical contact, adding evidence that it was the lichen species, not locality, that was important in determining cyanobiont identity. Comparable diversity of *Nostoc* genotypes to that observed in *P. venosa* had not been encountered previously, and this was also the first time when several *Nostoc* genotypes were found from different cephalodia of a single thallus of a tripartite cyanolichen. The authors discussed reasons for the apparent promiscuity in symbiont choice and proposed that it might be explained by the unusual development and ecology of *P. venosa*. Its special features include cephalodia on the lower surface of the thallus, the capability of forming different thallus types or developmental stages under different environmental conditions and, possibly, an additional ability to exhibit different degrees of lichenization with different *Nostoc* strains. Finally, the trnL sequence from *Nephroma resupinatum* focused attention to the apparent difference in *Nostoc* genotypes of bipartite *Nephroma* and *Peltigera* species, and to variation in the occurrence of peculiar indels in the P6b region of trnL sequences. Since then these have been reoccurring themes in several studies.

Paulsrud et al. (2001) took a closer look at cyanobacterial specificity in *Peltigera aphthosa* (Fig. 1C) by attempting to experimentally introduce foreign cyanobacterial genotypes into established lichen thalli in the field. All *Nostoc* strains used in the experiment were identifiable on the basis of trnL sequences. The experiment relied on the fact that cephalodia on the upper surface of *P. aphthosa* could be experimentally removed without killing the lichen: under favorable conditions new cephalodia would develop within a few weeks. Thalli of *P. aphthosa* were manipulated by removing all cephalodia and then inoculating the thalli with cultured cyanobacteria. Five different lichen-symbiotic *Nostoc* strains were used in the inoculations, originating from *Peltigera aphthosa* (both of the two *Nostoc* genotypes previously found from different *P. aphthosa* thalli in Sweden and Finland), *P. canina*, *P. membranacea*, and *Nephroma resupinatum*. In addition to the lichen-symbiotic strains, also two laboratory strains of *Nostoc* were used. After inoculation and subsequent growth, 80 new cephalodia and seven epiphytic *Nostoc* colonies were collected and analyzed. All cephalodia were found to contain the same *Nostoc* genotype, this being identical to that identified from the cephalodia excised from the lichen thalli at the beginning of the experiment. Thus, the original *Nostoc* symbiont turned up in every new cephalodium despite massive inoculations of six other *Nostoc* strains. Interestingly, two inoculated *Nostoc* strains, those isolated from *P. canina* and *P. membranacea*, could live and reproduce on the surface of manipulated lichens. Thus, while never finding their way into new cephalodia, by the end of the experiment they had formed thriving epiphytic colonies on the upper surfaces of some *P. aphthosa* thalli.

Summerfield et al. (2002) presented the first molecular study of cyanobacterial diversity in lichens of the southern hemisphere. They identified *Nostoc* trnL genotypes from specimens of four species of *Pseudocyphellaria* (Fig. 3B) collected from different sites in the South Island, New Zealand. *Nostoc* strains were isolated from some thalli and the identity of the isolates was confirmed by using trnL and partial 16S rDNA sequences. Also the identity of the fungal hosts was determined on the basis of fungal ITS sequences. The results showed that *P. crocata* and *P. neglecta* always housed different *Nostoc* symbionts. Furthermore, one *Nostoc* strain was again found to associate with more than one mycobiont, in this case *P. crocata* and *P. maculata*. Interestingly, the *Nostoc* genotype of *P. crocata* from New Zealand was nearly sequence identical to that previously reported from *Nephroma resupinatum* in central Finland. The authors concluded that the cyanobiont association of the two bipartite *Pseudocyphellaria* species was highly specific, and that a similar specificity had thus now been demonstrated in lichens belonging to three families of the suborder Peltigerineae from both the northern and southern hemispheres.

Costa et al. (2002) used the trnL method to analyze cyanobacterial diversity in two thalloid bryophytes, the hornwort *Anthoceros fusiformis* from western North America and the liverwort *Blasia pusilla* from central Finland (Figs 6A–B). The results showed that several different *Nostoc* genotypes were involved in both bryophyte symbioses and the level cyanobacterial diversity within individual bryophyte thalli was quite variable. Some *Nostoc* genotypes were detected from *Blasia* thalli collected from different sites and in different years, indicating a moderate level of spatial and temporal continuity in this symbiosis. One *Nostoc* genotype that was identified several times from *Blasia* thalli was identical to one of the symbionts that had previously been identified from the bipartite cyanolichen *Peltigera neopolydactyla* in central Finland. Also the sequences of several other bryophyte-associated *Nostoc* genotypes were very similar to those of lichen symbionts indicating that both symbionts belonged to the same linage. One new *Nostoc* genotype was identified from a *Peltigera didactyla* thallus growing amongst *Blasia* thalli. It differed slightly from *Nostoc* genotypes in neighboring bryophytes, but was nearly identical to a *Nostoc* symbiont that had been previously found from a *Peltigera britannica* cephalodium in western North America.

Costa et al. (2002) analyzed the variation in cyanobacterial trnL intron sequences in more detail and described how it was not random but strongly restricted by the secondary and tertiary structure of the transcribed intron. All *Nostoc* sequences analyzed shared high sequence similarity, but major differences were commonly seen in the P6b region, corresponding to one stem-loop in the transcribed intron. As already described in Paulsrud and Lindblad (1998), the *Nostoc* trnL sequences could be grouped into two classes, corresponding to distinct base pairing heptanucleotide repeats that built up the P6b region. Length variation in the sequences was mainly caused by different numbers of repeats, but some *Nostoc* strains also contained additional sequences in this region not following the heptanucleotide repeat motif. Several sequences showing similarity with these additional sequences were identified in the recently published *Nostoc punctiforme* genome. Furthermore, the regions flanking these sequences contained similar

heptanucleotide repeats as those flanking the corresponding sequences in the intron. It was proposed that slipped strand mispairing during replication and homologous recombination among different loci in the genome would have been important processes causing the present variation between intron types.

Oksanen et al. (2002) studied cyanobacterial diversity in a series of small, interconnected rock-pools on a limestone pavement in western Ireland. The purpose was to determine whether or not the cyanobacterial symbionts of *Collema multipartitum* and neighboring free-living *Nostoc* colonies belong to the same genotype (Figs 2B–C). One *Nostoc* trnL genotype was identified from three different *Collema* specimens, while another *Collema* specimen contained a second genotype. A third *Nostoc* genotype was isolated into culture from an additional lichen thallus. The presence of fungal DNA was confirmed in all these specimens. Five different *Nostoc* genotypes were identified from free-living colonies and no fungal DNA was obtained from any of them. The consistent difference between the *Nostoc* genotypes in *Collema* thalli and free-living colonies indicated that a different group of *Nostoc* was associated with each biological system in the restricted rock-pool environment. While the *Nostoc* genotypes that formed large colonies in deep depressions appeared to be free-living, some of them might still have been potentially lichen symbiotic. This was indicated by the fact that one free-living *Nostoc* genotype was sequence identical with another that had previously been identified from a thallus of *Peltigera neopolydactyla* in Oregon.

Rikkinen et al. (2002) compared cyanobacterial diversity in cyanolichen specimens collected from boreal forests of northern Europe and temperate forests of western North America and central China (Figs 5B–C). Epiphytic lichen communities in two boreal forests in central Finland were studied in some detail, and there some cyanobacteria were also isolated from epiphytic substrates. Cyanobacterial 16S rDNA sequences were used to resolve phylogenetic relationships, and trnL sequences were used to identify *Nostoc* genotypes. Most epiphytic cyanolichens in central Finland were found to associate with a small group of closely related *Nostoc* trnL genotypes. One particular genotype was identified from all six research sites, and it was shared extensively by three different *Nephroma* species and *Parmeliella triptophylla*. Several epiphytic cyanolichen specimens from western North American and central China contained similar *Nostoc* trnL genotypes, and the phylogenetic relationship between the genotypes was confirmed by the 16S rDNA phylogeny. A different set of *Nostoc* trnL genotypes was found from Finnish *Peltigera* specimens and from *Nostoc* isolates cultured from substrate samples. A congruent pattern was again seen in lichen specimens collected from Asia and North America, and the existence of two distinctive groups of *Nostoc* trnL genotypes was also confirmed by 16S rDNA data.

The new findings suggested that some cyanolichens could express their *Nostoc* specificity on a community scale (Fig. 4). Thus, in central Finland the predominately epiphytic, bipartite *Nephroma* species and *P. triptophylla* seemed to rely on a common pool of cyanobacterial symbionts and potentially form a horizontally linked system, the *Nephroma* guild. Conversely, some more predominately terricolous cyanolichen species, like *Peltigera praetextata* housed a completely different group of related *Nostoc*

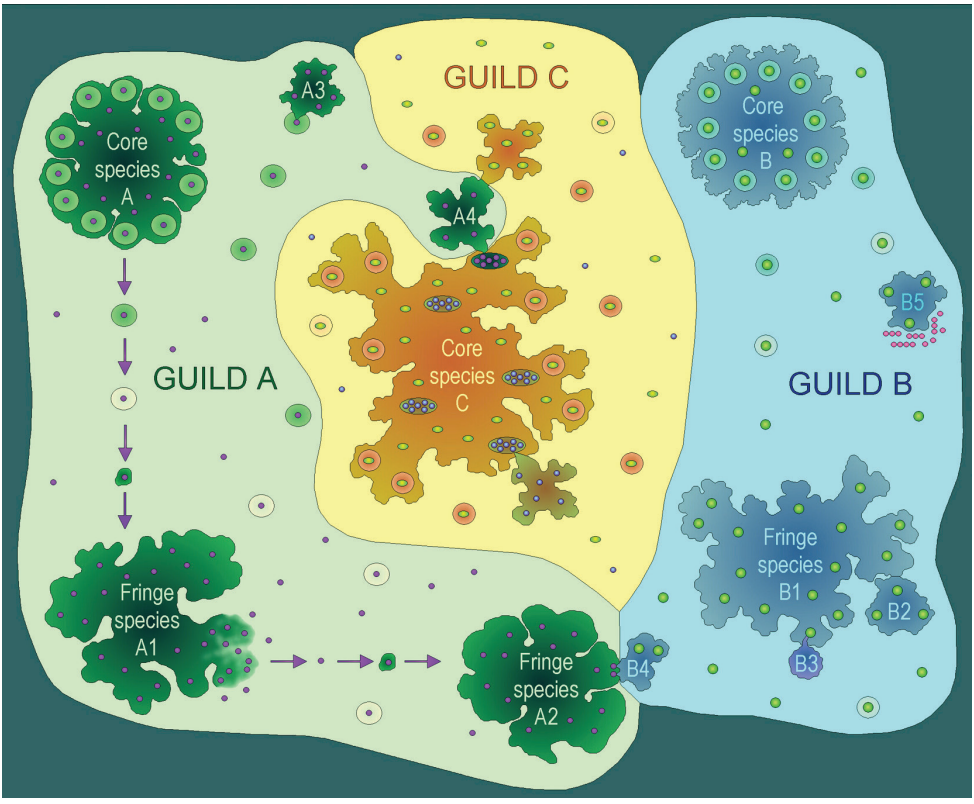

Figure 4. Photobiont-mediated guilds in lichens (modified from Rikkinen 2003).The lichen-forming fungi belong to three different guilds, one centring around cyanobacterial symbionts (**A**), the second around green algal symbionts (**B**), and the third around another genus of green algal symbionts (**C**). The lichen in the middle of the picture houses both green algae and cyanobacteria (in cephalodia), meaning that its fungal symbiont can operate in two different guilds (**C** and **A**). As the symbiotic propagules of this lichen only contain the fungus and green algal photobiont, the fungus is a core species in guild **C** and a fringe species in guild **A**. Under certain conditions this fungus may give rise to cyanobacterial morphotypes (**A4**) and/or green algal thallus lobes. The core species of the lichen guilds produce innumerable symbiotic propagules, most of which will never develop into mature thalli of that lichen species. Germinating spores of fringe species (**A1–A4**, **B1–B5**) may commonly acquire their photobionts from small free-living populations that originate from disintegrating symbiotic propagules of the core species. At the latest when the thallus of a fringe species dies and disintegrates (**A1**), some of the photobionts are released back to the local environment for the common benefit of all fungi of the same guild. However, without the ability to produce symbiotic propagules, the fringe species cannot effectively disperse appropriate photobionts into new habitats. Some fringe species are aggressive enough to steal photobionts from juvenile stages or weakened thalli of other lichen species (**A3**), or live as lichenicolous lichens (**B2**) on other lichens of the same guild. The juvenile stages of some green algal lichens establish loose cyanotrophic associations with free-living cyanobacteria (**B5**) and/or cyanolichens (**B4**). Some lichenicolous fungi (**B3**) have evolved from lichen-forming ancestors and in many cases also their host ranges still appreciate guild boundaries.

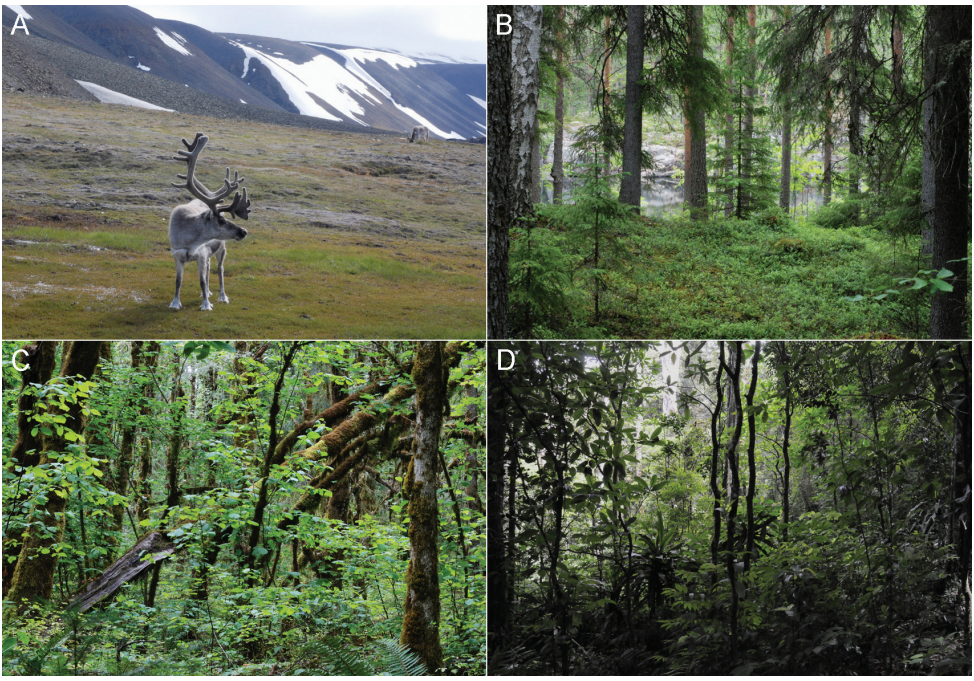

Figure 5. Environments sampled in molecular studies of cyanobacterial diversity. **A** Arctic tundra on Svalbard; so far only two studies have included cyanolichen specimens from polar environments (Wirth et al. 2003, Kaasalainen et al. 2012) **B** Boreal forest in central Finland; cyanolichens from boreal forests have been examined in several studies (e.g. Paulsrud and Lindblad 1998, Myllys et al. 2007, Fedrowitz et al. 2011) **C** Temperate forest in western North America; also cyanolichens from temperate forests have been analyzed in several studies (e.g. Rikkinen et al. 2002, Summerfield et al. 2002, Fedrowitz et al. 2011) **D** Tropical montane forest in East Africa; so far only two studies have included cyanolichen specimens from tropical ecosystems (Lücking et al. 2009, Kaasalainen et al. 2012).

strains, thus forming the *Peltigera* guild. It was also proposed that the dispersal ecology of such lichen guilds could involve "core species" which produce large amounts of symbiotic propagules. "Fringe species" would in turn only propagate via fungal spores and thus partly depend on core species for the dispersal of appropriate symbionts. In any case, the high sequence similarity between *Nostoc* symbionts of phylogenetically distant but ecologically similar cyanolichens in different parts of the world suggested that photobiont-mediated guilds may have played an important role in the evolution of the presently observed diversity among lichen symbiotic organisms.

Lohtander et al. (2002, 2003) expanded studies on the diversity of symbiotic *Nostoc* in the cyanolichen genus *Nephroma* (Figs 1B, 1D). First, phylogenetic analyses confirmed that all European *Nephroma* species, as traditionally circumscribed, could be identified on the basis of fungal ITS sequences. The results showed that the tripartite species did not form a monophyletic group within the genus. Concurrently, transitions from a bipartite symbiosis to a tripartite symbiosis, or vice versa, must have occurred repeatedly during the evolution of *Nephroma*. Next, the genetic diversity of

green algal and cyanobacterial symbionts in *Nephroma* was examined. Minimal variation was found in green algal ITS genotypes of the tripartite species *N. arcticum* and *N. expallidum*. Almost identical sequences were also obtained from thalli of two tripartite *Peltigera* species (*P. aphthosa* and *P. leucophlebia*). In contrast, the diversity of symbiotic *Nostoc* in *Nephroma* was found to be high. In accordance with earlier results, a phylogenetic analysis of partial 16S rDNA sequences demonstrated that all symbiotic *Nostoc* formed a monophyletic group with two main subgroups. The first of these corresponded to the previously identified *Nephroma* guild cyanobionts, and this included the *Nostoc* symbionts of all bipartite *Nephroma* species. The second group included the cyanobionts of many terricolous lichens, including several *Peltigera* species, but also the *Nostoc* symbionts of the two tripartite *Nephroma* species analyzed. This was interesting as it implied that within *Nephroma*, evolutionary transitions between bi- and tripartite symbioses could not have been achieved simply via the acquisition or loss of the green algal photobiont; they must have also required a concurrent switch of cyanobacterial symbionts. This may account for the apparently poor fitness of *N. arcticum* photosymbiodemes, for example. While these bipartite cyanomorphs have lost their green algal symbionts, they may still continue to house the "wrong" cyanobacterial genotype. As a whole, the new findings confirmed that lichen symbiotic *Nostoc* genotypes do not always group according to the species identities of their fungal hosts. For example, while *N. resupinatum* formed a sister group to all the other *Nephroma* species analyzed, the *Nostoc* symbionts of this species were often sequence-identical to those identified from other bipartite *Nephroma* species.

Linke et al. (2003) identified trnL genotypes of cyanobacteria from three *Peltigera* species and two *Nostoc* isolates obtained from named culture collections. The *Peltigera* species examined included a specimen of the aquatic species *P. hydrothyria* from western North America. The *Nostoc* genotypes obtained were closely related to those previously identified from other cyanolichens and from environmental isolates, demonstrating the power of trnL sequences as a tool of identifying lichen symbiotic *Nostoc* strains. The close relationship between lichen symbionts and named laboratory strains indicated that cyanobacterial genotypes belonging to several different botanical *Nostoc* species were present in different cyanolichens.

The geographical scope of earlier studies was expanded considerably by Wirtz et al. (2003) who identified cyanobacterial trnL genotypes from five cyanolichen species and free-living *Nostoc* colonies collected from several localities in two ice-free inland habitats on Livingston Island, maritime Antarctica. Lichen specimens from polar environments had not been analyzed in previous studies (Fig. 5A). Two of the lichens (*Leptogium puberulum* and *Massalongia carnosa*) were bipartite lichens, while the other three were tripartite species (*Placopsis contortuplicata*, *Placopsis parellina* and *Psoroma cinnamomeum*). In total, 64 specimens were examined, including four specimens of *P. parellina* collected in Argentina. The results revealed that Antarctic *Nostoc* genotypes formed two major groups, corresponding to the two different heptanucleotide repeat classes. The *Nostoc* genotypes from specific lichen species did not group according to their fungal hosts but several genotypes were widely intermixed between different

hosts. Three different genotypes were identified from the free-living *Nostoc* colonies and two of these were also found from some lichen thalli. The cyanobionts identified form Argentine populations of *P. parellina* were similar to each other but differed clearly from the Antarctic cyanobionts. The authors proposed that the low photobiont specificity observed might have been due to a limited number of lichen symbiotic *Nostoc* strains available, as a result of the extreme Antarctic environmental conditions. Accordingly, lichen mycobionts could survive only if they were relatively non-specific in their choice of cyanobacterial symbionts. Also a decrease in the number of cyanolichen species with increasing latitude might, in part, reflect the effects of a strong selection pressure against highly specific lichen mycobionts in extreme environments.

Oksanen, Lohtander et al. (2004) studied the evolution of trnL introns in heterocystous cyanobacteria and compared the utility of trnL sequences as a phylogenetic marker to that of 16S rRNA gene, the conventional marker in Eubacteria. Previous findings had supported the presumption that studies based on trnL sequences and 16S rDNA sequences of lichen symbiotic *Nostoc* genotypes produced qualitatively similar results – the resolution of the two markers was somewhat different, but the diversity patterns revealed were broadly congruent. However, as the evolution of trnL introns was known to be complex (Kuhsel et al. 1990, Xu et al. 1990, Paquin et al. 1997, Besendahl et al. 2000) and controversial (Rudi and Jakobsen 1997, 1999, Rudi et al. 2002), and because the evolution of the P6b region was particularly puzzling, this type of analysis was of considerable interest. The results confirmed that nucleotide differences in the P6b region of *Nostoc* trnL intron sequences were tricky to use in phylogenetic analyses. In the more conserved parts of these sequences, the small number of informative characters restricted phylogenetic group formation, this being related to short sequence length and the stable secondary and tertiary structure required by the self-splicing property of the group I intron. Hence, the conserved parts of trnL sequences alone did not provide enough sequence variation for hierarchical analyses. Nevertheless, phylogenetic analyses of the conserved parts of trnL sequences generally resulted in results similar to those based on the 16S rRNA gene, and thus provided additional support for 16S rDNA phylogenies. It was also noted that one should not explicitly trust 16S rRNA gene phylogenies alone without knowledge of possible variation among different gene copies.

Rikkinen (2004) reviewed all cyanobacterial trnL sequences that had become available by that time. The data set of 606 sequences was analyzed with multivariate methods, as a step towards putting all identified *Nostoc* trnL genotypes into meaningful contexts. The data set included many *Nostoc* genotypes from lichens and bryophytes, but also those identified from cycad roots (Costa et al. 1999, 2004) and free-living colonies (Wright et al. 2001). The results showed that most *Nostoc* trnL genotypes were clearly distinguished from those of other nostocalean genera. Three main sequence types, the Muscorum-, Commune- and Punctiformis-type, were delimited from the main cluster of *Nostoc* intron sequences. All sequences amplified from lichens had so far belonged to the latter two types. It was also noted that the sampling of 16S rRNA genes had been seriously skewed towards *Nostoc* genotypes with Punctiformis-

type trnL sequences. This drew into question whether all relevant subgroups of symbiotic *Nostoc* had been adequately sampled when constructing 16S rDNA trees. It was concluded that while the sequence types offered only a heuristic classification, they were not in conflict with phylogenetic divisions based on the 16S rRNA gene and conserved parts of the trnL intron. Furthermore, the identified groups seemed to broadly correspond with some botanical species within the genus *Nostoc*, which have been characterized on the basis of morphological characters and life-history traits. The findings confirmed that while informative sites in the conserved parts of cyanobacterial trnL sequences were useful for distinguishing between different genera and distantly related *Nostoc* species, characteristics of the P6b region could only be used for comparing closely related genotypes. In other words, the conspicuous features of this region, such as the presence of one of two contrasting heptanucleotide repeat motifs, was indicative of close phylogenetic relationship only if other shared signature characteristics in the more conserved parts of the intron sequence also indicated that the genotypes were closely related.

Oksanen, Jokela et al. (2004) reported that one *Nostoc* strain isolated from the bipartite cyanolichen *Pannaria pezizoides* (Fig. 3G) collected from southern Finland produced six different microcystins in culture. Microcystins are hepatotoxic cyclic heptapeptides which had previously been almost exclusively reported from planktonic cyanobacteria. The presence of the microcystin synthetase gene complex in the *Nostoc* strain was confirmed by sequencing and the gene sequences were compared of other microcystin-producing cyanobacteria. The phylogenetic position of the toxin-producing *Nostoc* strain among lichen symbiotic and other cyanobacteria was determined on the basis of full length 16S rDNA sequences. This dataset was largely the same as had been previously used for studying the utility of trnL sequences as a phylogenetic marker. The results showed that the toxin-producing *Nostoc* strain belonged to a monophyletic group of symbiotic *Nostoc* genotypes, which also included the reference strain *N. punctiforme* PCC 73102 (originally isolated from the cycad *Macrozamia* in Australia). Among all available *Nostoc* 16S rDNA sequences, the closest relative was another laboratory strain originally isolated from an unspecified lichen species in Scotland.

Svenning et al. (2005) studied the phylogeny of symbiotic and free-living *Nostoc* strains on the basis of full length 16S rDNA sequences. Their sampling included a selection of nostocalean cyanobacteria with an emphasis on symbiotic *Nostoc* strains isolated from the rhizomes of the angiosperm genus *Gunnera* (Fig. 6F). An enlarged dataset also included sequences of many *Nostoc* genotypes identified from lichen symbioses. The results were congruent with earlier findings. All the *Nostoc* genotypes obtained from *Peltigera* guild lichens by Rikkinen et al. (2002) fell into one well supported group. This monophyletic group included both free-living and symbiotic *Nostoc* strains from many different environments. While none of the new sequences were included in the previously described group of *Nostoc* symbionts from *Nephroma* guild lichens, also this group was recovered in the extended analysis with strong support. On a general level, the results illustrated a mix of cyanobacteria named as *Nostoc* within the genus *Anabaena* and vice versa. The authors discussed this problem and pointed

Figure 6. Plant hosts of symbiotic cyanobacteria; some of which can share *Nostoc* symbionts with cyanolichens. **A** The liverwort *Blasia pusilla* has *Nostoc* symbionts in auricles (Rikkinen and Virtanen 2008) **B** Hornworts house *Nostoc* symbionts in slime cavities (Costa et al. 2002) **C** The cyanobacterial symbiont of the water fern *Azolla* is not closely related to lichen symbiotic cyanobacteria (Ran et al. 2010) **D** All cycads associate with cyanobacteria, mainly *Nostoc* (Costa et al. 1999) **E** The cyanobacterial symbionts of cycads are housed in specialized roots (Costa et al. 2004, Gehringer et al. 2010, Yamada et al. 2012) **F** *Gunnera* species have endosymbiotic *Nostoc* in creeping rhizomes (Nilsson et al. 2000).

out that there were several candidate clades to which the genus name *Nostoc* could be attached. They also suggested their favorite – a well-supported clade including both major groups of lichen symbionts identified in Rikkinen et al. (2002), in addition to related symbiotic and free-living genotypes.

O'Brien et al. (2005) used phylogenetic analyses of partial 16S rDNA, rbcLX, and trnL sequences to study the degree of host specialization of *Nostoc* genotypes associated with four closely related species of *Peltigera*, and to compare their symbionts with cyanobacteria associated with other lichens and plant hosts, as well as free-living strains of *Nostoc* and related cyanobacteria. The sequences were obtained from five to seven specimens each of *Peltigera canina*, *P. didactyla*, *P. membranacea*, and *P. rufescens*, numerous other cyanolichens, and from cyanobacterial isolates from environmental samples, thalloid bryophytes, cycads, *Gunnera*, and *Geosiphon pyriforme*. The lichen specimens were mainly from North America and Europe, with smaller sets from other parts of the world. The results were broadly in line with those of earlier studies. The genus *Nostoc*, as presently circumscribed, comprised two divergent lineages, one with several laboratory strains, and the second with many symbiotic genotypes intermixed with free-living strains from environmental samples. A clade of *Nostoc* symbionts corresponding to those generally found in *Nephroma* guild lichens was recovered in all three analyses. The cyanobacteria of individual *Peltigera* species were often more closely related to *Nostoc* genotypes identified from other lichen species, plant hosts and/or environmental samples than to each other. This indicated that host specialization among symbiotic *Nostoc* genotypes was low, and that opportunities for coevolution with different partners appeared to have been rare. There were several cases of *Nostoc* genotypes from different continents having identical sequences at one or two loci, but cyanobacteria sharing identical genotypes at all three loci were always from the same continent, suggesting that there was some population differentiation at intercontinental scales.

The phylogenetic position of the Pacific North American endemic cyanolichen, *Nephroma occultum* was analyzed by Piercey-Normore et al. (2006), who identified *Nostoc* symbionts from two thalli collected from British Columbia. Also the symbiont of a *Nephroma isidiosum* thallus from the same region was studied. Even though *N. occultum* is endemic to moist forests in western North America, it fell into the same clade with South American temperate rainforest species. The two closely related *Nostoc* trnL genotypes identified were novel but belonged to the same group of symbionts as that of *N. isidiosum*, and those previously identified from many other *Nephroma* guild lichens. The authors proposed that a bottleneck that limited genetic variation in the *Nostoc* photobiont could in part account for the endemism and the decline of *N. occultum*, and pointed out that a larger number of populations should be analyzed to shed more light on variation in cyanobacterial composition and the possibility of a genetic bottleneck in the *Nostoc* symbionts.

Stenroos et al. (2006) studied the phylogeny of symbiotic and free-living *Nostoc* strains on the basis of partial 16S rDNA, rbcLX, and trnL sequences. Their sampling included a wide selection of lichen symbiotic and free-living cyanobacteria, with many new sequences from South American specimens of *Pseudocyphellaria* (Fig. 3B). Data-

sets from the different gene loci were combined into a single analysis using direct optimisation. The results generally confirmed findings made in earlier studies: many lichen hosts appeared to be strongly selective towards their cyanobionts, while others were generalists, being able to associate with a spectrum of different *Nostoc* genotypes. Free-living and plant-associated *Nostoc* genotypes were not clearly separated from the lichen symbiotic ones, and also this analysis confirmed the existence of the distinct lineage of *Nostoc* genotypes that are typically found in *Nephroma* guild lichens (now referred to as the *Pseudocyphellaria* group). In the discussion, the authors interpreted the apparent lack of parallel cladogenesis between different hosts and *Nostoc* symbionts as evidence of no co-evolution between the symbiotic partners. They also reviewed previous finding regarding the difficulties of using trnL sequences in phylogenetic analysis, and argued that their study was the first one to prove cyanobiont selectivity in lichens.

Studies on the cyanobacterial diversity of *Pseudocyphellaria* (Fig. 3B) were continued by Summerfield et al. (2006) who identified *Nostoc* trnL genotypes from several specimens of *P. crocata* (New Zealand, Australia, Chile, and British Columbia), *P. neglecta* (New Zealand and Australia), and *P. perpetua* (British Columbia). Five different *Nostoc* genotypes were found from the lichens analyzed, two of these in a number of specimens, and three each from a single lichen thallus. Signature characteristics in the trnL sequences show that all these genotypes were *Nephroma* guild cyanobionts. As no connection was found between genetic diversity of the fungal hosts and symbiont choice, all three *Pseudocyphellaria* taxa seemed to rely on a common pool of cyanobionts.

Myllys et al. (2007) examined cyanobiont selectivity in eight epiphytic cyanolichen species, two of these tripartite lichens (*Lobaria pulmonaria*, *Peltigera leucophlebia*) and the other five bipartite lichens (*Nephroma bellum*, *N. parile*, *N. resupinatum*, *Parmeliella triptophylla*, *Peltigera praetextata*). The sampling focused on one old-growth forest in southern Finland, and phylogenetic relationships between cyanobacterial symbionts were analyzed using partial 16S rDNA and rbcLX sequences and direct optimisation. The results were again in line with those of earlier studies: two clades of *Nostoc* symbionts, corresponding to *Nephroma* guild symbionts (here named Cade II) and *Peltigera* guild symbionts (here named Clade I), were recovered in all phylogenetic trees. The latter group also included free-living and plant symbiotic *Nostoc* strains. Within these two main lineages each lichen species associated with only one subclade of *Nostoc* symbionts, indicating that the fungi were discriminative in their choice of cyanobionts. The main exception was *Lobaria pulmonaria* (Fig. 3A), which seemed to associate with a wide range of different *Nostoc* genotypes. *Nostoc* symbionts exhibited a much lower degree of selectivity towards their fungal partners, and the lichen associations as a whole could not be described as highly specific. The authors found evidence of cyanobiont sharing between several lichen species and also noted that different *Nostoc* genotypes were present in different *Peltigera leucophlebia* specimens.

Elvebakk et al. (2008) focused on phylogenetic patterns among *Nostoc* cyanobionts within bi- and tripartite lichens of the genus *Pannaria*. The study was based on full length 16S rRNA gene sequences amplified from specimens of 21 *Pannaria* species collected in seven countries in the northern and northern hemispheres. Also two

specimens of *Pseudocyphellaria* from the Juan Fernández Islands were studied. The new *Nostoc* genotypes were compared to those previously found from various other symbioses and environmental samples. The results showed that while only one *Nostoc* genotype was present in all bipartite *Pannaria* thalli, several specimens of tripartite lichens had different *Nostoc* genotypes in different cephalodia. All the newly identified *Nostoc* genotypes belonged to one well supported lineage, with the phylogenetic tree showing a gradual transition from the lowermost nodes, corresponding to *Nostoc* genotypes of the *Peltigera* guild, to the uppermost, corresponding to *Nostoc* genotypes of the *Nephroma* guild. Several subgroups within the major branches gained good support and the *Pannaria* cyanobionts were placed into three relatively well defined groups. Some bipartite and tripartite lichen species seemed to share similar *Nephroma* guild symbionts, while other groups of *Nostoc* genotypes were exclusively found from either bi- or tripartite *Pannaria* species. After a detailed discussion of the observed diversity patterns the author concluded that while the distributions of some symbiotic *Nostoc* genotypes were correlated with mycobiont taxonomy at the species or genus level, the distribution patterns of others were best explained by habitat ecology. They also identified two possible cases of coevolution between symbiotic partners.

Papaefthimiou et al. (2008) compared phylogeny and morphology of cyanobacteria originating from different plant symbioses (*Anthoceros*, *Azolla*, several cycads, and *Gunnera*) with free-living *Nostoc* isolates from different habitats (Fig. 6). While their analysis did not include any cyanobacterial isolates from lichens, some of the plant symbionts included were very closely related to lichen symbiotic strains. The phylogenetic analysis indicated that two distinct patterns of evolution of symbiotic behavior existed within nostocacean cyanobacteria, one leading to the symbioses of *Nostoc* species with a variety of plants (and lichen-forming fungi), and the other leading to the association of a unique cyanobacterial taxon with the water fern *Azolla*. The authors suggested that the frequent occurrence of symbiotic strains within *Nostoc* s.str. would be linked to the intensive hormogonia production that was observed in many of the strains studied.

Rikkinen and Virtanen (2008) returned to *Nostoc* diversity in thalloid liverworts by identifying cyanobacteria associated with *Blasia pusilla* (Fig. 6A) in Finland and *Cavicularia densa* in Japan. The focus was on *Nostoc* trnL genotypes isolated from vegetative propagules, since both bryophytes studied could promote the persistence of their symbiosis with specialized gemmae, which facilitate the simultaneous dispersal of both symbiotic partners. The results showed that the predominant *Nostoc* trnL genotypes identified from both bryophytes were closely related and also closely related to those that had been identified from hornworts, cycads and many terricolous cyanolichens. For example, one *Nostoc* trnL genotype found from *B. pusilla* in Finland had previously been found from *Peltigera occidentalis* in western North America. Another genotype had been identified from *Peltigera degenii* (Canada), *P. canina* (Germany), *P. neopolydactyla* (Oregon), *P. praetextata* (Finland), and P. *membranacea* (Sweden, Canada, and Oregon). Within this context, Rikkinen (2009) gave a short summary of *Nostoc* diversity patterns in lichens and argued that symbiont-switches between lichen-forming fungi and plant hosts may have played a role in the evolution of some extant cyanobacterial symbioses.

Kaasalainen et al. (2009) studied cyanobacterial diversity in *Peltigera leucophlebia* and confirmed the presence of a microcystin-producing *Nostoc* strain in the cephalodia of this tripartite lichen species. Cyanobacteria were identified on the basis of full length 16S rDNA sequences and also the presence of the microcystin synthetase gene mcyE was confirmed. A wide variety of different *Nostoc* 16S rRNA genotypes were detected from lichen cephalodia, but only one was found in toxin-producing cultures. In phylogenetic analysis, most *Nostoc* genotypes from the lichen specimen were placed into three closely related groups among sequences previously obtained from various *Peltigera* guild lichens and plant symbioses. The closest relatives of the microcystin-producing genotype were the toxin-producing *Nostoc* strain previously isolated from *Protopannaria pezizoides* and related *Nostoc* genotypes from the liverwort *Blasia pusilla*. It was concluded that the wide diversity of *Nostoc* genotypes observed may have been partly due to the sampling method used. In order to get enough biomass for chemical analysis, hundreds of minute cephalodia from the upper surface of the lichen had to be pooled into one sample. While it is thus possible that some of the *Nostoc* genotypes identified might have been epiphytes from the thallus surface, such cyanobacteria could not have produced the high concentration of microcystins detected. Furthermore, all nine *Nostoc* strains isolated from *P. leucophlebia* cephalodia produced microcystins in culture and belonged to the one genotype that was dominant among those identified directly from cephalodia.

The taxonomic and geographic scope of earlier studies was expanded by Lücking et al. (2009) who investigated the phylogenetic relationships of presumed *Scytonema* and *Chroococcus* cyanobionts in tropical lichen species collected from Costa Rica (Figs 3H, 5D). The study was based of partial 16S rDNA sequences. The fungal hosts included both Ascomycota (*Coccocarpia*) and Basidiomycota (*Acantholichen*, *Dictyonema*), and the symbionts were compared with those isolated from several cyanobacterial genera. The results demonstrated that the filamentous or nearly unicellular cyanobacteria of all lichens studied belonged to the same phylogenetic lineage. However, they did not group together with *Scytonema* isolates, but rather represented a previously unrecognized lineage of lichen symbionts corresponding to the botanical name *Rhizonema*. The novel lineage was found to be present in at least twelve species and four genera of lichen- forming fungi representing a wide range of morphological variation and systematic affinities. The results also suggested that many *Rhizonema* genotypes were not host specific, but appeared to have evolved through wide photobiont sharing between unrelated but ecologically similar fungi, i.e. within a photobiont-mediated guild. While discussing specificity and selectivity patterns in lichens the authors noted that the apparent selection of well adopted photobiont strains and subsequent horizontal transfer between unrelated fungi was a phenomenon not very unlike crop domestication in human civilizations.

Otalora et al. (2010) presented a phylogenetic study based on new *Nostoc* rbcLXS sequences from 24 species of gelatinous cyanolichens of the Collemataceae and many *Nostoc* sequences obtained in earlier studies (Figs 3E–F). The results showed that there were two contrasting patterns of specificity between collemataceous fungi and *Nos-*

toc. The first corresponded to the commonly observed situation for lichens where a monophyletic group of cyanobacteria associated with multiple species of lichen-forming fungi, and where individual fungal species associated with multiple photobiont genotype groups. However, five species of *Leptogium* and *Collema* exhibited a different pattern where each monophyletic *Nostoc* genotype group was associated with only one species of lichen-forming fungus, and this fungus only associated with that unique *Nostoc* group, indicating strong reciprocal specificity by both symbiotic partners. The five lichen species had in common a largely asexual mode of reproduction where the symbiotic propagules contain both symbionts, allowing a vertical transmission of the photobiont from one fungal generation to the next. All these species also appeared to have specialized to live in relatively narrow ecological niches. The authors concluded that each of the five distinct monophyletic *Nostoc* groups, associated with the five specific mycobiont species, seemed to represent independent transitions from a generalist state during the evolution of both partners. The transitions might be explained by shifts to asexual fungal reproduction, involving vertical symbiont transmission, and narrowing of ecological niches.

Fedrowitz et al. (2011) studied the diversity patterns of *Nostoc* trnL genotypes in three epiphytic *Nephroma* species within a boreal forest landscape in Finland (Fig. 5B). Cyanobacterial 16S rRNA gene sequences were also amplified from a subset of lichen specimens and the fungal ITS gene was used to confirm fungal species identities of critical lichen specimens. The results indicated high photobiont specificity and selectivity within the local forest landscape: only five closely related *Nostoc* trnL genotypes were identified from the 232 *Nephroma* thalli analyzed. Two *Nostoc* genotypes were widely shared by *N. bellum* and *N. resupinatum*, while the thalli of *N. parile* always contained one of two different genotypes. On individual tree trunks all thalli of each *Nephroma* species usually contained the same *Nostoc* genotype. Furthermore, the two lichen species that mainly disperse via fungal spores (*N. bellum* and *N. resupinatum*) tended to have identical photobionts, while the symbiotically dispersing *N. parile* clearly relied on its own symbiont pool. On a landscape level, the distribution of all *Nostoc* genotypes seemed to have been influenced by strong founder effects presumably caused by relatively low colonization rates from one tree to another.

Fedrowitz et al. (2012) continued studies on the spatial aspects of symbiont specificity in *Nephroma* by examining the genetic diversity of fungal hosts and associated *Nostoc* photobionts within a global framework. The data set consisted of 271 *Nephroma* ITS sequences and 358 *Nostoc* trnL intron sequences, with over 150 sequence pairs originating from single lichen thalli. The results reconfirmed that all bipartite *Nephroma* species associate with one group of *Nostoc* different from the cyanobacterial symbionts found in cephalodia of tripartite *Nephroma* species. Most bipartite *Nephroma* species shared their *Nostoc* symbionts with at least one other species, and none of them associated with a single *Nostoc* genotype, generally supporting the existence of wide symbiont sharing. While the existence of specific associations between some symbiont pairs could be observed over vast geographical distances, genetic differences in both groups of symbionts tended to increase with increasing geographical distance. It

was concluded that symbiont selectivity patterns among the *Nephroma* species studied were best described as a geographic mosaic, with higher selectivity locally than globally. This mosaic of symbiotic interactions probably partly reflects regional coadaptation of specific symbiont combinations to particular environmental conditions, but also the influence of random effects during dispersal history.

The structural evolution in the P6b region of trnL sequences was studied further by Olsson et al. (2012). All the analyzed sequences were amplified from the monophyletic group of lichen symbiotic *Nostoc* that characterizes all lichens of the *Nephroma* guild. Phylogenetic analyses of 16 rDNA and trnL intron sequences, and secondary structure reconstructions of transcribed P6b stem loops were used to study the replication mechanisms and to gain new insights into distribution of indels not corresponding to the typical tandem repeat motif of these sequences. The results indicated that indel events were usually accompanied by specific single nucleotide changes in the P6b region and had occurred several times independently. In spite of this, the P6b sequences were found to provide useful phylogenetic information within this group of closely related *Nostoc* genotypes. Elhai et al. (2008) had previously reported that the short segments of non-repeating DNA interrupting the tandem repeats of trnL introns are dispersed throughout cyanobacterial genomes. The analyses of such sequences in context with their surroundings gave new information of the possible mechanistic basis of their dispersal, apparently distinct from mechanisms thus far described.

Finally, Kaasalainen et al. (2012) identified microcystin-producing cyanobacteria from cyanolichens by amplifying a part of the gene cluster encoding the enzyme complex responsible for toxin production and detecting the compounds directly from lichen thalli. The data set included 803 cyanolichen specimens originating from five different continents. Also cyanobacterial 16S rRNA sequences were obtained from all toxin producing thalli. The results showed that lichens with cyanobacterial toxins are common and found all over the globe. The cyanobacterial mcyE gene was amplified from nearly a hundred lichen specimens. Thirty different mcyE gene sequences were identified and these grouped together with the mcyE genes previously sequenced from other heterocystous cyanobacteria. 41 *Nostoc* 16S rDNA genotypes were found from the same set of specimens, always only one genotype from each lichen specimen. The phylogenetic trees constructed using mcyE and 16S rDNA sequences were not congruent, but both displayed a similar sporadic distribution of toxin-producing *Nostoc* symbionts among different cyanolichen groups and geographical origins. It was proposed that from an evolutionary perspective the high diversity of mcyE genes in lichen symbiotic cyanobacteria would be partly explained by their symbiotic way of life, especially the packaging of cyanobacterial symbionts into small vertically transmitted populations that must be relatively prone to random events, disruptive selection and genetic drift. In other words, when compartmentalized into the symbiotic propagules, the *Nostoc* symbionts invariably experience genetic bottlenecks where population sizes are severely reduced. At the same time, the symbiotic association with a fungal host may favor different chemical traits from those typically seen in many free-living *Nostoc*. The recurrent bottlenecks and other population shaping effects may thus account

for the high genetic and chemical diversity observed. Concurrently, lichen symbioses may have been an important environment for the diversification of toxin-producing cyanobacteria.

In addition to the primary research articles reviewed above, relevant discussions concerning the genetic diversity of lichen symbiotic cyanobacteria have also been published in several PhD theses, including those of Per Paulsrud (2002), Ilona Oksanen (2004) and Katja Fedrowitz (2011).

Conclusions and future perspectives

Per Paulsrud and Peter Lindblad (1998) ended their pioneering study on the diversity of lichen symbiotic *Nostoc* with the following words. "*This study is the first step in a more extensive survey of the genetic diversity of symbiotic cyanobacteria. We have addressed several important questions concerning lichen biology and presented a method which opens up the possibility of studying these, and other, symbiotic systems in more detail. Examining other symbiotic systems by the same approach will not only provide information concerning the biology of these systems but will also reveal similarities and differences between cyanobacterial symbioses in general.*"

Now, fifteen years and many interesting steps later, we are still only beginning to understand the complex network of biological interactions and evolutionary processes in which symbiotic cyanobacteria and their fungal partners live and diversify. During the coming years we can undoubtedly enjoy the results of many intriguing case studies of cyanobacterial diversity in new taxonomic groups and in different ecological settings. The genetic diversity of several important lineages of lichen symbiotic cyanobacteria remains totally unknown and the same applies to many biomes rich in cyanolichen species. When will we see the first in-depth studies on the cyanobacterial symbionts of Lichinomycetes, and on the hyperdiverse cyanolichen communities of tropical montane rainforests, for example?

Three different loci have so far been used in molecular studies of lichen symbiotic cyanobacteria: 16S rDNA, trnL, and rbcLXS. These markers have been used singly or in combination, and they all have their positive and negative aspects (e.g. Han et al. 2009). For example, the 16S rDNA gene is too conserved for many uses and trnL sequences are difficult to use in phylogenetic analyses. In some cases the different markers have also given partly incongruent results. For example, O'Brien et al. (2005) reported that while DNA extractions from *Peltigera degenii* and *P. horizontalis*, respectively, gave identical trnL sequences, there were substantial differences in partial 16S rDNA and rbcLX sequences. In two further cases DNA extractions produced identical 16S sequences and similar rbcLX sequences, but trnL intron sequences which had different repeat motifs in the P6b region. In eleven pairwise comparisons, the DNA extractions containing identical trnL sequences differed at both other loci and there were five and three comparisons, respectively, where only the 16S rDNA or the rbcLX sequences were identical.

These observations underline the problems one can encounter for example when trying to place a set of *Nostoc* trnL genotypes from different geographical regions and cyanolichen species into a phylogenetic context. On the other hand, there are also some inherent problems in screening multiple gene loci from complex biological material such as lichen thalli. A square millimetre of lichen may first appear like a small sample, but in the world of bacteria it is relatively huge. Extracts from lichen specimens will commonly contain DNA from thousands of cyanobacterial cells – potentially belonging to several different genotypes, species or even genera. Naturally the desired locus of the dominant DNA is usually amplified, but not necessarily in all cases. This is a particular problem in studies where several different loci are amplified: how can we know for sure that all the loci were amplified from the DNA of the same cyanobacterium? This is one reason why multi-gene analyses from field specimens should eventually be supplemented by data from unialgal laboratory cultures.

All bipartite cyanolichen species studied so far have appeared to house only one cyanobacterial genotype in each thallus. However, attempts to isolate symbionts into culture have often revealed the presence of several different genotypes in the same material. For example, Summerfield et al. (2002) found that *Nostoc* isolates from three *Pseudocyphellaria* species were different to those identified directly from lichen thalli, and did not seem to represent the primary symbionts of the lichens. Indeed, attempts to culture the correct symbiont from a lichen thallus can often be confused by the common presence of epiphytic cyanobacteria or their propagules. It will be interesting to see whether further studies will keep reconfirming the dominance of only one symbiont genotype in each bipartite cyanolichen thallus or reveal that also minor symbionts can sometimes be present. The latter option would not be all that surprising in the light of comparable findings from other symbioses, such as green algal lichens (e.g. Casano et al. 2011) and corals (e.g. Silverstein et al. 2012). It is quite feasible that the observed symbiont diversity in some cyanolichen species reflects one mechanism by which these symbioses respond to habitat variability and environmental change: it may allow some fungal hosts to associate with the cyanobacterial genotypes that are optimally adapted to the prevailing conditions. In some cases such processes might well operate on the level of single cyanolichen thalli – especially in the case of cephalodiate species.

Already Paulsrud and Lindblad (1998) noted that in order to determine to what extent fungal hosts really discriminate between *Nostoc* genotypes, we would need a much better knowledge of the diversity of cyanobacteria in the soil. The almost total lack of information on the diversity of aposymbiotic cyanobacteria in lichen habitats is still a major problem, seriously hindering attempts to understand the dispersal ecology of cyanolichens, among many other questions. The few studies in which cyanobacteria have been cultured from bark and soil have generally revealed high diversity in the substrate (e.g. Rikkinen et al. 2002, Rikkinen and Virtanen 2008). However, the possible connections between free-living cyanobacteria and their lichen symbiotic relatives remain unknown.

Some cases where different cyanobacteria are consistently found from different thalli of single cyanolichen species are explained by previously unrecognized heterogeneity

within the fungal host. However, in many cases one fungal species can associate with more than one symbiont and these are often also shared by several other cyanolichen species. The concept of photobiont-mediated guilds in lichens gained attention after it was published by Rikkinen et al. (2002) and refined by Rikkinen (2003). However, the basic idea was already presented in Rikkinen (1995) while trying to explain the apparent lack of interspecific competition observed in many lichen communities. As such, the concept has clear roots in the community ecology of the late 1900's (e.g. Hawkins and MacMahon 1989, Simberloff and Dayan 1991, Wilson 1999). While numerous ecological studies have since examined how coevolutionary interactions are modified within ecological communities (e.g. Thrall et al. 2007, Crowley and Cox 2011), the only synthetic theory presented so far is that of Thompson (2005). It is rewarding to see that the author chose to use lichens as an example when describing how, so long as some horizontal transmission of symbionts is possible, geographic mosaics of coevolution tend to produce variable degrees of reciprocal specificity in coevolving mutualistic symbioses.

Finally, it is important to note that molecular studies on the diversity of symbiotic cyanobacteria in lichens have not developed in a vacuum, but in close conceptual interaction with comparable studies on plant symbiotic and non-symbiotic cyanobacteria, lichen-symbiotic green algae, and lichen-forming fungi. Multidisciplinary influences have been crucial and will continue to be so in the future. For example, the recent description of the highly eroded genome of the cyanobacterial symbiont in *Azolla* (Ran et al. 2010) raises many interesting questions regarding the potential nature of some lichen symbiotic cyanobacteria that have not been successfully isolated into culture. In any case, the new genomic approaches will open many intriguing questions and provide novel approaches for future research.

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Biogeography and ecology of *Cetraria aculeata*, a widely distributed lichen with a bipolar distribution

Christian Printzen[1], Stephanie Domaschke[2], Fernando Fernández-Mendoza[2], Sergio Pérez-Ortega[3]

1 *Senckenberg Forschungsinstitut und Naturmuseum and Biodiversität und Klima Forschunszentrum (LOEWE BiK-F), Abt. Botanik und Molekulare Evolutionsforschung, Senckenberganlage 25, D-60325 Frankfurt am Main, Germany* **2** *Biodiversität und Klima Forschungszentrum (LOEWE BiK-F), Senckenberganlage 25, D-60325 Frankfurt am Main, Germany* **3** *Museo Nacional de Ciencias Naturales, MNCN-CSIC, c/ Serrano 115 dpdo, E-28006 Madrid, Spain*

Corresponding author: *Christian Printzen* (cprintzen@senckenberg.de)

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Abstract

Ecological and historical biogeography of lichens have rarely been studied in a concerted effort, but both aspects have to be taken into consideration when explaining the distributional patterns of species. This review summarizes, partly preliminary, results from a series of studies on phylogeography, ecophysiology and symbiotic interactions of the lichen *Cetraria aculeata*. This species is not only widespread but also occupies a very wide ecological niche. Evidence suggests that *Cetraria aculeata* has evolved and diversified in the Northern Hemisphere and colonised the Southern Hemisphere during the Pleistocene. Genetic isolation of populations indicates the absence of ongoing long range dispersal and genetic exchange between geographically isolated populations. We observe a hitherto unrecognized genetic diversity that may indicate ecotypic differentiation and speciation processes. Mediterranean and Polar populations differ not only genetically, but also in ecophysiological properties. Ongoing common garden experiments will have to show whether genetically fixed adaptation or acclimation is responsible for these differences. The genetic structure of the photobiont is best explained by climatic differences between localities, but co-dispersal with the mycobiont plays an important role as well. Taken together, these results indicate that a photobiont switch in the past enabled *C. aculeata* to widen its ecological niche, with subsequent genetic isolation of populations. Photobiont switches may play a crucial role in speciation processes of lichens. A combination of ecophysiological and phylogeographic studies with experimental approaches is necessary to better understand the reaction of lichens to changing environmental conditions.

Keywords
Ecological biogeography, phylogeography, population genetics, symbiont interactions, lichens, *Cetraria*

Introduction

Biogeography aims at explaining the distributions of species, which are basically shaped by two factors: their ecological niches and their evolutionary history. Of the two fundamental subdisciplines of biogeography (Cox and Moore 2010), ecological biogeography explains the restricted occurrence of species in terms of environmental conditions (climate, properties of the substratum etc.) and the adaptation or acclimation of the species to these conditions. Historical biogeography, the second important subdiscipline, is focussed on events or processes in the past (plate tectonics, dispersal events etc.) that shaped the extant ranges of species. It is obvious that neither ecological aspects nor history alone are sufficient to understand the geographical patterns that we observe in nature. The close connection between both factors was recognized from the very beginning (e.g. Schimper 1898). Darwin (1872) already invoked glacial climate changes as explanations for the bipolar distribution of taxa. Over the last decades, fossil and genetic data provided compelling evidence for the hypothesis that the distributional ranges of most temperate species shifted widely during the Pleistocene glacial cycles (Hewitt 1999). Their adaptation to certain ecological conditions forced species to migrate or disperse to follow the displacement of their habitats, i.e. ecology influenced the species' population history. On the other hand, adaptation occurs over evolutionary time scales. Ecotypic differentiation (Turesson 1922) in particular, the formation of ecotypes adapted to different ecological conditions within a species' range, may be a direct result of historical population processes, e.g. the fragmentation of habitats or dispersal to areas unconnected with the main distributional range.

Before molecular genetic data became available, historical biogeography relied on fossil evidence or comparisons of distributional ranges of closely related species, often in conjunction with paleoenvironmental reconstructions (e.g. Gray 1859, Firbas 1949). This approach largely excluded taxa for which fossil evidence was sparse. Since the 1990s molecular data have put historical biogeography on an entirely new basis. Phylogenetic methods and model-based approaches using population genetic data allow the reconstruction of past population structure and distributional ranges independent of fossil data, often with high spatial resolution (Ronquist and Sanmartín 2011, see chapter on demography and range shifts below for details). In the field of ecology, ecological niche modelling in combination with spatially explicit reconstructions of paleoclimates has increasingly been used to reconstruct the past distributional ranges of species, e.g. during the last glacial maximum. Comparisons of the results of both methods have shown that phylogeographic methods and approaches using ecological niche modelling often come to similar conclusions regarding the location of ancestral ranges (Waltari et al. 2007; Cordellier and Pfenninger 2009). This is of importance because it suggests that the ecological niches of the studied species have

not changed dramatically over the investigated time scales, a behaviour known as niche conservatism (Peterson et al. 1999; Wiens 2004).

Interpreting the distributional ranges of lichens has always been a challenging endeavour. Due to the sparse fossil record of lichens, historical biogeography was largely speculative before the advent of molecular markers (see e.g. the papers by Lynge 1941 or Poelt 1963). Their large distributional ranges were often attributed to the small size of their propagules that facilitated dispersal (e.g. Galloway and Aptroot 1995). For this reason, Fægri (1950) considered lichens unsuitable objects for historical biogeographical study, perhaps reflecting the microbiological dogma that „everything is everywhere, but the environment selects" (Baas-Becking 1934). Many lichens do not only display large geographical ranges, they also seem to have exceptionally wide ecological niches. The ranges of many predominantly polar species extend into the temperate zone and vice versa (Printzen 2008). Especially for crustose lichens, the true distributional ranges or even species circumscriptions are often not nearly clear, which makes biogeographic inferences difficult if not impossible.

To complicate matters further, the symbiotic nature of lichens impedes the interpretation of results. Distributional ranges may be limited not by ecological restrictions of the lichen or because of dispersal limitations of the mycobiont, but because suitable photobiont(s) are absent. Adaptations to local climatic conditions or acclimation may occur in only one or both of the symbionts. In symbiotic systems, adaptation may not even be the result of mutation and natural selection. A symbiotic host may "outsource" (Gilbert et al. 2010) parts of its stress response to symbiotic partners and respond to changing environmental conditions by habitat-adapted symbiont association (Rodriguez et al. 2008). Such a mechanism has, for example, led to the coral probiotic hypothesis (Reshef et al. 2006) and the hologenome concept (Rosenberg and Zilber-Rosenberg 2011). Many bacterial symbiotic communities are known to vary even in response to short-term environmental changes, e.g. heat stress in corals or the nutritional diet of insects (Littman et al. 2010; Feldhaar 2011). In the case of lichens, it has been demonstrated that species associate with different photobionts in different habitats (Blaha et al. 2006; Yahr et al. 2006) although nothing is known about the time scales over which mycobionts can switch their photobiont partners.

To sum up, the large ecological niches observed in some lichens may be the result of (1) the ability of one or both symbionts to acclimate to widely different ecological conditions, (2) ecotypic differentiation of populations in different parts of a species range, (3) „habitat-adapted symbiosis" by selective association of mycobionts with different photobionts or microbial symbionts, and (4) an overly broad species concept and the presence of unrecognized, possibly cryptic, species. The results summarized in this short review are based on ongoing studies on the phylogeography, population genetics, symbiont interactions and ecophysiology of the widespread bipolar lichen *Cetraria aculeata* (Schreb.) Fr. (Fig. 1). It will become evident that even after years of research focussing on a single species there are more questions open than answered. „Inferring the past to predict the future" (Cordellier and Pfenninger 2009) has been the motivation of many phylogeographic reconstructions. To our mind, it is becom-

Figure 1. *Cetraria aculeata*, habit.

ing increasingly evident that a combination of ecophysiological and phylogeographic approaches and more experimental studies are necessary to understand the reaction of symbiotic systems such as lichens to changing environmental conditions.

Delimiting species within the *Cetraria aculeata* group

Uncertain species delimitations can undermine population-level studies of lichens. If several unrecognized species are included in a study, the assumptions of null-models (e.g. panmixia or certain modes of range expansion) may be violated. Recognizing the presence of different species and restricting the dataset to one of them may lead to a different problem: erosion of the dataset, to an extent that may prevent meaningful statistical inferences. For example, Spribille (2011) found that *Mycoblastus sanguinarius* (L.) Norman, assumed to be a common, easily identified circumboreal species, consists of several genetically, morphologically and chemically distinct lineages. Further, Wirtz et al. (2008) showed that Western North American and Antarctic populations thought to represent the bipolar species *Usnea sphacelata* R. Br. belonged to a different species, *U. lambii* (Imshaug) Wirtz & Lumbsch.

Species delimitation within *Cetraria* s. str. is not unproblematic. *Cetraria aculeata* and *C. muricata* (Ach.) Eckfeldt can be extremely difficult to distinguish in the field. A molecular study by Thell et al. (2000) based on ITS and nucSSU group I intron sequences showed *C. muricata* embedded within a paraphyletic *C. aculeata*. This does

not mean that both belong to the same phylogenetic species (see Wirtz et al. 2012 for a brief outline of species delimitation problems). Several interpretations are possible, including the presence of more than two distinct lineages within a broadly circumscribed *C. aculeata*. Indeed, in a study using ITS and b-tubulin sequences (Thell et al. 2002), two individuals of *C. muricata* appeared as sister to clades of *C. aculeata* and *C. odontella* (Ach.) Ach. In the most recent molecular studies of cetrarioid lichens (Thell et al. 2009; Nelsen et al. 2011) based on more markers, *C. aculeata* and *C. muricata* were always treated as separate species. At least two additional close relatives of *C. aculeata* have so far been described, the corticolous *C. crespoae* (Barreno & Vázquez) Kärnefelt from western Spain and *C. steppae* (Savicz) Kärnefelt from Ukraine containing norstictic acid (Kärnefelt et al. 1993). The latter was later also reported from Spain (e.g. Maestre et al. 2011). The status of these four species has not yet been tested with molecular data. More problems with the circumscription of *C. aculeata* have been reported from Western North America (Goward 1999) and became obvious during our own field work in the region. Most ITS sequences generated in an unpublished population genetic study on *C. aculeata* in Alaska and the Yukon Territory proved to belong to a lineage more or less unrelated to *C. aculeata* (Seifried 2009).

Spanish authors have used the name *C. steppae* for morphologically distinct, vagrant forms of a *Cetraria* with thickened and sometimes almost foliose, unbranched thalli. Apparently, norstictic acid has not been reported from this Spanish material. In a detailed anatomical, physiological and population genetic study, Pérez-Ortega et al. (2012) described anatomical and physiological differences between these forms and „typical" *C. aculeata* from the same habitats. While vagrant morphs differed in several anatomical details (thicker cortex with multilayered cell walls and fibrous material, denser algal layer with smaller algal cells), photosynthetic response curves and dark respiration of both forms were almost identical. Vagrant morphs, however, had an increased water-holding capacity. The genetic comparison revealed that mycobionts and photobionts of both forms shared multilocus haplotypes, but tests of differentiation showed that vagrant haplotypes were not a random subsample of the investigated populations. At present it seems as if the distinct morphology of vagrant thalli is an adaptation to arid conditions, but that both morphs are not differentiated enough to warrant taxonomic delimitation from the typical morphs of *C. aculeata* found in the same locations. In order to further investigate the degree of genetic isolation between these morphs, markers with a higher resolution such as microsatellites will be necessary. Likewise, the degree of isolaton between *C. aculeata* and typical *C. steppae* or the sympatric *C. crespoae* has to be investigated using population genetic approaches. A different case is that of *C. aculeata* in Western North America. Figure 2 shows that the samples from Alaska and the Yukon Territory closely resembling *C. aculeata* that were studied by Seifried (2009) mostly belonged to a group of haplotypes that is well separated from two haplotype groups from Iceland and Svalbard. Only three out of 66 individuals from Beringia belonged to these two groups. In this case, genetic isolation seems to be almost complete.

So, how many species can we distinguish within the *C. aculeata* group and how can they be delimited? The species tree in Fig. 3 is based on a dataset comprising ITS,

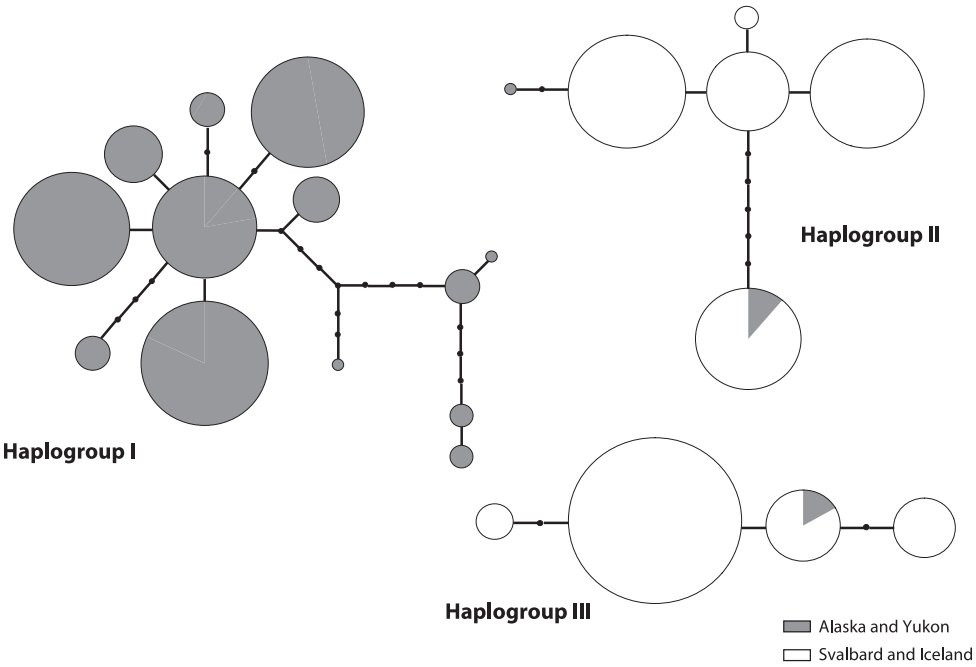

Figure 2. 95 % parsimony probability haplotype network based on ITS sequences of the mycobiont displaying genetic differences between Beringian samples of the *C. aculeata* group (grey) and individuals from two haplotype groups present on Iceland and Svalbard (white). Large circles represent haplotypes, each line a mutational step and black dots missing haplotypes. The size of the circles is proportional to the number of individuals sharing that haplotype. The presence of three unconnected haplogroups indicates that the genetic differences between them are too large to find a connection with a 95 % probability of being the most parsimonious one.

mtLSU and glyceraldehyde-3-phosphate dehydrogenase (GPD) DNA sequences and contains individuals of all currently accepted species of the *C. aculeata* group. Preliminary studies (Fernández-Mendoza et al. 2011; Domaschke et al. 2012) had shown considerable genetic diversity within the group with several relatively well supported clades. When sequences of *C. steppae* and *C. crespoae* were added to these datasets, they usually ended up among the different clades of *C. aculeata* s. lat. We therefore decided to treat each separate clade as a separate OTU (operational taxonomic unit) in a Bayesian species tree approach using *BEAST (for details on methods see below) instead of using a phylogenetic approach to infer the evolutionary relationships among single sequences. In addition to *C. australiensis* Kärnefelt, *C. crespoae, C. muricata, C. odontella* and *C. steppae* we distinguished seven different OTUs within what was potentially *C. aculeata*: (1) material from Western North America and South America, here provisionally called "*C. panamericana*", vagrant morphs from central Spain, Mediterranean and Arctic material from two genetically divergent ITS clades (haplogroups II and III in Figs. 2 and 3), and material from the southern hemisphere that in previous analyses (Fernández-Mendoza et al. 2011; Domaschke et al. 2012) had proved to form a

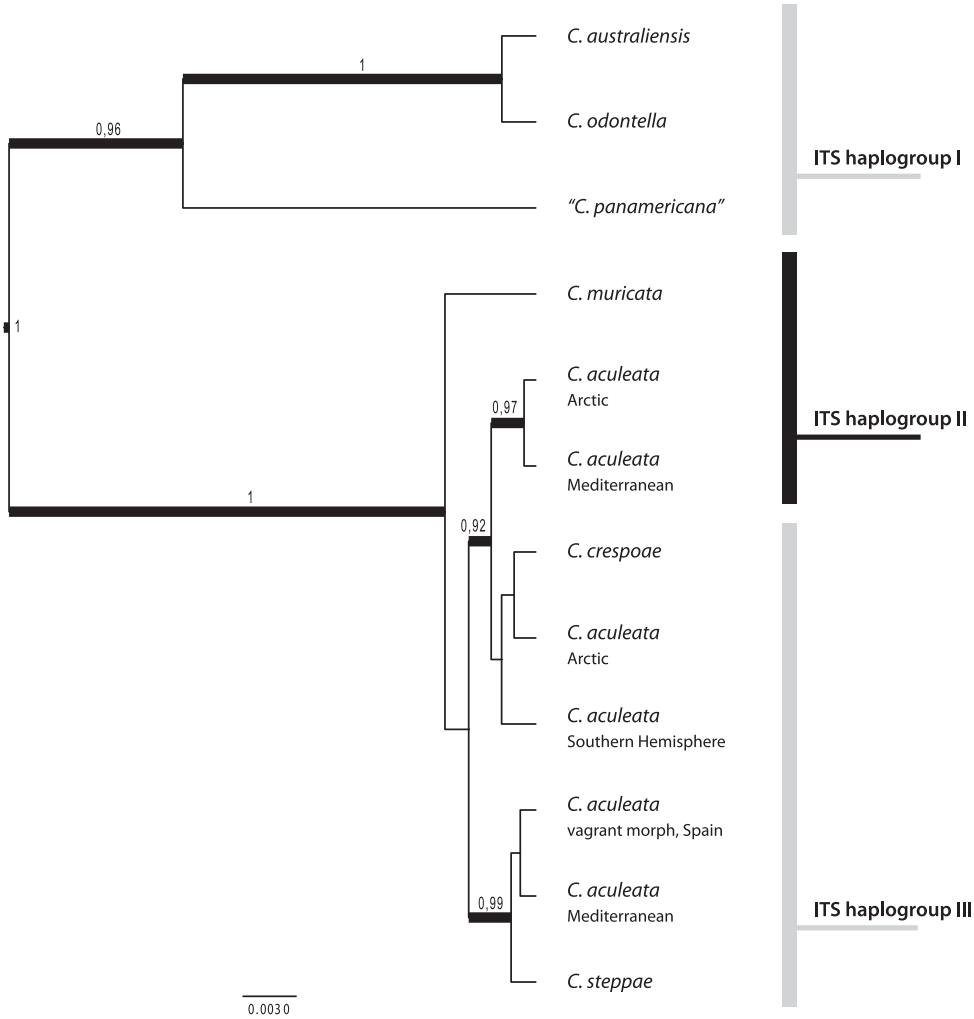

Figure 3. Species tree of the *C. aculeata* group inferred from ITS, GPD and mtLSU DNA sequences. For details of the analysis see under Material and methods.

monophyletic group. A species tree approach has the advantage that it does not require species to form monophyletic groups on gene trees and can account for the discordance between gene trees and species phylogeny that results from incomplete lineage sorting among closely related species.

With a high posterior probability, the Beringian and South American samples ("*C. panamericana*") are closely related to *C. odontella* and *C. australiensis* (Fig. 3). These two taxa are usually easily distinguished from other members of the *C. aculeata* group on account of their flattened thallus lobes. However, Kärnefelt (1986) already pointed out that some specimens from Alaska, Peru and Taiwan were lacking distinctly flattened lobes. We have so far not studied these specimens but assume that they belong to this group which is morphologically characterized by terete densely branched lobes

that are usually more blunt than in *C. aculeata* and have smaller pseudocyphellae. According to our field experience, typical *C. aculeata* is rare in Western North America. Six specimens identified by us as *C. muricata* were included in the analysis and formed an outgroup to the remaining OTUs. Two further clades within *C. aculeata* – *C. crespoae* – *C. steppae* receive high statistical support: (1) a group comprising *C. steppae*, the vagrant morphs and the Mediterranean samples from ITS haplogroup III, and (2) a group consisting of Arctic and Mediterranean individuals from ITS haplogroup II. *Cetraria crespoae*, Arctic samples from ITS haplogroup III and the southern hemispheric group form a third, poorly supported group that is nested between these two clades. The most likely candidate for a separate species within *C. aculeata* s. lat. appears to be a *C. steppae* that also comprises specimens without norstictic acid and the vagrant morphs from Spain. More data are necessary to decide the status of *C. crespoae* and the remaining groups within *C. aculeata*. Because the genetic distances between all of these groups are minimal, we treat them as "*C. aculeata* s. lat." for the time being.

Historical demography and range shifts

Questions about species delimitations and possible cryptic species put aside, the wide distribution of many lichens raises questions about how they managed to colonise their often enormous ranges. *Cetraria aculeata* is widespread in the Arctic and temperate regions of the northern hemisphere and common in Patagonia, Tierra del Fuego, some subantarctic islands and the maritime Antarctic. Furthermore, it has been reported from southeast Australia (Kantvilas 1994) and New Zealand (Galloway 2007) and also occurs at low latitudes in various tropical mountain ranges, including the central Andes (Kärnefelt 1986) and the East African Mountains (Swinscow and Krog 1988). Where did *C. aculeata* first evolve and how did it manage to move between the hemispheres? In the absence of a fossil record, population genetic data offers the most useful source of information about historical range shifts. The basic assumptions of the simplest approaches are that genetic diversity accumulates over time in stable populations and that population size bottlenecks reduce genetic diversity by removing alleles or haplotypes from a population (Nei et al. 1975). Bottlenecks can be caused by different historical events, for example migration or long range colonisation of areas by just a few individuals of a species, population size reductions by habitat fragmentation or climatic changes etc. Many studies have shown that large, refugial areas with a long historical continuity usually harbour more genetic diversity than newly colonised regions (Hewitt 1999). Comparisons of genetic diversity or allelic richness thus help to distinguish between old "source areas" and newly colonised regions within a species' range. The spatial distribution of certain haplotypes might then offer additional information that often helps in identifying dispersal routes.

The comparison of genetic variabilities of the mycobiont of *C. aculeata* (Domaschke et al. 2012) over much of its range showed highest diversity in the Arctic and a marked decline towards the Antarctic (Fig. 4) indicating that *C. aculeata* s. lat. evolved in the

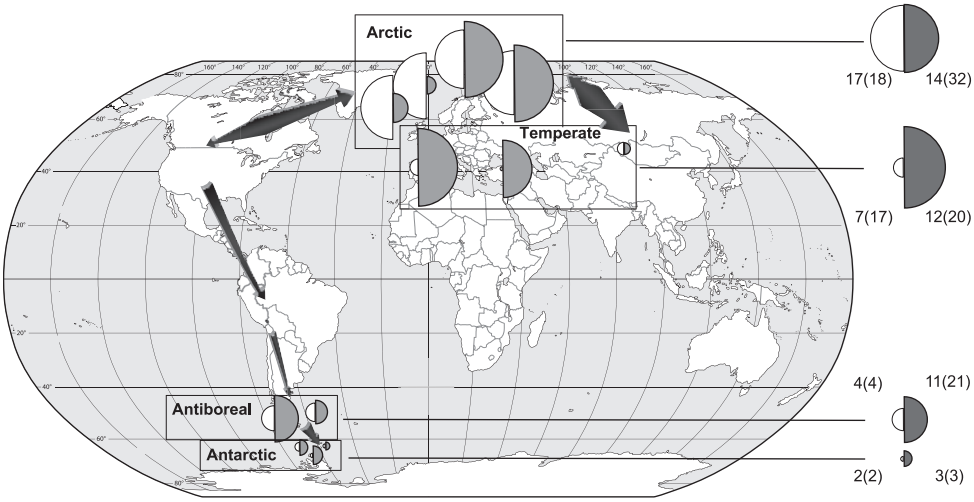

Figure 4. Nucleotide diversity (π) of different populations of photobionts (grey) and mycobionts (white) based on ITS sequences from 222 thalli of *C. aculeata* (data from Domaschke et al. 2012). Size of the semi-circles is proportional to the diversity level. Circles on the right summarize diversity levels within regions. Numbers of observed haplotypes and estimated absolute numbers following Chao 1 (in brackets) are added. Arrows indicate inferred historical migration events.

north and colonised the southern hemisphere from there. Antarctic populations are genetically almost uniform, which suggests that this continent was perhaps colonised in a single event and that genetic exchange with the genetically more diverse southern tip of South America is absent or very rare. The pattern for the photobiont is similar, although the Mediterranean part of the range is considerably more diverse than in the mycobiont. Mediterranean populations of both symbionts are genetically well separated from the rest of the populations (completely for the photobiont). The species tree in Fig. 3 with an entirely Mediterranean group at the base of the *C. aculeata* clade and the Mediterranean *C. crespoae* and ITS haplogroup II spread over the remainder of the clades suggest that *C. aculeata* s. lat. first evolved in the Mediterranean and spread to the Arctic from there.

Because only few haplotypes are shared among both Hemispheres (Fig. 3 in Fernández-Mendoza et al. 2011 and Fig. 1 in Domaschke et al. 2012), the disjunction was assumed to result from a single colonisation event. Fernández-Mendoza and Printzen (2013) have recently used coalescent-based migration modelling (Beerli and Palczewski 2010) and a modified version of stochastic character mapping that allows temporally explicit reconstruction of character changes to study the expansion of *C. aculeata* into its current range. They found evidence for a Pleistocene dispersive burst in which a population size expansion led to the acquisition of a South-American range that culminated in the colonization of the Antarctic. Their results suggest that the transition from the Arctic into Patagonia preceded that into the Central Andes. Either Patagonian populations became genetically isolated from an Andean dispersal pathway into South America, while the Central Andes still received immigrants from northern populations. Or the small and scattered patches of suitable habitat in the Central An-

des were extinguished during glacial maxima and later re-colonized from the North, while Patagonia with its widely ice-free areas east of the Andes maintained larger and genetically diverse populations of *C. aculeata*.

Adaptation or acclimation of *C. aculeata* to environmental conditions in temperate and polar environments

In contrast to most other polar organisms, which are highly adapted to the extreme climatic conditions in which they live, most polar lichens are not restricted to cold habitats. Many polar species are widespread at lower latitudes, some are confined to high mountains (Galloway and Aptroot 1995), while others extendend into a wide range of other habitats. *Cetraria aculeata* s. lat. is present not only in polar ecosystems, but also in coastal sand dunes, woodlands and steppes in temperate and semiarid regions around the Mediterranean and in Central Asia. Its occurrence in such widely differing biomes indicates an unusually wide ecological niche which can either result from an extreme physiological plasticity or from niche differentiation between sister taxa. At least to a certain degree, the wide ecological niches of some lichen species could be explained by their poikilohydric life style, avoiding environmental stress by simply deactivating their metabolism (Longton 1988; Pannewitz et al. 2003). However, an ecological niche like that of *C. aculeata* still requires that the species can modulate its physiological performance (Kappen 2000; Pannewitz et al. 2006) and maintain positive net photosynthesis during short phases of biological activity ("resource pulses") under very different climatic regimes (Pannewitz et al. 2003, 2006; Yang et al. 2008). It is tempting to assume that individuals of *C. aculeata* in different parts of its range are genetically adapted to the widely differing environmental conditions. Different temperature optima for photosynthesis have been reported for polar and temperate lichen species (Kappen 1988). Similarly, thalli of the same species sampled in different biomes differed in photosynthetic response and growth rate (Murtagh et al. 2002). Sonesson et al. (1992), for example, concluded that populations of *Nephroma arcticum* (L.) Torss. comprise different ecotypes and Schipperges et al. (1995) suggested high phenotypic plasticity and genetically determined ecophysiological differences between populations of *Flavocetraria nivalis* (L.) Kärnefelt & A. Thell.

So far, the only published ecophysiological study on *C. aculeata* is that by Pérez-Ortega et al. (2012) that compared morphologically divergent thalli within a few localities in Spain (see above). Domaschke and Printzen (2011) studied ecophysiological properties of *C. aculeata* thalli from Antarctica, Svalbard, Spain and Germany and found that Polar and temperate populations differed in maximal net photosynthesis rates (NPR), light compensation points, light saturation points and temperature optima (Fig. 5). NPR was significantly lower in the Polar than in the temperate populations, which could partly be explained by lower chlorophyll concentrations per unit dry mass in the Polar samples. The temperature optima,

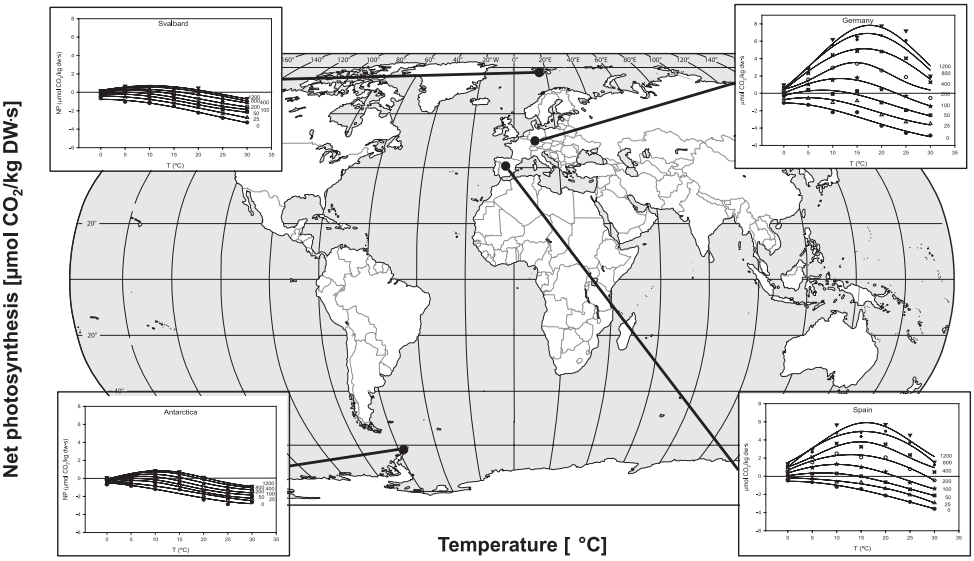

Figure 5. Dependency of net photosynthesis (NP) on temperature at various photon flux densities (0 to 1200 µmol m^{-2} s^{-1}) related to different geographical origins of *C. aculeata*. Figures are based on measurements of 7-8 thalli per location. Further details under Material and methods.

however, are largely unaffected by chlorophyll levels. Because whole lichen thalli were measured, it is not possible to disentangle the contributions of the mycobiont's respiration and the photobiont's photosynthesis and respiration to overall net photosynthesis. Experiments on isolated photobiont cultures are currently under way (Domaschke, in prep.) to assess whether one or both symbionts are responsible for the observed differences. Whether these physiological differences are genetically fixed adaptations or the result of acclimation can only be studied by transplantation experiments which are currently carried out (Domaschke, in prep.). At any rate, the available evidence is so far not in conflict with the hypothesis that genetically different individuals of *C. aculeata* in Polar and temperate biomes are adapted to local environmental conditions.

The symbiotic aspect

Molecular studies have demonstrated that lichen mycobionts can associate with different photobionts (Kroken and Taylor 2000; Opanowicz and Grube 2004; Piercey-Normore 2004; Blaha et al. 2006) and that photobiont switching is common (Piercey-Normore and Depriest 2001; O'Brien et al. 2005; Nelsen and Gargas 2008; Wornik and Grube 2010). It is evident that the association of mycobionts with locally adapted photobionts could contribute greatly to the ability of *C. aculeata* to colonise ecologically different biomes. Evidence for an ecological influence on symbiotic interactions in lichens is indeed accumulating (Blaha et al. 2006; Casano

et al. 2011; Fernández-Mendoza et al. 2011; Peksa and Skaloud 2011). A number of different hypotheses and theories have been developed to explain the dynamics of various symbiotic systems (Reshef et al. 2006; Rosenberg et al. 2007; Rodriguez et al. 2008; Gilbert et al. 2010). They all agree in that the symbiotic lifestyle is likely to increase the adaptive and evolutionary potential of symbiotic holobionts. A symbiotic host may adapt to changing environmental conditions by "outsourcing" (Gilbert et al. 2010) parts of its stress response to the symbiotic partners (habitat adapted symbiosis, Rodriguez et al. 2008). The observed shifting of symbiotic partners in coral-*Symbiodinium* associations has triggered the coral probiotic hypothesis (Reshef et al. 2006) and the hologenome theory of evolution (Rosenberg et al. 2007; Gilbert et al. 2010). At present, there is no evidence for fast and repeated photobiont switches in lichens. But rapid symbiont switches would certainly enable the mycobiont to react much faster to environmental changes than the slow evolutionary processes of mutation and selection.

The observation that North and South Polar populations of *C. aculeata* are genetically more similar to each other than to the temperate populations induced Fernández-Mendoza et al. (2011) to study whether the genetic structure of mycobiont and photobiont populations is best explained by geographic distances or by climatic differences between sampling localities. The major problem with such studies is that differences in climate and spatial distance may be correlated over large geographical distances. Variation partitioning (Borcard et al. 1992) showed that climate alone or in combination with geographical distance explained a major part of the genetic variability of the photobiont, but not the mycobiont (Fig. 6). On the other hand, *C. aculeata* is a largely asexual species. Supposedly, it propagates mainly by thallus fragments containing both symbionts. It can therefore be assumed that at least part of the genetic structure of the photobiont is correlated to that of the mycobiont. Indeed co-dispersal with the mycobiont accounts for an almost equally high proportion of the genetic structure of the photobiont (Fig. 6, bottom). This indicates that photobiont switches, which must obviously have occurred because of the partly incongruent genetic patterns of both symbionts, are not happening very often. The fact that the temperate populations of both symbionts are genetically isolated from Polar populations (see above), suggests that a historic photobiont switch enabled *C. aculeata* to colonise its wide ecological niche.

It has recently become clear that the lichen symbiosis also harbours a large diversity and abundance of lichen-associated bacterial communities (reviewed by Grube and Berg 2009). These communities are often dominated by *Alphaproteobacteria* (Cardinale et al. 2008; Bates et al. 2011; Hodkinson et al. 2012) although other bacterial groups may also be fairly abundant. Lichen-associated bacterial communities can be species-specific (Grube et al. 2009; Hodkinson et al. 2012) indicating that they might play an important role in the symbiosis. A preliminary analysis of *Alphaproteobacteria* associated with *C. aculeata* revealed that their community structure is similar to the genetical patterns observed in the photobiont with several OTUs being shared among North and South Polar populations (Printzen et al. 2012). This

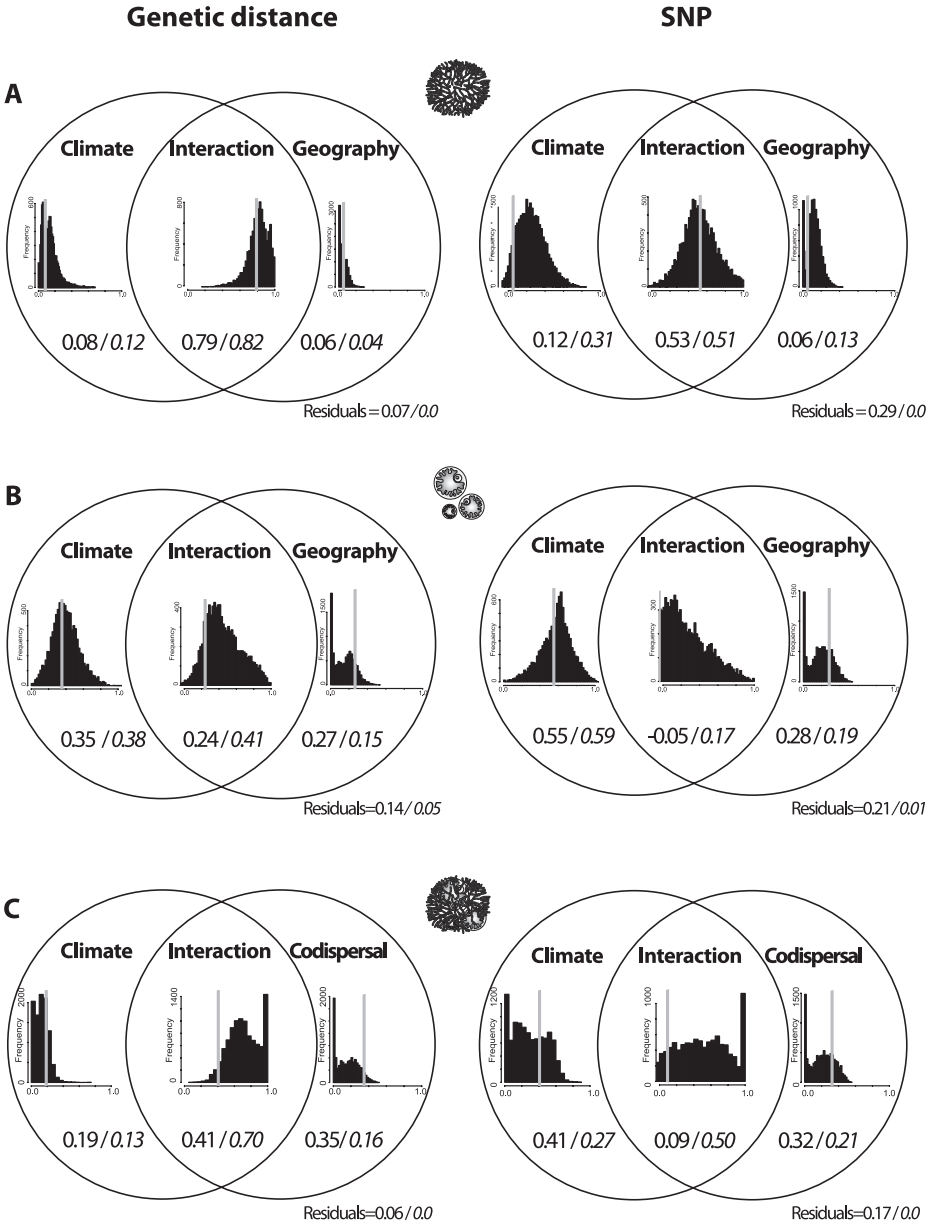

Figure 6. Variation partitioning diagrams using climate, geography and co-dispersal as explanatory components for the genetic structure of mycobiont and photobiont populations of *Cetraria aculeata*. Top, mycobiont; Center, photobiont, explanatory variables climate and spatial distances; Bottom, photobiont, explanatory variables climate and co-dispersal with the mycobiont. Left: genetic structure measured as pair-wise genetic distances between populations. Right: genetic structure measured as SNP composition. Numbers in the Venn diagrams indicate the fraction of the variation that is explained by the respective component or the intersection of components, with medians of the bootstrap analyses in italics. Results of the bootstrap analyses are also displayed in the bar charts with proportions of explained variation on the x-axes. Grey bars indicate results based on the original (non-resampled) data sets. From Fernández-Mendoza et al. (2011).

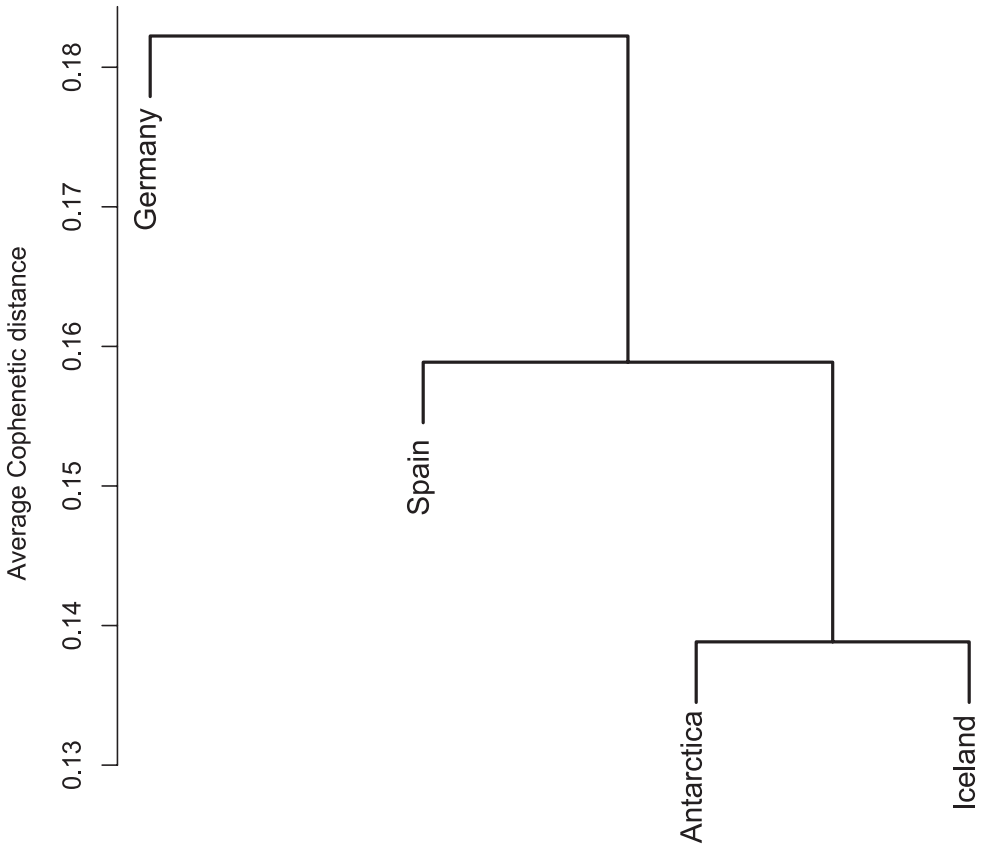

Figure 7. Community classification of four populations of Alphaproteobacteria associated with *C. aculeata*. The bar on the left indicates the value of the cophenetic distances between communities. From Printzen et al. (2012).

was at the same time the first evidence for bipolar distributions of lichen-associated bacteria. A community classification showed that bacterial populations from Iceland and the maritime Antarctic are more similar to each other than to temperate populations from Spain and Germany (Fig. 7).

Hill (2009) recently suggested that "although ... the mycobiont may acquire strains (species, varieties, forms or genotypes) [of photobionts] that are more suitable than others .. this is unlikely [to] have any evolutionary consequence." Our results show that acquiring a new photobiont may have wide-ranging evolutionary consequences for a mycobiont by extending its ecological niche. In the case of *C. aculeata* it has either enabled an ancestral species adapted to arctic conditions to colonise temperate and semi-arid biomes or vice versa. In spite of their relative geographic proximity, extant mycobiont populations of *C. aculeata* from the Mediterranean and the Arctic are genetically almost completely isolated. This suggests that habitat-adapted symbiosis (Rodriguez et al. 2008) might trigger speciation processes in lichens. The role of bacterial symbionts in this process is so far entirely unclear and requires more intensive attention.

Material and methods

Sampling, methods of DNA isolation, sequencing and PCR subcloning of bacterial communities as well as statistical analyses are outlined in Fernández-Mendoza et al. (2011), Domaschke et al. (2012) and Printzen et al. (2012).

Calculation of the species tree in Fig. 2 was based on 83 ITS, 71 mtLSU and 64 GPD sequences from the *C. aculeata* group. Sequences were assigned to eleven species and genetic lineages: i) *Cetraria odontella*, ii) *C. australiensis*, iii) *C. panamericana* ined., iv) *C. muricata*, v) *C. crespoae*, vi) *C. steppae*, and six geographic and genetic groups of *C. aculeata*; vii) Mediterranean haplogroup II, viii) Mediterranean haplogroup III, ix) Southern hemisphere, x) Northern boreoarctic haplogroup II and xi) Northern boreoarctic haplogroup III. We used the species tree ancestral reconstruction method *BEAST (Heled and Drummond 2010) implemented in BEAST v 1.7.1 (Drummond and Rambaut 2007), to reconstruct a mixed species/population tree. Each population/species was modelled under a separate coalescent prior with a constant root and a continuous population size. Optimum substitution models were estimated using jMODELTEST (Guindon and Gascuel 2003; Posada 2008). The analysis of exploratory runs for each locus suggested that using a site heterogeneity model (Gamma) was not adequate which hence was not used in the reconstructions. For all loci, nucleotide frequencies were also estimated in the analysis. Clock like evolutionary models were used without calibration points. The adequacy of imposing a strict molecular clock was assessed for each locus using the ML test implemented in MEGA5 (Tamura et al. 2011). We imposed a strict clock model for mtLSU and GPD, and an uncorrelated exponential relaxed clock for ITS. The analysis was run for 50×10^6 generations; parameters were sampled every 2.5×10^3th generation. Convergence and posterior parameter distributions were examined using Tracer v1.5 (Rambaut and Drummond 2007). The resulting distributions were combined in log-Combiner (Drummond and Rambaut 2007), and after an adequate burn-in fraction was selected, maximum clade credibility trees were calculated for the reconstructed species and gene trees.

CO_2 exchange measurements were performed on lichen thalli that were carefully washed with mineral water and cleaned from fragments of other lichen species. Dry thalli were reactivated for 72 hours in a climate chamber (light intensity 100 µmol m^{-2} s^{-1}, 12 hours light / dark cycle, 15 °C, humidification every 24 h with mineral water). CO_2 measurements were performed with a compact minicuvette system (CMS 400, WALZ, Effeltrich, Germany) under controlled temperature, light and humidity conditions. For each population 7–8 replicates were investigated. Prior to all measurements, samples were soaked in mineral water for 20 minutes to ensure full hydration of thalli. Optimal water contents were determined for 4 individuals per population by studying the change of the photosynthetic performance during the desiccation process of the lichen at a constant temperature of 15 °C and photon flux densities of 0 and 400 µmol m^{-2} s^{-1}. The response of net CO_2 exchange was measured at optimal water content at temperatures between 0 and 30°C and photosynthetic flux densities (PPFD) of 0, 25, 50, 100, 200, 400, 800 and 1200 µmol m^{-2} s^{-1}. PPFD response curves were analysed by statistical fitting of a Smith function (Green et al. 1997) using SigmaPlot 10.0 (Chicago, Illinois, USA).

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A molecular phylogeny of Graphidaceae (Ascomycota, Lecanoromycetes, Ostropales) including 428 species

Eimy Rivas Plata[1,2], Sittiporn Parnmen[1], Bettina Staiger[3], Armin Mangold[4], Andreas Frisch[5], Gothamie Weerakoon[6], Jesús E. Hernández M.[7], Marcela E. S. Cáceres[8], Klaus Kalb[3,9], Harrie J. M. Sipman[10], Ralph S. Common[11], Matthew P. Nelsen[1,12], Robert Lücking[1], H. Thorsten Lumbsch[1]

1 *Department of Botany, The Field Museum, 1400 South Lake Shore Drive, Chicago, Illinois 60605-2496, U.S.A.* **2** *Department of Biology, Duke University, 125 Science Drive, Durham, North Carolina 27708, U.S.A.* **3** *University of Regensburg, Institute for Botany, Universitätsstr. 31, D-93040 Regensburg, Germany* **4** *Universität Duisburg-Essen, Botanisches Institut, Universitätsstr. 5, D-45141 Essen, Germany* **5** *National Museum of Nature and Science, 4-1-1 Amakubo, Tsukuba, Ibaraki, 305-0005 Japan* **6** *Department of Botany, University of Sri Jayawardenepura, Sri Lanka* **7** *Fundación Instituto Botánico de Venezuela, Ave. Salvador Allende, Jardín Botánico de Caracas, Universidad Central de Venezuela, Apartado # 2156, Caracas 1010-A, Venezuela* **8** *Departamento de Biociências, Universidade Federal de Sergipe, CEP: 49.500-000, Itabaiana, Sergipe, Brasil* **9** *Lichenologisches Institut Neumarkt, Im Tal 12, D-92318 Neumarkt, Germany* **10** *Botanischer Garten and Botanisches Museum Berlin Dahlem, Königin-Luise-Strasse 6–8, D-14195 Berlin, Germany* **11** *534 Fenton St., Lansing, Michigan 48910, U.S.A.* **12** *Committee on Evolutionary Biology, University of Chicago, 1025 E. 57th Street, Chicago, Illinois 60637, U.S.A*

Corresponding author: *Robert Lücking* (rlucking@fieldmuseum.org)

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Abstract

We present a comprehensive molecular phylogeny of the lichen family Graphidaceae (subfamilies Graphidoideae and Fissurinoideae) based on partial sequences of the mtSSU, nuLSU rDNA, and *RPB2* loci. The phylogeny includes all currently available sequences in Genbank plus 897 newly generated sequences, from a total of 908 ingroup OTUs representing 428 species. The phylogeny supports the synomymy of Graphidaceae and Thelotremataceae and confirms that rounded and lirellate ascomata evolved multiple times in unrelated clades within the family. The phylogenetic distinctiveness of Fissurinoideae versus

Graphidoideae is also supported in our extended taxon sampling. The three-gene phylogeny suggest that in addition to the three tribes previously established for the major clades within subfamily Graphidoideae, several further clades exist that might represent additional tribes. Specifically, the *Leptotrema* clade is excluded from tribe Ocellularieae and the *Carbacanthographis*, *Heiomasia*, *Topeliopsis*, and *Wirthiotrema* clades are excluded from tribe Thelotremateae. The phylogenetic position of these clades remains unresolved but they are not supported as belonging to these larger tribes. Based on the results, the current status and problems are discussed for all clades and genera currently accepted within the family.

Key words
Asterothryiaceae, Fissurinoideae, Gomphillaceae, Graphideae

Introduction

Graphidaceae, which is currently accepted to include the previously independent families Thelotremataceae (Mangold et al. 2008a, Rivas Plata et al. 2012a), Gomphillaceae, Asterothyriaceae, and Solorinellaceae (Baloch et al. 2010, Rivas Plata et al. 2012a, b), is the second largest family of lichenized fungi and the dominant element of lichen communities in tropical regions, with over 1800 accepted species (Staiger 2002, Frisch et al. 2006a, Archer 2006, 2007, 2009, Lücking and Rivas Plata 2008, Lücking 2009, Lücking et al. 2008, 2009, Rivas Plata et al. 2008, 2012a, Mangold et al. 2009). Quantitative extrapolations and molecular data available for species complexes, as well as continuous new discoveries, suggest that the family may actually contain well over 2000 species (Lücking 2012, Rivas Plata and Lücking 2012, Rivas Plata et al. 2012b, Sipman et al. 2012).

For over 100 years, family and genus concepts in this group were based on apothecia and ascospore types, separating most of the species into groups with either rounded, lirellate, or pseudostromatic ascomata and, for each ascoma type, into four genera depending on whether ascospores were transversely septate or muriform and hyaline or pigmented (Müller 1887, Hale 1974, 1978, Wirth and Hale 1963, 1978, Staiger 2002, Frisch et al. 2006a): rounded (Thelotremataceae): *Ocellularia*, *Thelotrema*, *Phaeotrema*, *Leptotrema*; Graphidaceae: lirellate (Graphidaceae): *Graphis*, *Graphina*, *Phaeographis*, *Phaeographina*; pseudostromatic (Graphidaceae): *Glyphis*, *Medusulina*, *Sarcographa*, *Sarcographina*). This concept was highly schematic and challenged by several authors, e.g. in the former Thelotremataceae by Salisbury (1971, 1972, 1978) and Hale (1980, 1981). The latter author proposed an alternative generic concept with three genera based on excipular structures: carbonized and lacking periphysoids (*Ocellularia*), non-carbonized and lacking periphysoids (*Myriotrema*), and non-carbonized and with periphysoids (*Thelotrema*). However, delimitation of very large genera with well over 300 species each and the problems with generic assignment of aberrant taxa made this concept unsatisfactory. In addition, subsequent phylogenetic studies showed that these genera were not monophyletic (Frisch et al. 2006a, Mangold et al. 2008a). Also, no comparable solution was proposed for lirellate and stromatic species classified

in Graphidaceae, which until recently remained essentially unchanged from ascospore-based concepts established in the 19th century (Wirth and Hale 1963, 1978, Archer 1999, 2000, 2001a-d, 2002, Staiger 2002).

Major systematic revisions of both families only started after turn of the millenium, with two monographic treatments put forward by Staiger (2002) and Frisch et al. (2006a). In these, a more detailed approach to genus classification was employed, based on a combination of characters such as thallus structure, excipular structure, hamathecium type, ascospore type, and secondary chemistry. This refined classification, which resulted in many new genera (Staiger 2002; Frisch et al. 2006a, Kalb 2009, Rivas Plata et al. 2010a, Lücking et al. 2011, Cáceres et al. 2012, Sipman et al. 2012) has subsequently been tested using molecular approaches, with a growing amount of sequence data available (Staiger 2002, Kalb et al. 2004, Lumbsch et al. 2008, Frisch et al. 2006b, Staiger et al. 2006, Mangold et al. 2008a, Baloch et al. 2010, Rivas Plata and Lumbsch 2011, Rivas Plata et al. 2012a, b). Using molecular data, it was also shown that Graphidaceae and Thelotremataceae were non-monophyletic and consequently Thelotremataceae was included in Graphidaceae. Within Graphidaceae five major clades were distinguished based on supported monophyletic clades (Mangold et al. 2008a, Rivas Plata and Lumbsch 2011, Rivas Plata et al. 2012a). In addition, two separate studies supported three further families, Gomphillaceae, Asterothyriaceae, and Solorinellaceae, as part of Graphidaceae (Baloch et al. 2010, Rivas Plata et al. 2012a, b). The inclusion of the latter has been controversely discussed (Aptroot 2012, Rivas Plata et al. 2012a), since it appears to disrupt the more or less morphologically homogeneous entity formed by subfamilies Fissurinoideae and Graphidoideae; on the other hand, as pointed out by Sipman et al. (2012), the differences between Gomphilloideae and the remaining Graphidaceae are merely gradual in nature. It can be argued that Graphidaceae could be retained as a paraphyletic entity excluding Gomphillaceae (Aptroot 2012). However, the known topology does not support this view since Gomphilloideae is strongly supported as sister to Fissurinoideae on a long branch, i.e. Fissurinoideae is more closely related to Gomphilloideae than to Graphidoideae and hence, Graphidaceae after exclusion of Gomphillaceae would be polyphyletic rather than paraphyletic. In the present study, subfamily Gomphilloideae is not treated since only few species have been sequenced and this clade will be dealt with in a separate contribution.

In this paper, we present the most recent molecular phylogeny of Graphidaceae, focusing on subfamilies Fissurinoideae and Graphidaceae, using a supermatrix approach of all available and newly generated sequences of the mtSSU, nuLSU rDNA, and *RPB2* gene partitions. Based on the results, we discuss the progress and problems in the delimitation of infra-family and genus-level taxa in the family. We did not include subfamily Gomphilloideae in this analysis since this subfamily will be treated in detail in a separate paper and its inclusion or exclusion does not affect the topology of subfamilies Fissurinoideae and Graphidoideae.

Material and methods

Partial sequences of the mtSSU rDNA, nuLSU rDNA and the *RPB2* gene were obtained from 907 ingroup OTUs representing 428 species in Graphidaceae (subfamilies Fissurinoideae and Graphidoideae). Thirteen additional species from the ostropalean families Stictidaceae, Porinaceae, Gyalectaceae, and Coenogoniaceae, were used as outgroup. A total of 865 new ingroup sequences were newly generated (Appendix 1): 481 for the mtSSU, 260 for the nuLSU, and 124 for the RPB2 gene. In addition, 433 ingroup sequences were downloaded from GenBank (Appendix 1): 263 for the mtSSU, 151 for the nuLSU, and 19 for the *RPB2* gene. Unfortunately, for many OTUs, sequence representation was incomplete, either because original DNA extracts from GB sequences were unavailable or because PCR did not work, especially with nuLSU and *RPB2* primers. Thus, of a potential total of 907 × 3 = 2721 ingroup sequences, 1298 (48%) were assembled in the dataset. The mtSSU was most complete (744 out of 907 or 82%), followed by the nuLSU (411 out of 907 or 45%) and RPB2 (143 out of 907 or 16%). A total of 35 further sequences, particularly of the nuLSU, could not be used because of contaminants or else unexplained conflict.

New sequences were generated for this study using the Sigma REDExtract-N-Amp Plant PCR Kit (St. Louis, Missouri, SA) for DNA isolation following the manufacturer's instructions, except that 40 µL of extraction buffer and 40 µL dilution buffer were used. DNA dilutions (5x) were used in PCR reactions of the genes coding for the nuLSU, mtSSU and RPB2, respectively. Primers for amplification were: (a) for nuLSU: AL2R (Mangold et al. 2008a), and nu-LSU-1125-3' (= LR6) (Vilgalys and Hester 1990), (b) for mtSSU: mr-SSU1 and Mr-SSU3R (Zoller et al. 1999), and (c) for RPB2: fRPB2-7cF and fRPB2-11aR (Liu et al. 1999). PCR reactions contained 5.0 µL R4775 Sigma REDExtract-N-Amp™ PCR ReadyMix, 0.5 µL of each primer (10 µM), 2 µL genomic DNA extract and 2 µL distilled water for a total of 10 µL. Thermal cycling parameters were: (1) for nuLSU: initial denaturation for 5 min at 94°C, followed by 35 cycles of 30 s at 95°C, 30 s at 58°C, 1 min at 72°C, and a final elongation for 10 min at 72°C; (2) for mtSSU: initial denaturation for 5 min at 95°C, followed by 35 cycles of 45 s at 94°C, 1 min at 50°C, 1 min 30 s at 72°C, and a final elongation for 10 min at 72°C; and (3) for *RPB2*: initial denaturation for 3 min at 95°C, then 1 min at 95°C, and 37 cycles of 1 min at 57°C, 1 min at 58°C, 1 min at 59°C, 1 min at 60°C, 1 min at 61°C, 1 min at 62°C, 1 min at 63°C, 1 min at 64°C and 1.5 min at 72°C, and a final elongation for 10 min at 72°C. Samples were visualized on a 1% ethidium bromide-stained agarose gel under UV light and bands were gel extracted, heated at 70° C for 5 minutes, cooled to 45° C for 10 minutes, treated with 1 µL GELase (Epicentre Biotechnologies, Madison, WI, USA) and incubated at 45° C for at least 24 hours. The 10 µl cycle sequencing reactions consisted of 1–1.5 µl of Big Dye version 3.1 (Applied Biosystems, Foster City, California, U.S.A.), 2.5–3 µl of Big Dye buffer, 6 µM primer, 0.75–2 µl gelased PCR product and water. Samples were sequenced with PCR primers. The cycle sequencing conditions were as follows: 96° C for 1 minute, followed by 25 cycles of

96° C for 10 seconds, 50° C for 5 seconds and 60° C for 4 minutes. Samples were precipitated and sequenced using Applied Biosystems 3730 DNA Analyzer (Foster City, California, U.S.A.), sequences were assembled in SeqMan 4.03 (DNASTAR) and submitted to GenBank (Appendix 1).

Sequences were arranged into multiple sequence alignments (MSA) for each gene using BIOEDIT 7.09 (Hall 1999) and automatically aligned with MAFFT using the --auto option (Katoh and Toh 2005). The unaligned MSA for the mtSSU and nuLSU gene partitions were also submitted to the GUIDANCE web server at http://guidance.tau.ac.il to assess alignment confidence scores for each site (Penn et al. 2010a, b). GUIDANCE uses a MAFFT alignment and returns a colored MSA that allows delimiting ambiguously aligned portions of the MSA. These were then excluded from further analysis. Introns were deleted from the nuLSU gene partition because of their random occurrence but kept in the mtSSU partition if consistent within species or species groups. This resulted in alignments of 995 sites for the mtSSU, 972 sites for the nuLSU, and 951 for *RPB2*, for a total of 2918 sites in the combined dataset. After testing for supported topological conflicts (Mason-Gamer and Kellogg 1996, Miadlikowska and Lutzoni 2000, Kauff and Lutzoni 2002), the three genes were combined into a single supermatrix. Individual datasets and the combined supermatrix were subjected to maximum likelihood search using the RAxML-HPC BlackBox 7.3.2 on the Cipres Gateway server (Stamatakis 2006, Stamatakis et al. 2005, 2008; Miller et al. 2010; http://www.phylo.org/portal2/login!input.action), with parametric bootstrapping generating 350 replicates as automatically determined by RAxML using a saturation criterion. The universal GTR-Gamma model was chosen for the analysis.

Results

In our analysis, the core Graphidaceae is divided into two strongly supported clades representing subfamilies Fissurinoideae and Graphidoideae (Fig. 1, see Appendix 2 for entire, fully resolved tree). Subfamily Graphidoideae is further divided into six larger and smaller clades, some of which form unsupported clusters. These clades largely represent species with either lirellate or rounded ascomata, but no supported division into two clades representing either ascomata type is evident; also, subfamily Fissurinoideae includes both species with lirellate and rounded ascomata.

Within subfamily Fissurinoideae, species of *Fissurina* s.lat. form several clades, indicating that the genus as currently defined is polyphyletic (Fig. 2). Most of the clades correspond to particular morphotypes: the *F. humilis*, *F. nitidescens*, and *F. nigrolabiata* clades (*Fissurina* 2–4) represent species with carbonized lirellae, the *F. pseudostromatica* clade (*Fissurina* 6) species with pseudostromatic lirellae, the *F. astroisidiata* clade (*Fissurina* 7) a species with platythecioid lirellae, and the *F. dumastii* clade (*Fissurina* s.str.) species with uncarbonized lirellae. Nested within the backbone are the clades corresponding to the genera *Clandestinotrema*, *Cruentotrema*, *Dyplolabia*, *Enigmotrema*, and *Pycnotrema*. *Clandestinotrema* forms two sister clades, one

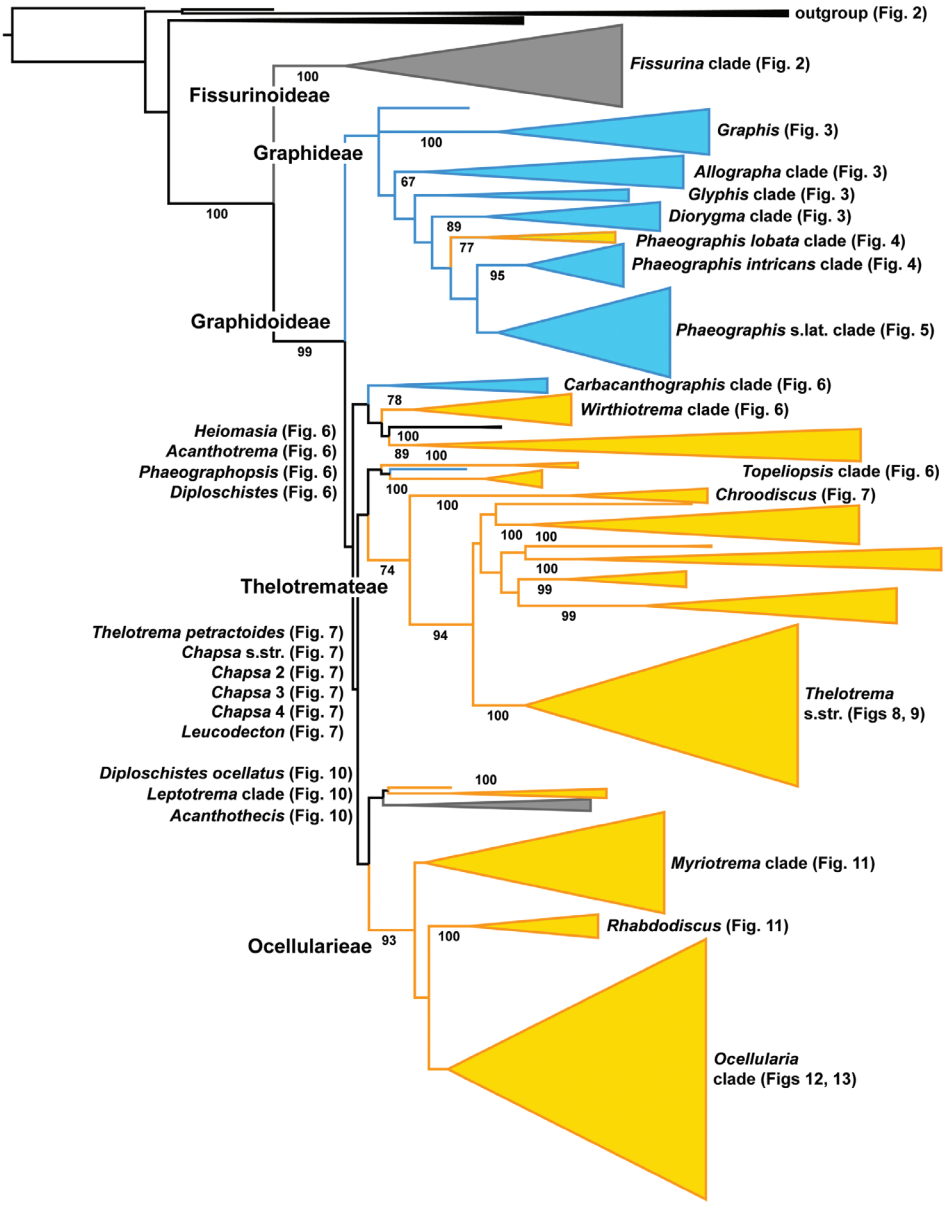

Figure 1. Cartoon tree showing the major clades distinguished within Graphidaceae with bootstrap support given next to branches. Blue clades indicate graphidoid taxa (lirellate or pseudostromatic ascomata), orange clades indicate thelotremoid taxa (rounded ascomata), and grey clades indicate mixed graphidoid and thelotremoid taxa. Bootstrap support is indicated for major clades and figures with detailed clade information are indicated for each clade. The entire, detailed tree is available as Appendix 2.

corresponding to species with narrow apothecial pores with entire margin and finger-like columella, represented by the type species, *C. clandestinum*, and the other to species with broadly open apothecia with fissured margin and broad-stump-shaped

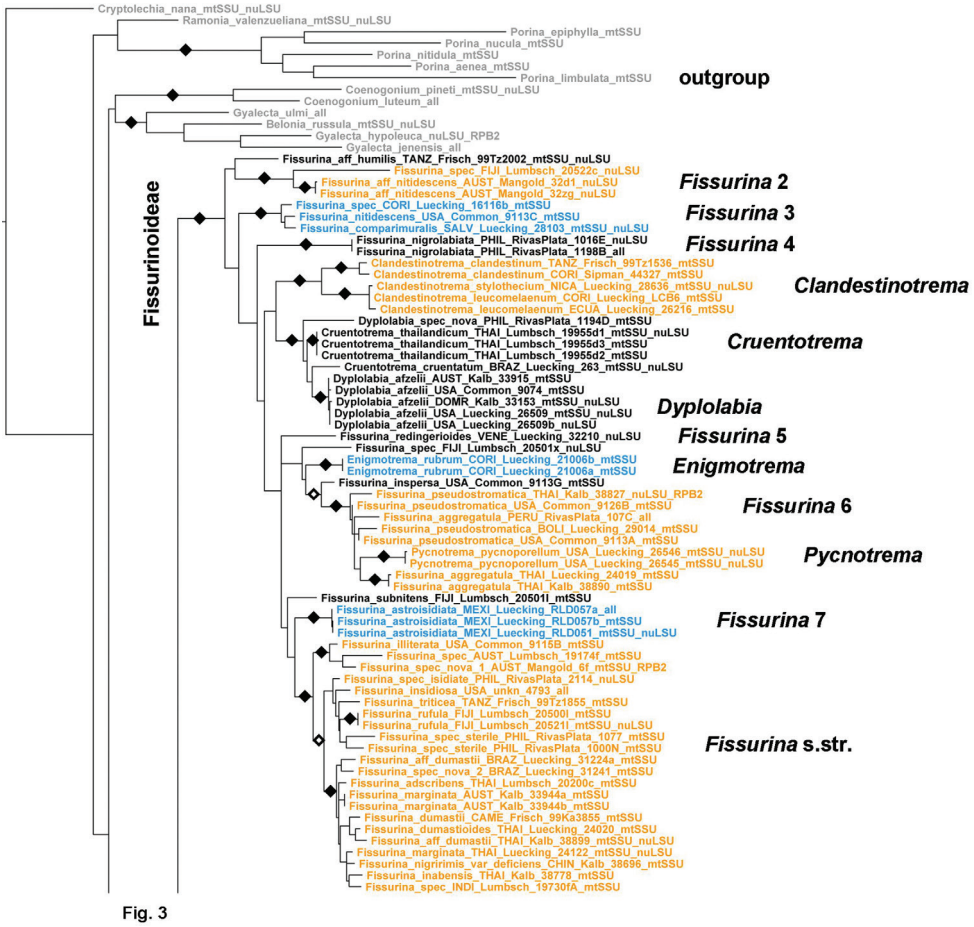

Figure 2. Detailed topology of the *Fissurina* clade (subfamily Fissurinoideae). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

columella, represented by *C. leucomelaenum* and *C. stylothecium*. *Cruentotrema* is nested within *Dyplolabia*, and each genus forms two separate clades each, which contradicts their pronounced morphological differences. *Enigmotrema* and *Pycnotrema* are nested within *Fissurina* s. lat.

The first clade in subfamily Graphidoideae represents the large tribe Graphideae (unsupported here but supported in previous studies), which can be divided into several larger and smaller clades (Figs 3–5). *Graphis* s.str., the type genus of the family, is the most unique clade phylogenetically (Fig. 3); it includes part of *Hemithecium* which corresponds to *Graphis* except in the uncarbonized lirellae. *Platythecium* is polyphyletic, forming two separate clades representing the *P. allosporellum* (*P. sphaerosporellum*) and *P. grammitis* groups. Both differ morphologically and chemically in that

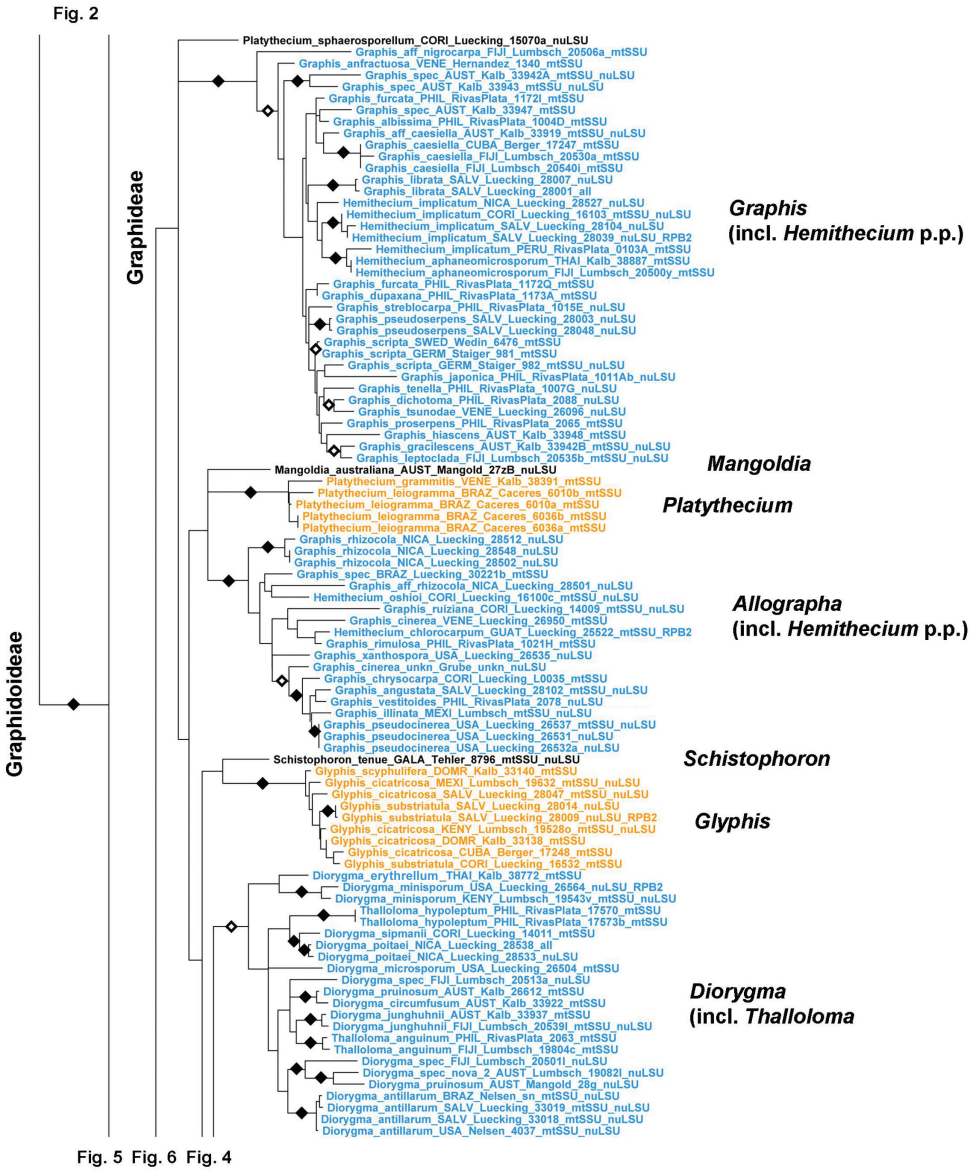

Figure 3. Detailed topology of the *Graphis*, *Platythecium*, *Allographa*, *Glyphis*, and *Diorygma* clades (subfamily Graphidoideae tribe Graphideae p.p.). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

the first group includes species with basally carbonized excipulum and the testacein chemosyndrome. The genus *Allographa* includes species of the *G. cinerea* group and part of *Hemithecium* with large ascospores. A new small genus, *Mangoldia* (described in a separate paper; Lücking et al. 2012), which is characterized by *Phaeographis le-*

Fig. 3

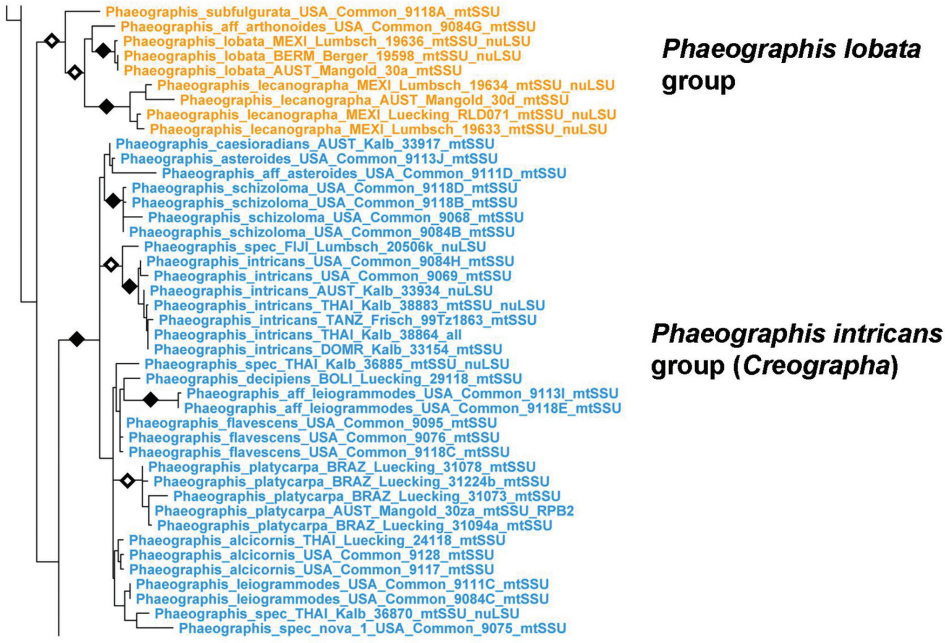

Fig. 5

Figure 4. Detailed topology of the *Phaeographis* s.lat. clade p.p. (subfamily Graphidoideae tribe Graphideae p.p.). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

canographa-like ascomata but *Graphis*-type ascospores, forms a separate clade with unresolved position. *Glyphis* is strongly supported as monophyletic and the mazaediate *Schistophoron* persistently clusters at the base of *Glyphis* but without support. *Diorygma* includes the genus *Thalloloma* and also a species formerly placed in the arthonioid genus *Herpothallon* but now recombined as *Diorygma antillarum* (Nelsen et al. 2012).

Phaeographis and allied genera (*Creographa*, *Halegrapha*, *Leiorreuma*, *Malmographina*, *Pallidogramme*, *Phlegographa*, *Platygramme*, *Sarcographa*, *Thecaria*) form a complex clade in which generic relationships appear diffuse, with many genera as currently defined being non-monophyletic (Figs 4–5). The *Phaeographis lobata* group, including *P. lecanographa* and allies, forms an unsupported sister-group relationship to the remaining species (Fig. 4). The clade itself is supported by both molecular data and morphological characters and deserves generic status. Species of the *Phaeographis intricans* group from a strongly supported clade sister to all remaining species, for which the name *Creographa* is available; however, one species with identical morphology, anatomy, and chemistry, *P.* aff. *intricans* (*Phaeographis* 4), clusters outside this clade, suggesting a remarkable case of parallel evolution. *Platygramme* s.str. appears to be monophyletic but some species currently classified in that genus, with a different

Fig. 4

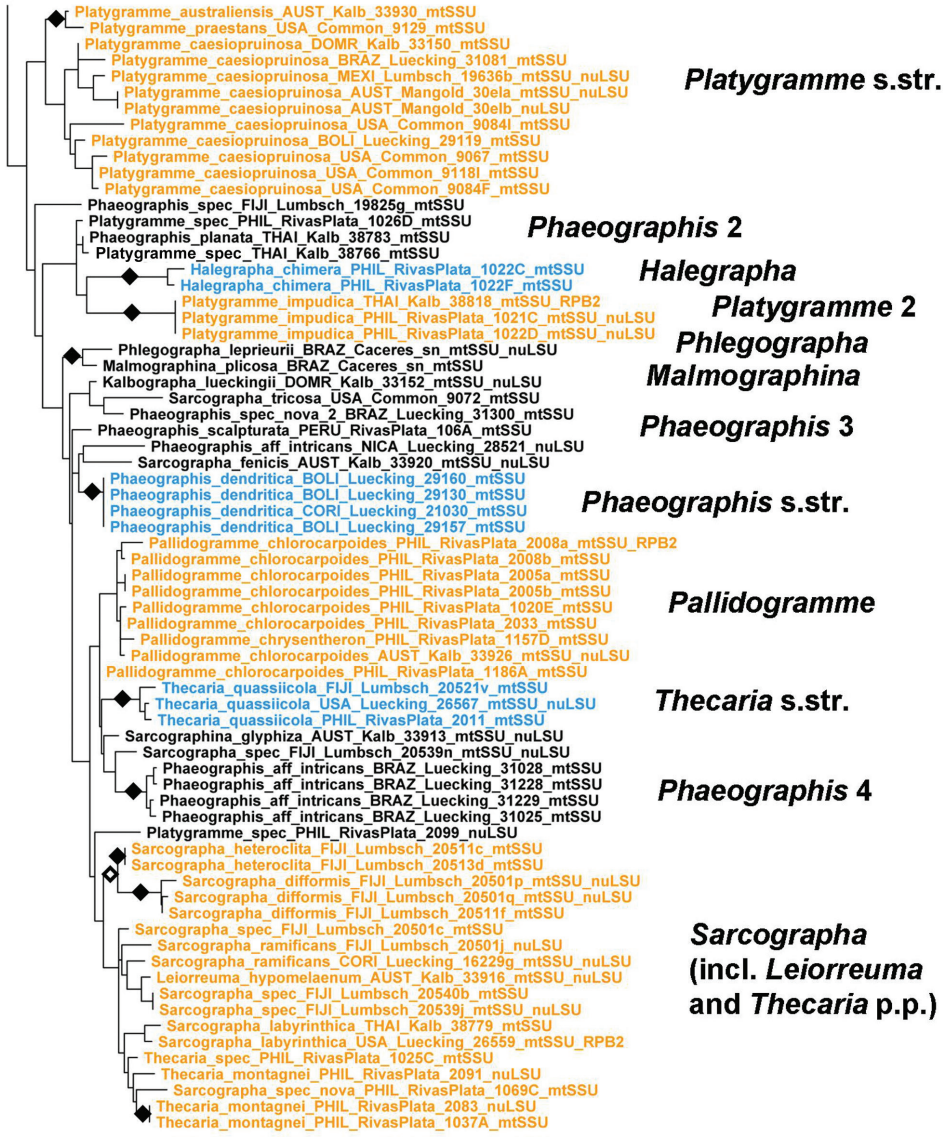

Figure 5. Detailed topology of the *Phaeographis* s.lat. clade p.p. (subfamily Graphidoideae tribe Graphideae p.p.). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

lirellae morphology (thick labia and concealed disc), such as *P. impucida* (*Platygramme* 2) cluster outside (Fig. 5). *Halegrapha* is supported as monophyletic, with an unsupported sister-group relationship to *Platygramme impudica* (*Platygramme* 2). Both share *Graphis*-like lirellae but differ in thallus morphology. Two morphologically unique

species classified in separate genera, *Malmographina plicosa* and *Phlegographa leprieurii*, form a supported sister group, unsupported sister to the remaining species of *Phaeographis* s.lat. *Malmographina* resembles *Allographa* in lirellae morphology, whereas *Phlegographa* is externally similar to *Glyphis*. The remaining species form several separate clades representing *Phaeographis* s.str., *Phaeographis* s.lat. (*Phaeographis* 2–4), *Pallidogramme*, *Sarcographa*, and *Thecaria*. The latter is polyphyletic, with *T. montagnei* nested within *Sarcographa*; also nested within that genus is *Leiorreuma hypomelaenum*, whereas *Sarcographina glyphiza* falls outside.

The next clades in subfamily Graphidoideae (Fig. 6) are small clades that were previously assigned to tribe Thelotremateae (Rivas Plata et al. 2012a) but in this study are not supported to form part of that tribe; their phylogenetic position remains unresolved. The *Carbacanthographis* clade includes two groups with lirellate ascomata that have apically spinulose periphysoids (*Carbacanthographis*) or paraphyses (*Acanthothecis* 2); the two lineages are otherwise separated by carbonized (*Carbacanthographis*) versus uncarbonized (*Acanthothecis* 2) lirellae, and the latter also differ in their corticate thallus and striate lirellae. This clade is phylogenetically and morphologically quite distinct from *Acanthothecis* s.str. (see below; Fig. 9) and deserves generic status. The *Wirthiotrema* clade includes genera and species previously mostly classified as *Thelotrema*, but apparently not related to *Thelotrema* s.str. The dominant secondary chemistry of this clade is stictic acid, present in nearly all species. *Wirthiotrema* itself is monophyletic and characterized by a dense, splitting cortex and non-amyloid ascospores with thickened septa. A second subclade is formed by *Thelotrema bicinctulum*, which resembles a *Myriotrema* with double margin but differs in its chemistry and rudimentary periphysoids. A third subclade is formed by *Chapsa platycarpa* and *Thelotrema leucophthalmum*, two species that, although previously assigned to different genera, are remarkably similar morphologically. The name *Asteristion* is available for this clade. Finally, the *Nadvornikia* subclade includes the mazaediate genus *Nadvornikia* as well as two non-mazaediate, lepadinoid species, *Leucodecton expallescens* and *Myriotrema peninsulae*. The latter is remarkably similar to *Nadvornikia* superficially except for the persistent, non-mazaediate hymenium. The next clade comprises the enigmatic, sterile genus *Heiomasia*. The next clade is formed by *Topeliopsis* and allied genera (*Melanotopelia* and *Schizotrema*), the genus *Schizotrema* apparently not being monophyletic, with one undescribed species forming a sister group relationship with *Topeliopsis* and another with *Melanotopelia*. *Chapsa lamellifera* is supported as nested within *Topeliopsis* but on a very long branch; morphologically it fits into that genus but differs by its very large apothecia. Finally, the last, unsupported clade comprises three subclades corresponding to three genera with unique morpho-ecological features: the chroodiscoid *Acanthotrema*, the mazaediate-lirellate *Phaeographopsis*, and the chroodiscoid-lecanoroid *Diploschistes*, which features a chlorococcoid photobiont and usually grows on soil or rock. These genera are morphologically and ecologically very disparate and their clustering might not reflect their true phylogenetic relationships, particularly since a long branch leads to each genus. *Diploschistes* appears non-monophyletic since *D. ocellatus* clusters outside the clade (see below; Fig. 10).

Fig. 3

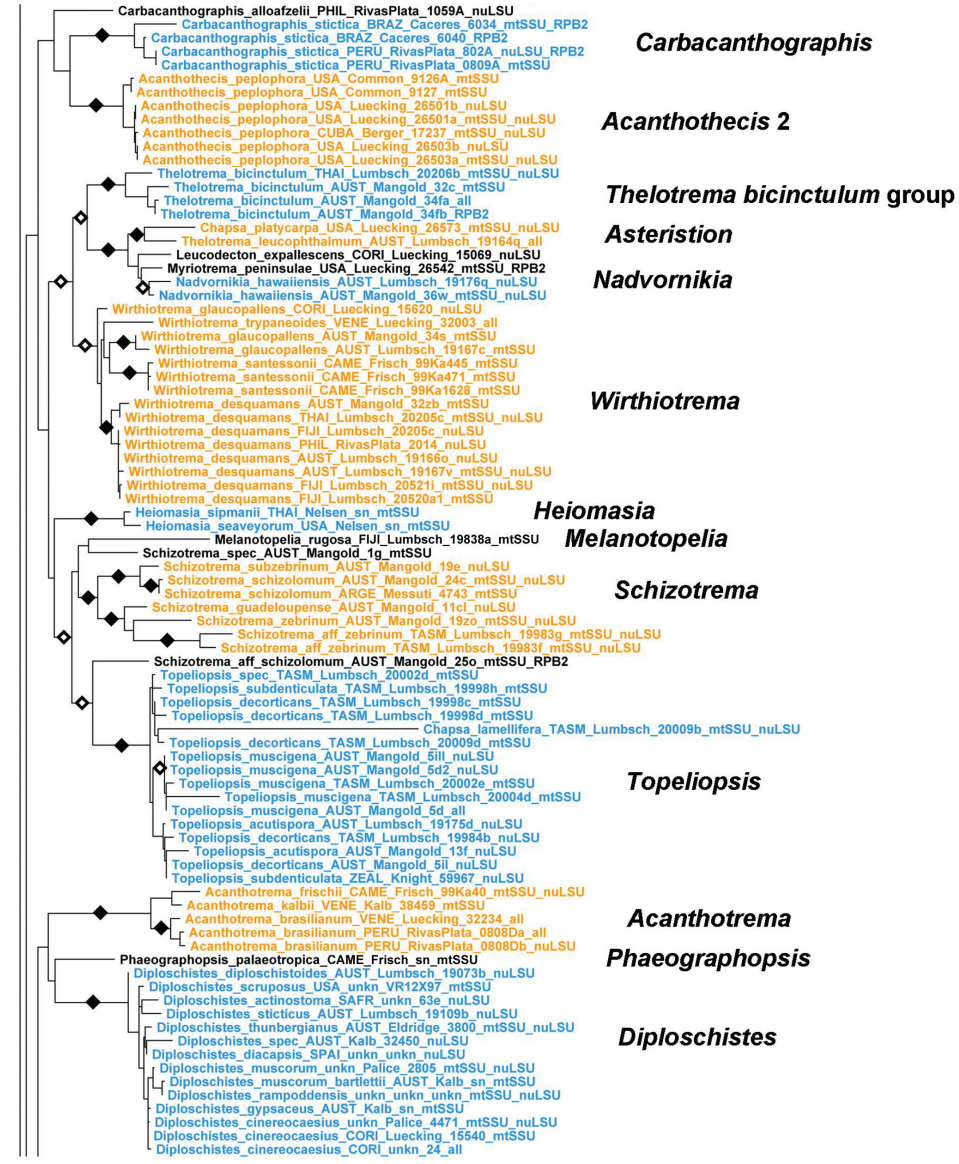

Fig. 7

Figure 6. Detailed topology of the *Acanthothecis* s. lat., *Acanthotrema*, *Carbacanthographis*, *Diploschistes*, *Heiomasia*, *Nadvornikia*, *Phaeographopsis*, *Schizotrema*, *Topeliopsis*, and *Wirthiotrema* clades (subfamily Graphidoideae). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

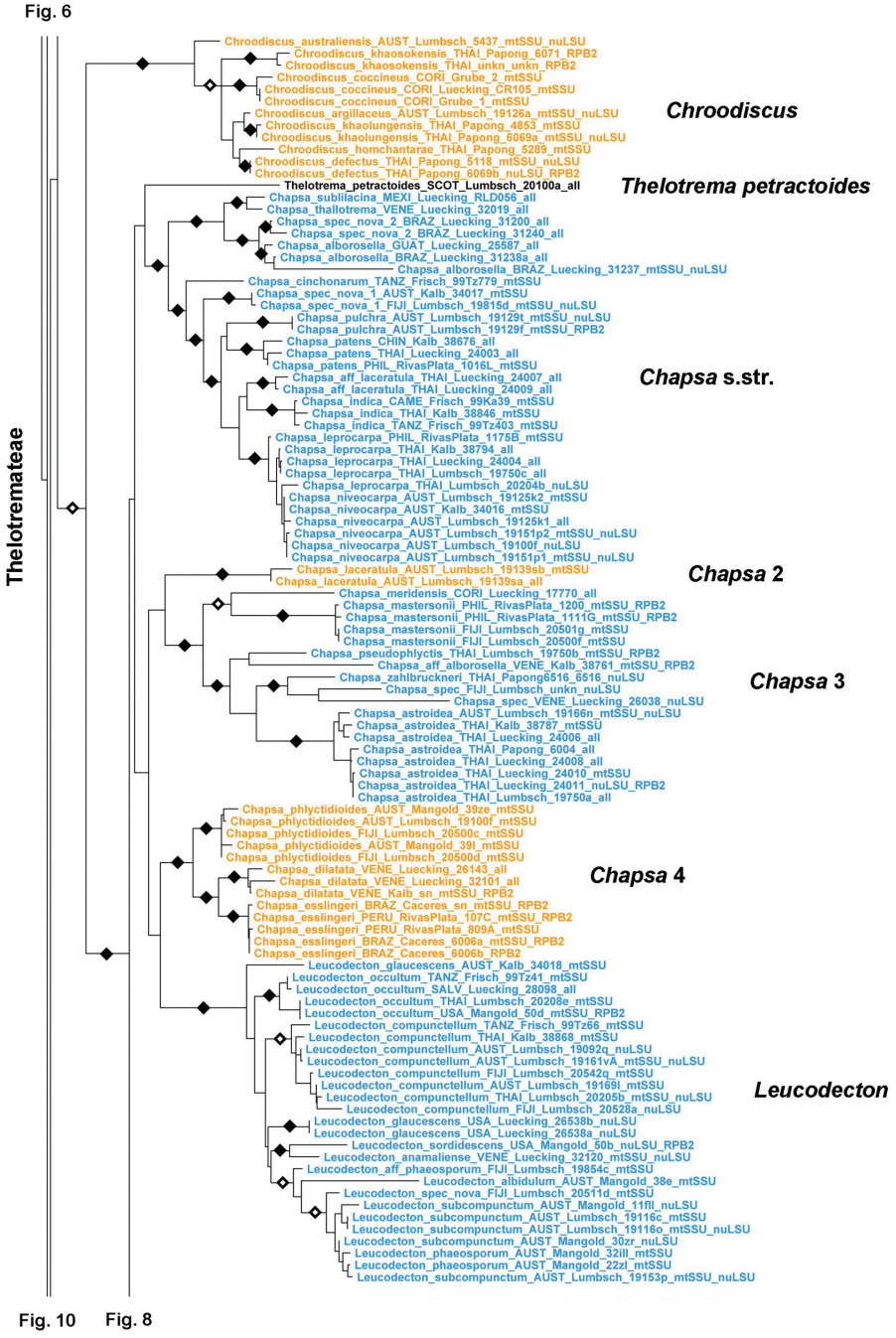

Figure 7. Detailed topology of the *Chroodiscus*, *Chapsa* s.lat., and *Leucodecton* clades (subfamily Graphidoideae tribe Thelotremateae p.p.). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

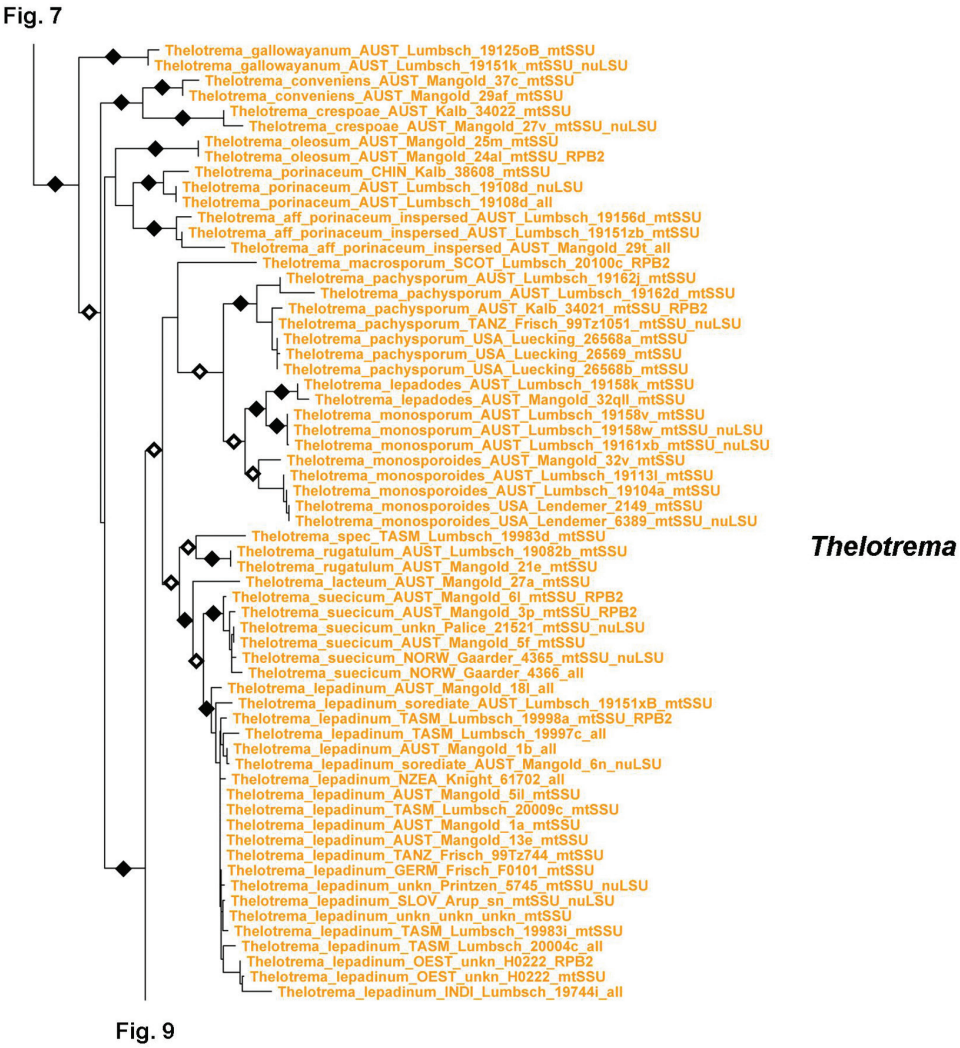

Figure 8. Detailed topology of the *Thelotrema* clade p.p. (subfamily Graphidoideae tribe Thelotremateae p.p.). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

Tribe Thelotremateae forms a large, monophyletic, well-supported clade including the genera *Chapsa*, *Chroodiscus*, *Leucodecton*, and *Thelotrema* (Figs 7–9). The latter three are monophyletic except *Thelotrema petractoides*, whereas *Chapsa* s.lat. is polyphyletic and can be divided into four clades (Fig. 7) depending on the criteria used to recognize each clade (branch length and support): *Chapsa* s.str., including the type species, *C. indica*; the *C. laceratula* clade (*Chapsa* 2), with *Topeliopsis*-like apothecia, the *C. astroidea* clade (*Chapsa* 3), and the *C. dilatata* clade (*Chapsa* 4). Species of *Chapsa* s.lat. also appear in other clades, such as *C. platycarpa* in the *Wirthiotrema* clade (see

Fig. 8

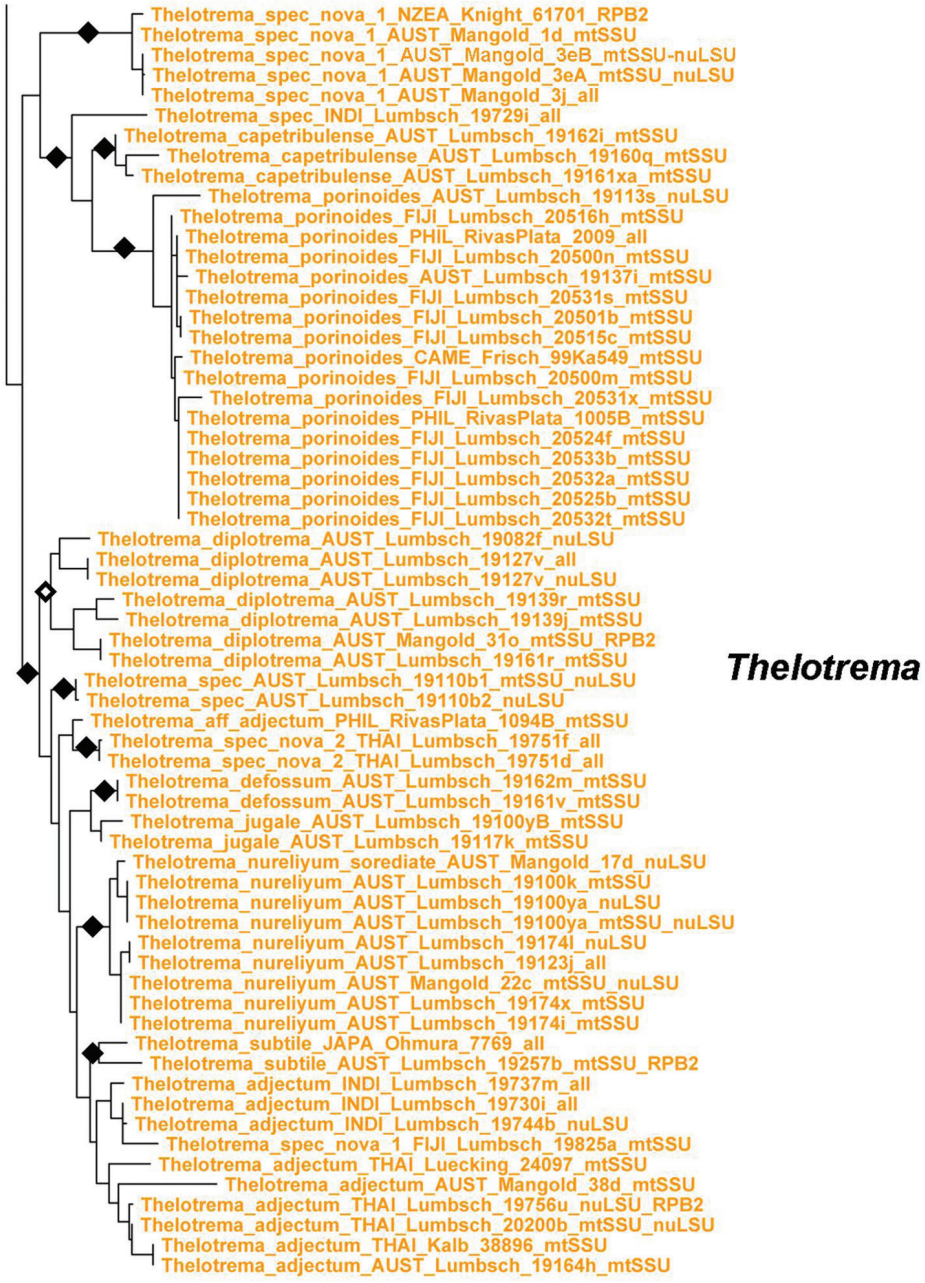

Figure 9. Detailed topology of the *Thelotrema* clade p.p. (subfamily Graphidoideae tribe Thelotremateae p.p.). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

above; Fig. 6) and *C. leprieurii* in tribe Ocellularieae (see below; Fig. 12). *Thelotrema* s.str. is currently the most well-represented clade in terms of sequenced species and geographic cover (Figs 8–9); it shows a well-resolved phylogenetic structure, with *T. gallowayanum*, *T. conveniens*, *T. oleosum* and *T. porinaceum* forming supported basal branches in the genus (Fig. 8). All have very narrow pores with the ascomata resembling those of *Leucodecton compunctellum* and certain *Ocellularia* s.lat. species (e.g. *O. profunda*). The *T. monosporum* and *T. lepadinum* groups form a supported sister group relationship, which is remarkable since both differ markedly in morphological and anatomical features: ecorticate, white thalli and brown ascospores in the *T. monosporum* group versus corticate, yellowish thalli and colorless ascospores in the *T. lepadinum* group. These two clades also support the concept of sporomorphs, species with identical morphology that differ in ascospore type only, since the species in the *T. monosporum* group are morphologically indistinguishable and also *T. suecicum* (small, transversely septate ascospores) and *T. lepadinum* (large, muriform ascospores) agree in external morphology. Species separated chiefly by their chemistry, such as *T. porinoides* and *T. diplotrema*, are supported as separate clades (Fig. 9). The same applies to species with clear versus inspersed hymenium, such as *T. porinoides* versus *T.* aff. *porinoides* (Fig. 8), suggesting that secondary chemistry and hymenium inspersion are important species-level characters even if not accompanied by other morphological differences.

The next three clades (Fig. 10) are partially unsupported and their position within the backbone of the family is unresolved. *Diploschistes ocellatus*, which differs from *Diploschistes* s.str. in chemistry (norstictic acid) and morphology (apothecia lecanoroid) is here not supported as part of the latter genus (see above; Fig. 6). The *Leptotrema* clade, which includes the genera *Leptotrema* and *Reimnitzia*, had been previously assigned to tribe Ocellularieae (Rivas Plata et al. 2012a) but is here not supported as part of that tribe, although it usually clusters at the base of the latter. The *Acanthothecis* clade, including the type species, *A. hololeucoides*, is not well-supported and also includes two species previously assigned to *Topeliopsis* but with a morphology similar to *Acanthothecis*, *T. darlingtonii* and *T. elixii*. This clade requires further study.

The last clade represents the large tribe Ocellularieae (Figs 11–13). It includes most of the species traditionally assigned to *Myriotrema* and *Ocellularia* but apparently corresponds to a much larger number of genus-level clades, some of them already recognized as *Fibrillithecis*, *Melanotrema*, *Redingeria*, *Rhabdodiscus*, and *Stegobolus* (Frisch et al. 2006) and others, such as *Compositrema*, *Glaucotrema*, and *Rhabdodiscus*, established recently as part of a detailed study of this clade (Rivas Plata et al. 2012b). The residual *Myriotrema* and *Ocellularia* as defined by Frisch et al. (2006) are still highly polyphyletic. *Myriotrema* can be divided into *Myriotrema* s.str., the *M. album* group (for which the name *Ocellis* is available), and the *M. glaucophaenum* group (recently separated as *Glaucotrema*). *Ocellularia* forms a large clade here named *Ocellularia* s.lat. (Figs 12–13) but also several smaller clades more closely related to *Myriotrema* s.lat., such as *O. conformalis*, *O. inturgescens*, *O. praestans*, *O. profunda*, and *O. pyrenuloides* (Fig. 11). *Stegobolus* s.lat. is divided into the distantly related clades *Stegobolus* s.str. (ascomata uncarbonized with thick, fuzzy proper margin) and *Rhabdodiscus* (asco-

Fig. 7

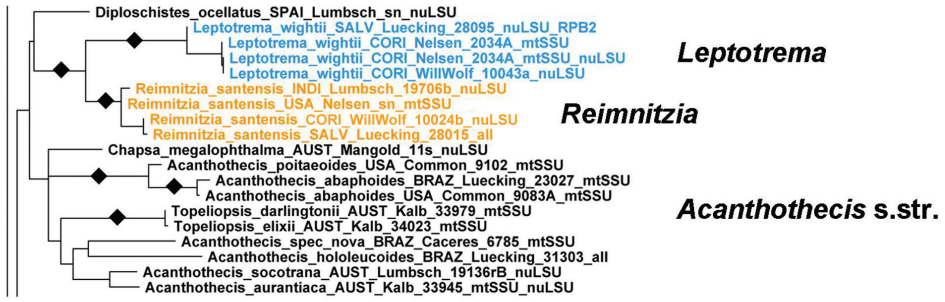

Fig. 11

Figure 10. Detailed topology of the *Diploschistes ocellatus*, *Leptotrema*, and *Phaeographopsis* clades (subfamily Graphidoideae). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

mata carbonized with thin, compact and smooth proper margin), the latter forming an unsupported sister to *Ocellularia* s.lat. (Fig. 11). The *Ocellularia* s.lat. clade is highly structured, including several strongly supported subclades with homogeneous morpho-chemical features (Figs 12–13): the *O. terebrata* group with psoromic acid and olive-green thalli; the *O. wirthii* group with psoromic acid and white thalli; the *O. baileyi* group with nornotatic acid; the *O. microstoma* group (for which the name *Macropyrenium* is available) with large, annulate ascomata and large, muriform ascospores, but variable chemistry; the *O. bahiana* group (for which the name *Stigmagora* is available) with often grainy thalli due to columnar clusters of crystals, an often irregular pseudocolumella, and protocetraric acid as predominant secondary compound; the *O. perforata* group with small, often myriotremoid ascomata and psoromic and protocetraric acid or no substances; the *O. dolichotata* group, lacking substances but with large, transversely septate ascospores; and the *O. eumorpha* group, with large ascomata and ascospores and hypoprotocetraric acid. *Ampliotrema*, with ecolumellate, often pigmented ascomata, inspersed hymenium, and protocetraric acid, appears as a paraphyletic residual basal to the *O. eumorpha* clade and *Ocellularia* s.str. The latter is unsupported but always monophyletic and chiefly includes species with pigmented medulla and hypoprotocetraric or nornotatic acid or cinchonarum or other unknowns. *Gyrotrema* is nested within *Ocellularia* s.str., although its chroodiscoid ascomata are quite different from the ocellularioid ascomata of the other species. Another gyrotremoid-stegoboloid species, *O. percolumellata*, is also nested in this clade. Perhaps the most unexpected surprise of this study is the supported placement of *Chapsa leprieurii* within *Ocellularia* s.lat.; except for the corticate, olive-brown thallus, this species agrees with *Chapsa* s.lat. in ascomata features.

Of the 428 species included in this analysis, 185 (42%) are represented by two or more (up to 21 in the case of *Thelotrema lepadinum*) OTUs (total of 658 out of 922

Fig. 10

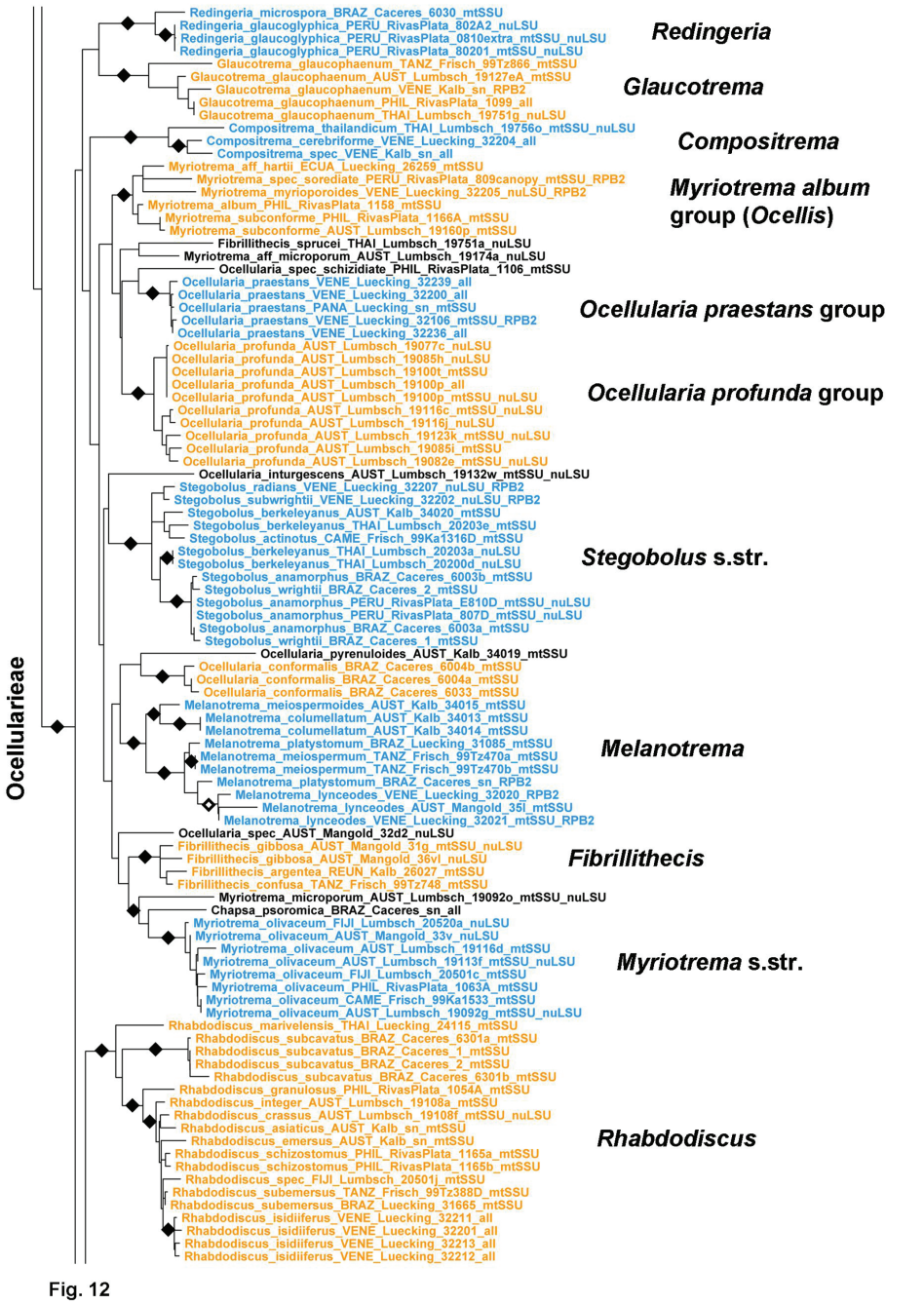

Fig. 12

Figure 11. Detailed topology of the *Myriotrema* s.lat. clade and relatives (subfamily Graphidoideae tribe Ocellularieae p.p.). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

Fig. 11

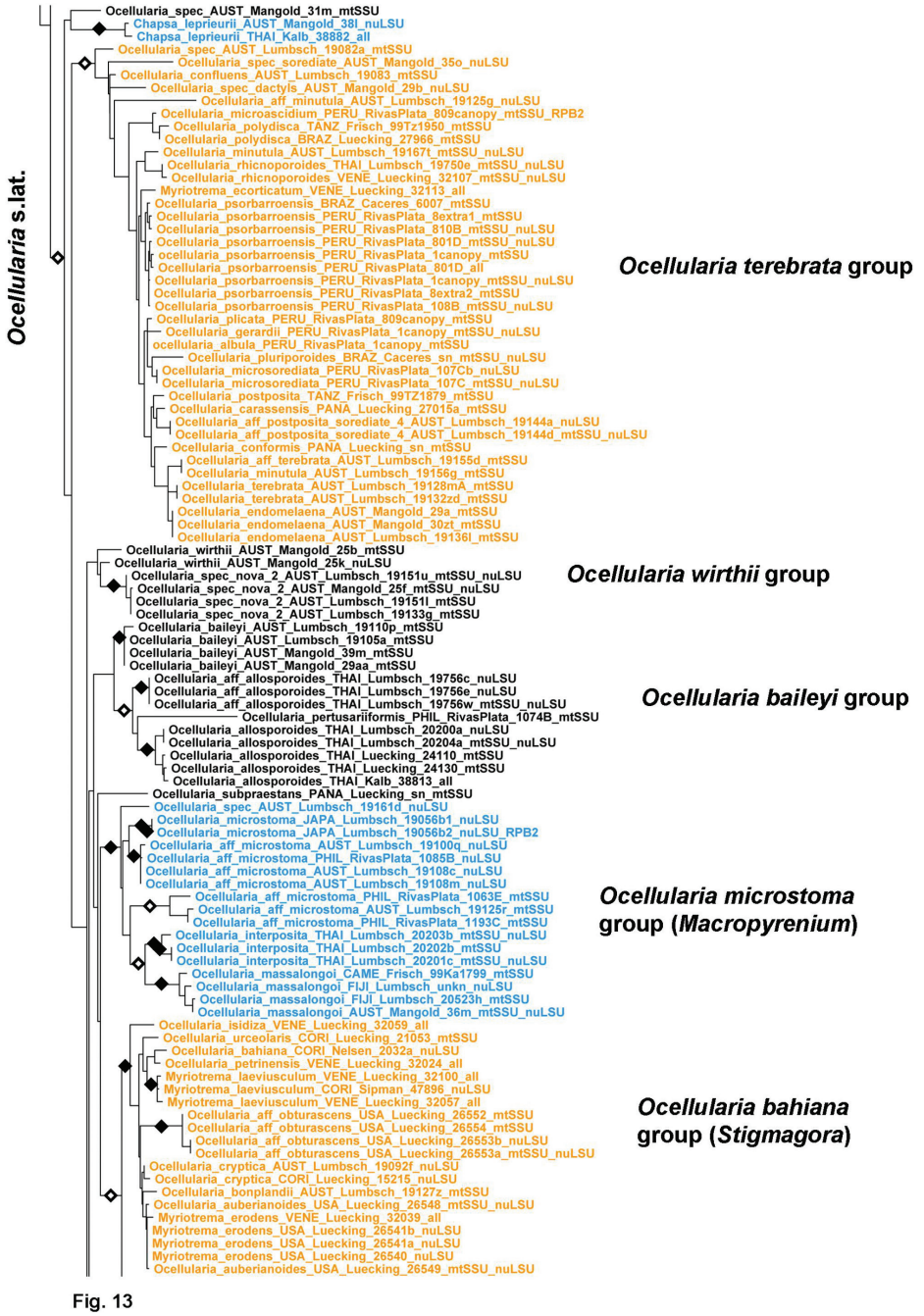

Fig. 13

Figure 12. Detailed topology of the *Ocellularia* s.lat. clade p.p. (subfamily Graphidoideae tribe Ocellularieae p.p.). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

Fig. 12

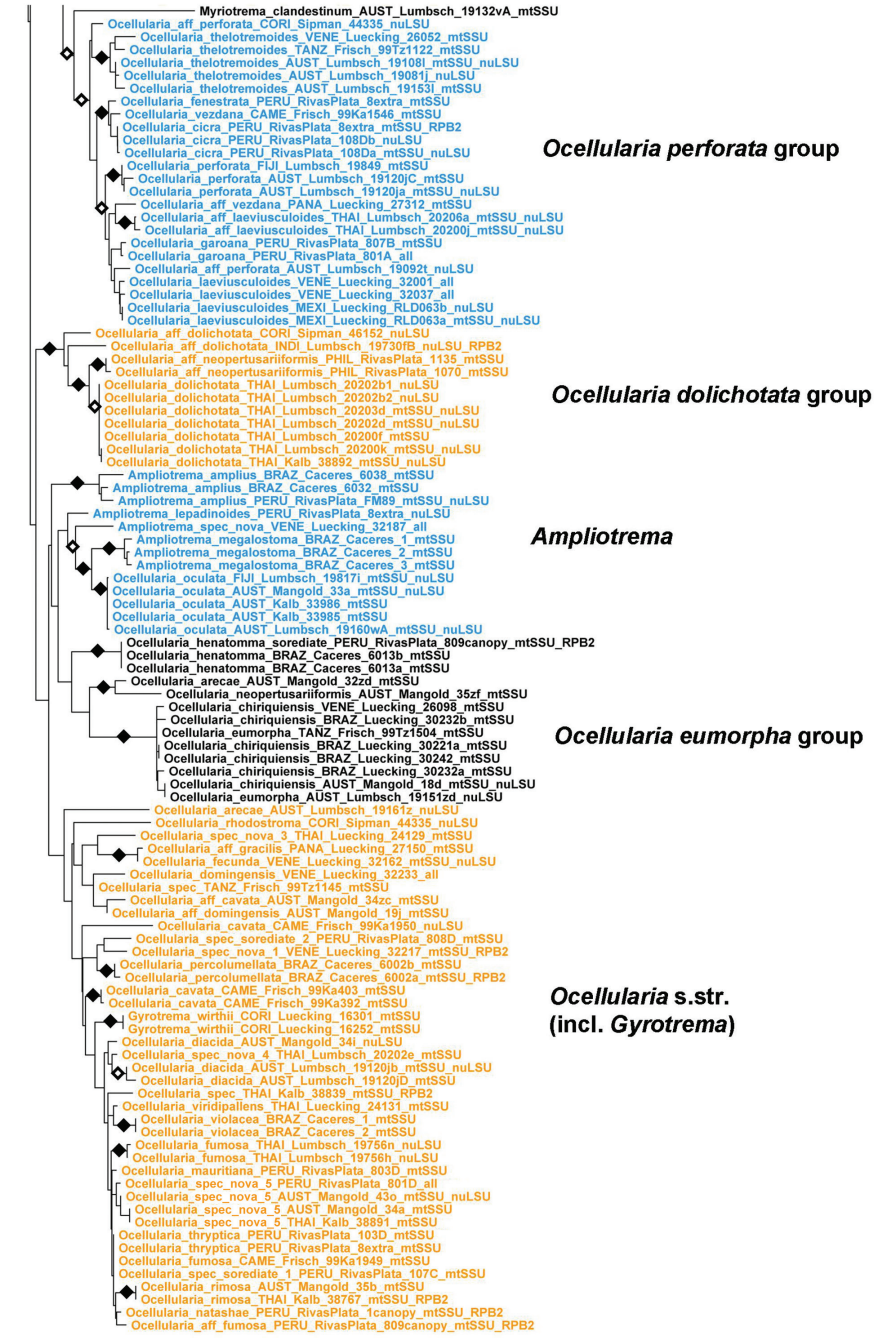

Figure 13. Detailed topology of the *Ocellularia* s.lat. clade p.p. (subfamily Graphidoideae tribe Ocellularieae p.p.). Genus level clades are highlighted. Bootstrap support is indicated as black (90% and higher) and black-and-white (70% and higher) symbols. For specimen information and GenBank numbers see Appendix 1. The entire, fully resolved tree with detailed bootstrap support values is available as Appendix 2.

OTUs), providing a base for a first test of species circumscriptions. The identifications used in this analysis are based on critical cross-examination of all available material including comparison to authentic type specimens, meaning that specimens bearing the same name were found to be morphologically identical. Of the 185 species with more than one sample, 148 (80%) were monophyletic; 20 were paraphyletic, and 17 were polyphyletic. There are several reasons to explain species-level paraphyly, such as insufficient resolution in closely related species complexes, especially in such a large phylogeny where terminal clades suffer from the effect of gap-rich alignment portions. Also, incomplete lineage sorting could cause paraphyly. Polyphyly can be explained by incomplete and non-overlapping gene sampling between OTUs of the same species, as well as incorrect species concepts suggesting (semi-)cryptic species. In the present case, two cases of paraphyly (*Hemithecium implicatum*, *Ocellularia wirthii*) and eleven cases of polyphyly (*Diorygma pruinosum*, *Glyphis substriatula*, *Leucodecton glaucescens*, *Melanotrema platystomum*, *Ocellularia* aff. *microstoma*, *Ocellularia arecae*, *Ocellularia cavata*, *Ocellularia fumosa*, *Stegobolus berkeleyanus*, *Topeliopsis subdenticulata*, *Wirthiotrema glaucopallens*) can be explained by incomplete gene sampling, e.g. mtSSU available for one OTU of a species and nuLSU for another, but not both for the same OTU. Another 12 cases of paraphyly (*Chapsa leprocarpa*, *Clandestinotrema leucomelaenum*, *Fibrillithecis gibbosa*, *Leucodecton phaeosporum*, *L. subcompunctum*, *Myriotrema erodens*, *Ocellularia auberianoides*, *O. chiriquiensis*, *O. eumorpha*, *Platythecium leiogramma*, *Stegobolus anamorphus*, *S. wrightii*) are potentially explained by insufficient resolution at the terminal clade level or incomplete lineage sorting or a combination of both. This leaves six cases of paraphyly (*Fissurina pseudostromatica*, *Ocellularia diacida*, *Pallidogramme chlorocarpoides*, *Sarcographa ramificans*, *Thecaria montagnei*, *Thelotrema adjectum*) and another six of polyphyly (*Fissurina aggregatula*, *F. marginata*, *Glyphis cicatricosa*, *Graphis scripta*, *Topeliopsis acutispora*, *Topeliopsis decorticans*) that cannot be explained by these causes and thus appear to indicate issues with current species circumscriptions.

The species level approach also allowed for testing the existence of widely distributed, intercontinental, pantropical, and cosmopolitan species. Of the 148 monophyletically resolved species, based on the available molecular data, two were confirmed as cosmopolitan taxa (the closely related sister species *Thelotrema lepadinum* and *T. suecicum*), 20 as pantropical (*Dyplolabia afzelii*, *Glaucotrema glaucophaenum*, *Graphis caesiella*, *Leucodecton occultum*, *Melanotrema lynceodes*, *Ocellularia albocincta*, *O. cryptica*, *O. rhicnoporoides*, *O. thelotremoides*, *Phaeographis alcicornis*, *P. intricans*, *P. lecanographa*, *P. lobata*, *P. platycarpa*, *Platygramme caesiopruinosa*, *Reimnitzia santensis*, *Sarcographa labyrinthica*, *Thecaria quassiicola*, *Thelotrema monosporoides*, *T. pachysporum*), four as at least Gondwanan (Neotropics and tropical Africa) but possibly pantropical (*Clandestinotrema clandestinum*, *Diorygma minisporum*, *Ocellularia polydisca*, *Rhabdodiscus subemersus*), four as at least paleotropical (tropical Africa, Asia and Australia) but possibly in part pantropical (*Chapsa indica*, *Leucodecton compunctellum*, *Myriotrema olivaceum*, *Ocellularia massalongoi*), and one austral (Argentina and Australia: *Schizotrema schizolomum*).

Discussion

The phylogeny presented here is the most comprehensive analysis of Graphidaceae known to date compared to previous studies (Staiger et al. 2006, Mangold et al. 2008a, Rivas Plata and Lumbsch 2011). The taxon sampling, with 428 species and 908 ingroup OTUs, represents about 25% of the currently accepted species and hence provides substantial insight into the phylogenetic relationships of major clades, genera, and species. It is obvious that the evolution within this family is much more complex than reflected by previous classifications which were in use until about ten years ago (Wirth and Hale 1963, 1978; Hale 1974, 1978, 1980, 1981; Archer 1999, 2000, 2001a–d, 2002). Many of the relationships found based on molecular data would have been impossible to predict using morphological characters, such as the split into subfamilies Fissurinoideae and Graphidoideae or the polyphyly of the large genera *Chapsa* sensu Frisch et al. (2006), *Graphis* sensu Staiger (2002) and *Ocellularia* sensu Frisch et al. (2006a). Also, the inclusion of myriotremoid and ocellularioid lineages, such as *Clandestinotrema*, *Cruentotrema*, and *Pycnotrema*, within *Fissurina* s.lat., or the separation of morphologically similar taxa like *Leptotrema wightii*, *Leucodecton phaeosporum*, and *Myriotrema laeviusculum*, in unrelated lineages came quite unexpected (Rivas Plata and Lumbsch 2011, Rivas Plata et al. 2012a). Another novel clade detected in the present study is the *Nadvornikia-Wirthiotrema* clade, which contains the mazaedioid *Nadvornikia* amidst an assemblage of *Thelotrema*-like species that are unrelated to *Thelotrema* s.str. While apparently most morphological characters such as ascomata shape, excipular carbonization, and the presence of a columella, evolved multiple times within the family in distantly related clades (Rivas Plata and Lumbsch 2011), surprisingly one of the most conserved character complexes turned out to be secondary chemistry. Most clades have a predominance of particular chemosyndromes, such as stictic and norstictic acids in the tribes Graphideae and Thelotremateae and various smaller clades falling outside these tribes, as well as psoromic, protocetraric, and hypoprotocetraric acids, and cinchonarum unknowns, in tribe Ocellularieae.

The most controversially discussed higher-level changes in the classification of Graphidaceae were the inclusion of Thelotremataceae, Gomphillaceae, and Asterothyriaceae (= Solorinellaceae) in this family (Aptroot 2012; Hodkinson 2012, Rivas Plata et al. 2012a; Sipman et al. 2012). The non-monophyly of Graphidaceae versus Thelotremataceae was already indicated in early phylogenetic studies (Staiger et al. 2006) and later confirmed by Mangold et al. (2008a). The current, much larger dataset clearly supports the conclusion that Graphidaceae and Thelotremataceae cannot be separated at the family level. In order to maintain the name Thelotremataceae, it would have to be restricted to tribe Thelotremateae, which would require to establish several separate families for tribes Graphideae and Ocellularieae, as well as for a number of smaller clades. Since the only argument separating the two families historically was ascomata shape (round in Thelotremataceae versus lirellate or pseudostromatic in Graphidaceae), a classification restricting the name Thelotremataceae to tribe Thelotremateae would be misleading and would be an effort to maintain a well-known

name, rather than reflecting a scientific advance. In the light of multiple evolution of ascomata types in different clades, merging the two families and instead delimiting infrafamily-level clades reflects the phylogenetic relationships much better.

A more difficult issue is the inclusion of Gomphillaceae and Asterothyriaceae within Graphidaceae. At first glance, this appears to be a purely phylogenetic requirement when applying the concept of strict monophyly. According to Aptroot (2012), Graphidaceae could technically be maintained as paraphyletic residual giving origin to a monophyletic Gomphillaceae (including Asterothyriaceae and Solorinellaceae). The problem of paraphyly is controversely discussed and no widely accepted solution has been proposed (Podani 2010, Hörandl and Stuessy 2010). However, the topology of the three clades proposed as subfamilies of Graphidaceae, Graphidoideae, Fissurinoideae, and Gomphilloideae (Rivas Plata et al. 2012a) does not reflect what is widely understood as paraphyly: a comb-shaped backbone from which individual clades emerge in different positions. On the contrary, the three subfamilies form three strongly supported clades on long stem branches each, and multigene studies show strong support for Fissurinoideae and Gomphilloideae being sister to each other and together sister to Graphidoideae (Baloch et al. 2010, Rivas Plata et al. 2012a, b, Nelsen et al. 2012). Therefore, splitting Fissurinoideae and the more closely related Gomphilloidae at the family level while keeping Fissurinoideae and the more distantly related Graphidoideae in a single family Graphidaceae would not create a paraphyletic taxon but would strongly disagree with the phylogenetic relationships of these taxa. Another alternative would be the recognition of three families, Gomphillaceae, Fissurinaceae, and Graphidaceae, as suggested by Hodkinson (2012). However, such a classification would not actually solve the dilemma of the apparent discrepancy between molecular phylogeny and morphological character evolution, since it would separate two clades, Fissurinoideae and Graphidoideae, at the family level with virtually no phenotypic character supporting such a separation.

One criterion that could be used to compare the alternative classifications is information content. Obviously, merging all clades into one family has the information content zero at the family level. Therefore, splitting them into three families makes only sense if the information content is greater than zero. This can be tested by using a pairwise comparison: assuming that Gomphilloideae (GO) are morphologically distinct from both Fissurinoideae (FI) and Graphidoideae (GR), whereas the latter two are not distinguishable, the pairwise comparison would result in two comparisons that are informative (GO versus FI and GO versus GR) and one comparison that is not informative (FI versus GR). Setting informative pairwise comparisons to +1 and non-informative to –1, the total would be +1, thus greater than zero, which would favor the 3-family solution partially suggested by Hodkinson (2012) over the 1-family solution suggested by Rivas Plata et al. (2012a). However, the distinction of Gomphillaceae from the other two families is not as straightforward as considered by Hodkinson (2012) and its inclusion within a wider Graphidaceae not as counterintuitive as it appears at first glance (Sipman et al. 2012). Gomphillaceae is largely separated from Graphidaceae by a chlorococcoid versus trentepohlioid photobiont, the anastomosing

versus unbranched paraphyses, the thin-walled and non-amyloid versus graphidoid and amyloid ascospores, and the hyphophorous conidiomata, as well as the predominantly foliicolous growth habit (Lücking et al. 2004, Lücking 2008). However, these differences disappear when considering the entire range of variation in both families: Graphidaceae includes taxa with chlorococcoid photobiont (*Diploschistes*) and species with thin-walled, non-amyloid ascospores (*Chroodiscus*, *Acanthotrema*). Other groups, such as *Diorygma*, *Dyplolabia*, and *Ocellularia* s.lat., have at least partially anastomosing paraphyses. Hyphophores are not present in all species of Gomphillaceae and are absent in the genera *Asterothyrium*, *Gyalidea*, and *Psorotheciopsis* (Henssen and Lücking 2002, Lücking 2008). Many Gomphillaceae, particularly in the genera *Echinoplaca* and *Gyalideopsis*, are corticolous, and in Graphidaceae, genera such as *Chroodiscus* are exclusively foliicolous. In addition, both groups share important features, such as the usually zeorine, hemiangiocarpous ascomata with a strong tendency to become lobate-lirellate, and the graphidoid ascus type. Therefore, considering the whole range of variation, there is no clear limit between these two families. Notably, *Aulaxina* was maintained in Graphidaceae by Santesson (1952) and *Asterothyrium* at some point was suggested to belong in Thelotremataceae and being related to *Chroodiscus* (Vezda and Poelt 1987; Aptroot et al. 1994). This view is also supported by the undisputed inclusion of *Diploschistes* in Graphidaceae, a genus that is ecologically very different from the remaining Graphidaceae and also genetically distinct, and which had been included in a separate family, Diploschistaceae, in the past (Zahlbruckner 1905). Hence we prefer to maintain a larger circumscription of Graphidaceae at this point.

Several genera currently placed in Graphidaceae have not yet been sequenced. These include *Amazonotrema*, *Anomalographis*, *Anomomorpha*, *Diaphorographis*, *Gymnographopsis*, *Kalbographa*, and *Sarcographina* (Staiger 2002, Lücking 2007, Kalb 2009, Rivas Plata et al. 2012a). The type of *Sarcographina* is unrelated to the sequenced *S. glyphiza* which therefore does not represent that genus. Also, the placement of *Kalbographa lueckingii* is doubtful since this species is morphologically and chemically closer to *Phaeographis dendritica* that to *Kalbographa* s.str. We therefore consider *Kalbographa* as of uncertain phylogenetic placement. Most of these genera have thin-walled ascospores different from most Graphidaceae but similar to, for example, *Phaeographopsis*. Since the latter forms a single clade with unresolved position, we also anticipate these unsequenced genera to represent further separate clades within the family. *Anomomorpha* is expected to be closely related to *Platythecium* str., since it shares morphological and chemical characteristics with that genus (Staiger 2002).

Clades and taxa: progress and problems

Subfamily Fissurinoideae

This subfamily currently includes six accepted genera. The core genus, *Fissurina*, includes more than 70 known species (Staiger 2002, Lücking, in prep.) and is highly

polyphyletic, as obvious from more than half of the species sequenced. The largest, supported clade is centered around the type species, *F. dumastii*. Another large clade includes predominantly pseudostromatic species centered around *F. pseudostromatica*; for this clade, the name *Medusulina* is potentially available, but more species need to be sequenced to test whether this clade corresponds to pseudostromatic species only. Species with carbonized excipulum form at least three supported clades at the base of the subfamily; they differ chiefly in ascomata morphology: fissurinoid to chroodiscoid in the *F. humilis* and *F. nitidescens* clades and graphidoid in the *F. nigrolabiata* clade. Several other species of *Fissurina* do not cluster with these larger clades. A few more critical species require sequencing and detailed morpho-anatomical studies are necessary to elucidate whether all these clades can be recognized as separate genera. For instance, species of *Fissurina* s.lat. partly feature warty paraphyses and also vary in ascospore amyloidity (Staiger 2002) and these could be features distinguishing between clades.

Clandestinotrema is strongly supported as a monophyletic clade, distinguished from other taxa in this subfamily by the whitish, often loosely corticate or ecorticate thallus and ocellularioid, usually carbonized and often columellate ascomata. The taxonomy of this genus is well-resolved (Rivas Plata et al. 2012a, Sipman et al. 2012), but a few more of the currently accepted 12 species need to be sequenced to confirm monophyly of this clade. There is some indication that species with pore-like versus broadly open apothecia form two subclades that each might deserve genus or subgenus rank, but more sequences are required to test this assumption. A peculiar character shared with *Cruentotrema*, *Dyplolabia*, and several species of *Fissurina*, are the astrothelioid ascospores, which also help to distinguish this genus from similar species in *Melanotrema* and *Ocellularia* s.lat. (Rivas Plata and Lumbsch 2011).

Cruentotrema and *Dyplolabia* are two very closely related genera, although morphologically quite disparate, being either chroodiscoid with bright red medullary pigment or in the latter graphidoid with thick white pruina containing lecanoric acid (Kalb and Staiger 2000; Rivas Plata and Lumbsch 2011; Rivas Plata et al. 2012a). Each genus currently contains three species and two species each have been sequenced. In the best-scoring ML tree, *Cruentotrema* appears nested within *Dyplolabia*, but without support, and SH testing performed on a subset of the data including the *Cruentotrema-Dyplolabia* clade and its sister group does not reject the possibility of both genera being monophyletic (results not shown). Considering the morphological differences between the two genera and the infrageneric uniformity within each of them, non-monophyly seems indeed unlikely and we are planning sequence additional loci to elucidate the relationships of these genera. However, this could be an ideal group to test the potential effect of incomplete lineage sorting on incongruence between gene phylogenies and actual taxon-level lineages.

The recently described, monospecific genus *Enigmotrema* is very similar to *Cruentotrema* but its apothecia remain closed for a long time and are bright red from the outside, resembling the pyrenocarpous lichen *Pyrenula cruenta* (Sipman et al. 2012). Inspite of its similarities with *Cruentotrema*, the genus clusters outside the *Cruentotrema-Dyplolabia* clade and appears more closely related to the *Fissurina pseudostro-*

matica clade. Nested within this clade is another monospecific genus, *Pycnotrema*, which resembles species of *Myriotrema* with its small, rounded apothecial pores (Rivas Plata et al. 2012a). Its nested position within the *Fissurina pseudostromatica* clade needs to be examined further.

Subfamily Graphidoideae: tribe Graphideae

This is the largest clade within the family, with possibly over 600 species. It includes the bulk of the former Graphidaceae with lirellate ascomata, with the exception of *Fissurina* s.lat., *Carbacanthographis*, and *Acanthothecis*. The major clades within this tribe correlate largely with ascospore type (hyaline-amyloid versus brown-hemiamyloid), whereas characters such as excipulum carbonization play a minor role, as shown by the inclusion of species of *Hemithecium* in either *Graphis* or *Allographa*. Thallus morphology also appears to be a good predictor of clade relationships, with white, strongly crystalline thalli characteristic of *Graphis* and *Allographa*, ecorticate thalli found mostly in the *Diorygma-Thalloloma* clade, and olive-green to yellow-brown thalli often lacking crystal clusters in *Glyphis* and the *Phaeographis* s.lat. clade. Graphideae are divided into two genetically distinct clades, one corresponding to *Graphis* s.str. and the other to all other taxa. The genetic uniqueness of *Graphis* s.str. was only recently established (Rivas Plata et al. 2011, Berger et al. 2011). A large number of species previously included in the revised concept of *Graphis* sensu Staiger (2002) turned out to belong in a separate genus, *Allographa*, which is more closely related to the remaining Graphideae. A formal reclassification reflecting this phylogeny has not yet been presented since species with uncarbonized excipulum previously separated in *Hemithecium* also fall into either *Graphis* or *Allographa* and more species of *Hemithecium*, including its type, need to be sequenced in order to correctly reallocate them. Rivas Plata et al. (2011) already showed that *Hemithecium* sensu Staiger (2002) was highly polyphyletic and formed at least five unrelated clades. Two of them clustered with either *Graphis* or *Allographa*, whereas another two fell into the vicinity of *Phaeographis* and are currently recognized as *Malmographina* and *Pallidogramme*, respectively (Lücking et al. 2008, Cáceres et al. 2012). The fifth clade, consisting of *H. rufopallidum*, apparently formed an isolated clade related to *Allographa* (Rivas Plata et al. 2011). In our current analysis, the species *Platythecium grammitis* fell into this clade, and re-examination of the material of *H. rufopallidum* revealed that our previous identification was incorrect: while this species was present in the voucher material, the sequenced taxon actually belonged to the superficially similar *Platythecium leiogramma*, thus supporting *Platythecium* as a separate genus.

The genus *Glyphis* remains a strongly supported clade within Graphideae, including species with rounded, lirellate, and pseudostromatic ascomata. However, species relationships require further study since the pseudostromatic *G. cicatricosa* does not appear to be monophyletic. The mazaediate genus *Schistophoron*, which resembles species of *Allographa*, consistently comes out as sister to *Glyphis*, but lacking support. Both genera are morphologically and anatomically quite different. *Diorygma* and *Thalloloma*, two gen-

era with similar thallus and ascomata morphology but with different chemical profiles (Staiger 2002, Kalb et al. 2004) appear to be nested within each other and most probably will have to be merged into a single genus. Supposed differences such as hamathecium structure and amyloidity (Staiger 2002, Kalb et al. 2004) do not seem to correlate with the topology found in this clade. On the other hand, *Platythecium* sensu Staiger (2002) appears to be polyphyletic, corresponding to two morphologically and chemically distinct clades.

The remaining genera all have brown, hemiamyloid ascospores and consistently form a monophyletic clade (Lücking et al. 2011, Rivas Plata et al. 2011, Cáceres et al. 2012), although support was lacking in the present analysis. However, genus-level relationships and genus delimitation within this clade are in need of major revision. Most of the currently accepted genera (Staiger 2002) are para- or polyphyletic at some level, whereas supported monophyletic clades have not been recognized at genus level. Among the latter are the *Phaeographis lobata-lecanographa* group and the *P. intricans* group, for which the name *Creographa* is potentially available. *Platygramme* s.str. is monophyletic but its delimitation towards *Phaeographis* s.lat. is uncertain, since species with distinctly carbonized labia, such as *P. impudica*, appear elsewhere in this clade. Most other genera, such as *Halegrapha*, *Malmographina*, *Pallidogramme*, *Phlegographa*, and *Thecaria*, show a similar pattern, with the type species and its close relatives forming monophyletic clades but nested within a paraphyletic *Phaeographis* s.lat. grade. *Thecaria montagnei* is apparently not close to the type species, *T. quassiicola*, and the generic name *Pliariona* is potentially available for this species. *Leiorreuma* and *Sarcographa* appear both non-monophyletic, with the core groups forming a single clade in a derived position. *Phaeographis* s.str. thus far appears to be restricted to the type species, *P. dendritica*, which is characterized by its white, ecorticate thallus and non-pruinose ascomata. Most species of *Phaeographis* s.lat. have a corticate thallus and pruinose ascomata, and the name *Ectographis* is potentially available for these. However, many more species need to be sequenced in these groups to establish a solid phylogeny and generic concept for *Phaeographis* and its allies. The most important characters separating clades within this assemblage are ascomata organization (solitary versu pseudostromatic), disc visibility (exposed versus concealed), hymenium inspersion (inspersed versus clear), and excipular and hypothecium carbonization (uncarbonized versus carbonized). On their own, these clades appear to be quite distinctive; however, exactly the same level of variation is found in Graphis and Allographa, two clades that form single genera including between 100 and over 200 species each, and therefore, it would not appear illogical to actually merge *Creographa*, *Ectographis*, *Halegrapha*, *Leiorreuma*, *Malmographina*, *Pallidogramme*, *Phaeographis* s.str., *Phlegographa*, *Platygramme*, *Pliariona*, *Sarcographa*, and *Thecaria*, into a single, large genus *Phaeographis*.

A unique, novel lineage was detected clustering between *Platythecium* and *Allographa*. The new genus, *Mangoldia*, from Australia combines a *Phaeographis* morphology with *Graphis* type hymenium and ascospores, a previously unknown combination of characters (Lücking et al. 2012).

Subfamily Graphidoideae: *Acanthothecis* clade

This clade includes species of *Acanthothecis* s.str. with weakly corticate or ecorticate thallus and rounded ascomata, as well as some corticolous species currently classified in *Topeliopsis*: *T. darlingtonii* and *T. elixii*. The morphology of these taxa is quite similar and most likely they should all be included within *Acanthothecis*, although not all have the apically spinulose paraphyses and periphysoids characteristic of that genus (Staiger and Kalb 1999, Staiger 2002). Species of *Acanthothecis* s.lat. with corticate thallus and lirellate ascomata do not belong here but cluster next to *Carbacanthographis*.

Subfamily Graphidoideae: *Acanthotrema* clade

Acanthotrema is a small genus of four species characterized by a corticate thallus, chroodiscoid ascomata, and thin-walled ascospores of the *Chroodiscus* type (Rivas Plata et al. 2010b). This genus, which is found on shaded tree trunks in the lowland rain forest, appears rather isolated within the family on a very long branch and its exact position remains unresolved. Depending on taxon sampling, it either clusters close to tribe Thelotremateae or the *Diploschistes* clade (Papong et al. 2009, Rivas Plata et al. 2012a), but always lacking support for either placement.

Subfamily Graphidoideae: *Carbacanthographis* clade

This clade includes two strongly supported genus-level clades: *Carbacanthographis* and *Acanthothecis* 2, in addition to a further species of *Carbacanthographis* forming an unsupported sister clade. *Carbacanthographis* has graphidoid ascomata but is not closely related to *Graphis* and allies, whereas *Acanthothecis* 2 has fissurinoid-hemithecioid ascomata, while not being closely related to either *Fissurina* or *Hemithecium* (Staiger and Kalb 1999, Staiger 2002). *Carbacanthographis* currently includes close to 20 species, but only two have been sequenced; molecular data for more species are required to confirm monophyly of this genus. Only one species of *Acanthothecis* falling into this clade, *A. peplophora*, has been sequenced so far, but its morphology suggests that this clade includes species of *Acanthothecis* with corticate thallus and striate lirellae, such as the widespread tropical *A. subclavulifera* (Staiger and Kalb 1999, Staiger 2002). This clade will eventually require a new genus name; it is not closely related to *Acanthothecis* s.str., based on *A. hololeucoides*.

Subfamily Graphidoideae: *Diploschistes* clade

Diploschistes is a medium-sized genus of about 30 currently accepted species (Lumbsch 1989, Guderley and Lumbsch 1996, Lumbsch and Elix 2003, Rivas Plata et al. 2010b).

It is unique in the family in having a chlorococcoid photobiont and due to its ecology and chemistry, forming well-developed, thick thalli usually on soil or rock surfaces or sometimes on bryophytes with the depsides gyrophoric, lecanoric, and diploschistesic acids as main compounds. The genus usually clusters close to tribe Thelotremateae but this position is not supported. The very long stem branch together with the very short terminal branches suggest that this genus radiated very recently, which is remarkable considering that it is cosmopolitan in distribution. The placement of the norstictic acid containing, lecanoroid *D. ocellatus* requires additional studies (see below). Another genus supposedly related to *Diploschistes*, *Ingvariella* (Guderley et al. 1997), has been shown to belong in Stictidaceae (Fernández-Brime et al. 2011).

Subfamily Graphidoideae: *Diploschistes ocellatus* clade

Diploschistes ocellatus differs from other species of *Diploschistes* in the lecanoroid ascomata and norstictic acid as major compound in the thallus. Its phylogenetic position is unresolved, although it usually clusters close to or at the base of the *Diploschistes* clade (Fernández-Brime et al. 2011). Additional loci need to be sequenced to address monophyly of *Diploschistes* as currently circumscribed.

Subfamily Graphidoideae: *Heiomasia* clade

This clade includes a single genus with two known species, *Heiomasia seaveyorum* and *H. sipmanii*. It was recently described and both species are only known with isidioid propagules but lack ascomata (Nelsen et al. 2010). Additional loci need to be sequenced to address the relationships of this isolated clade but it might well represent a relict clade with no close extant relative, similar to the case of *Phaeographopsis*.

Subfamily Graphidoideae: *Phaeographopsis* clade

Phaeographopsis is a lowland rain forest genus. It resembles species of *Diorygma* in the green, ecorticate thallus and pruinose, lirellate ascomata, but differs in the thin-walled, dark brown ascospores which accumulate above the hymenium and form weakly developed mazaedia (Aptroot et al. 1997, 2007, Kalb 2004, Lücking and Rivas Plata 2008). The genus, which includes two species, consistently clusters close to *Diploschistes* but without support; therefore its phylogenetic position is unresolved. The unique morphological features and long branch suggest this to represent another relict taxon in Graphidaceae.

Subfamily Graphidoideae: *Topeliopsis* clade

This clade was previously included in tribe Thelotremateae (Rivas Plata et al. 2012a) but with this larger dataset falls outside that tribe. It includes the bulk of taxa with topeliopsidoid-schizotremoid ascomata, that is, apothecia with layered (striate) excipulum; many species occur in the southern Hemisphere or are tropical-montane and often grow on bryophytes (Frisch and Kalb 2006, Mangold et al. 2008b, Lumbsch et al. 2010). Within this clade, both *Schizotrema* s.str. (excipulum carbonized, ascomata erumpent over tree bark) and *Topeliopsis* s.str. (excipulum non-carbonized, ascomata mostly sessile on bryophytes) form monophyletic and well-supported clades. *Melanotopelia* (excipulum carbonized, ascomata mostly sessile on bryophytes) is also potentially monophyletic, but only one of the four currently accepted species has been sequenced so far. Two species of *Schizotrema* s.lat. from separate clades and their relationships need to be studied further. Two further species of *Topeliopsis* cluster within to *Acanthothecis* s. str.

Subfamily Graphidoideae: *Wirthiotrema* clade

This well-supported clade includes species previously classified in *Thelotrema* but apparently not related to the latter genus. In addition, the mazaediate *Nadvornikia hawaiiensis* is nested within this clade. Three supported subclades can be distinguished: a clade consisting of *Thelotrema bicinctulum*, a corticate species morphologically intermediate between *Thelotrema* and *Myriotrema*; a clade consisting of *Chapsa platycarpa* and *Thelotrema leucophthalmum*; a clade consisting of *Myriotrema peninsulae*, *Leudodecton expallescens*, and *Nadvornikia hawaiiensis*; and a well-supported clade representing the recently segregated genus *Wirthiotrema* (Rivas Plata et al. 2010a). This entire clade will be treated in detail in a separate, forthcoming paper.

Subfamily Graphidoideae: tribe Thelotremateae

This tribe is well-supported in all analyses and contains four currently accepted genera, including a monophyletic *Chroodiscus* forming sister to the remaining genera, a monophyletic *Leucodecton*, and a monophyletic *Thelotrema* s.str., all strongly supported. These three genera differ consistently in ascomata morphology: *Chroodiscus* has chroodiscoid ascomata lacking periphysoids, *Leucodecton* also lacks periphysoids but its ascomata are mostly myriotremoid-porinoid, and *Thelotrema* features mostly lepadinoid ascomata with periphysoids and double margin (proper excipulum free). On the other hand, the genus *Chapsa*, with chroodiscoid ascomata and periphysoids, is polyphyletic, forming several well-supported clades. There is some correlation with morphological features in these clades but these need to be studied further, and this clade is currently being analyzed in a separate study (Parnmen et al. 2012). Also, several species of *Chapsa* s.lat. fall outside this tribe, such as *C. platycarpa* and *C. leprieurii*.

The well-supported placement of the latter as sister to *Ocellularia* s.lat. is another surprising find of this study.

Subfamily Graphidoideae: *Leptotrema* clade

This clade was previously included in tribe Ocellulariae as basal sister group to Ocellularieae s.str. (Rivas Plata and Lumbsch 2011, Rivas Plata et al. 2012a), but the larger dataset analysed here does not support this relationship, although the clade consistently comes out as sister to Ocellularieae. The clade includes two genera, *Leptotrema* and *Reimnitzia*, which agree in general thallus morphology and ascus and ascospore type (Frisch et al. 2006a), but differ in the myriotremoid versus chroodiscoid ascomata.

Subfamily Graphidoideae: tribe Ocellularieae

This tribe is being treated in detail in a parallel paper based on roughly the same dataset (Rivas Plata et al. 2012b) and therefore is not further discussed here. It includes well over 300 species currently assigned to up to 12 genera: *Ampliotrema*, *Compositrema*, *Fibrillithecis*, *Glaucotrema*, *Gyrotrema*, *Melanotrema*, *Myriotrema*, *Ocellis*, *Ocellularia* s.lat., *Redingeria*, *Rhabdodiscus*, and *Stegobolus*. However, several more lineages exist deserving generic status and some genera appear to be nested within others, such as *Gyrotrema* within *Ocellularia* s.str. We expect that a more complete dataset in terms of complete sampling of genes will help to resolve this clade. Very unexpected is the placement of *Chapsa leprieurii* in *Ocellularia* s.lat.; this species has *Chapsa*-like ascomata with lateral periphysoids, a character otherwise absent in the entire Ocellularieae. Yet, its placement is supported by several sequences representing all three genes from two different specimens. We have no explanation for this placement, other than recognizing it as yet another remarkable case of parallel evolution in Graphidaceae (Rivas Plata and Lumbsch 2011).

Species delimitations in Graphidaceae

Although the sampling for this study was focused in targeting as many species as possible, with generally few repeats per species, the data allows a first approach in testing species concepts. The identifications made for the purpose of this study already represent a refined species concept as result of cross-examination of all available material and comparison with authentic type specimens. Thus, for example, previous identifications made using a more coarse species concept had already been corrected prior to this analysis, particularly in the genera *Thelotrema* and *Ocellularia* s.lat. (e.g. Rivas Plata et al. 2012b). Still, of the 185 species represented by at least two (and up to 21) OTUs, 37 turned out to be paraphyletic or polyphyletic. Of these, 12 cases can be explained by insufficient

gene sampling (non-overlapping genes for different OTUs of the same species). Another 13 cases can potentially be resolved by analysing the corresponding clades separately, since for terminal clades, gap-rich regions caused by aligning a multitude of sequenced across a large group of species will mask the phylogenetic signal contained in these regions. Thus, the remaining 12 out of 185 species appear to present problems in terms of species concepts. These may either represent cases of too narrow species concepts (e.g. *Pallidogramme chlorocarpoides* versus *P. chrysenteron*, which differ only in ascospore size and number) or cases of cryptic species (e.g. *Fissurina marginata*, *O. diacida*, *Thelotrema adjectum*). In some cases, such as *Glyphis cicatricosa* and *Graphis scripta*, the sequenced material could not be completely tested, but available data suggest that more than one species are hidden under these names. For example, *G. cicatricosa* includes forms with different ascomata configuration (rounded versus lirellate-stellate), which might explain the presence of more than one species. In the case of *Graphis scripta*, a recent revision (Neuwirth and Aptroot 2012) already divided this taxon into at least four species, and the molecular data seem to confirm this view. Other species, such as *Fissurina aggregatula* versus *F. pseudostromatica*, or taxa such as *S. ramificans* and *Thecaria montagnei*, include specimens with subtle morphological differences and these are obviously not yet fully understood. Notable is the case of *Topeliopsis acutispora* versus *T. decorticans*, two morphologically similar species that have quite different ascospores; the clade including these species obviously requires more scrutinity. In spite of these unresolved cases, it however seems that the combination of molecular analysis and critical revision of phenotype characters has improved the species concept in Graphidaceae considerably.

It was generally assumed that many lichens have a wide geographic distribution, much wider than vascular plants (Jørgensen 1983, Galloway 1988, Tibell 1994, Lücking 2003, Feuerer and Hawksworth 2007). However, molecular phylogenetic studies have challenged this view for many taxa (Tehler et al. 2009, 2010, Del Prado et al. 2011, Molina et al. 2011), and Graphidaceae are no exception. While previous studies assumed a proportion of 30–50% intercontinental, pantropical, or even cosmopolitan species (Wirth and Hale 1963, 1978, Hale 1974, 1978, 1981), the present analysis only confirmed 31 out of 148 monophyletic species (21%) as belonging to one of these categories, and only 20 as pantropical. In part this is a sampling artifact, and with more sequence data available, more species, such as *Leucodecton compunctellum* and *Myriotrema olivaceum* (for which unsequenced material from the Neotropics is known), will be confirmed to have a wide distribution. However, this is probably more than counterbalanced by the large number of new species with apparently narrow distribution ranges discovered in recent, detailed inventories (Kalb 2009, Rivas Plata and Lücking 2012, Rivas Plata et al. 2012b, Sipman et al. 2012). It also appears that some of the pantropical species, such as *Glaucotrema glaucophaenum*, *Platygramme caesiopruinosa*, *Thelotrema diplotrema*, and *T. pachysporum*, exhibit a highly structured phylogeny, suggesting that these might represent species complexes with separate, (semi-)cryptic species occurring in different geographic regions. Therefore, it appears that the proportion of species with narrow distribution ranges in Graphidaceae, and in lichens in general, is much higher than previously assumed.

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Appendix 1

GenBank numbers and voucher information for specimens and sequences used in this study, sorted alphabetically by taxon, country, and collector. (doi: 10.3897/mycokeys.6.3482.app1) Microsoft Word Document (doc).

Citation: Rivas Plata E, Parnmen S, Staiger B, Mangold A, Frisch A, Weerakoon G, Hernández M JE, Cáceres MES, Kalb K, Sipman HJM, Common RS, Nelsen MP, Lücking R, Lumbsch HT (2012) A molecular phylogeny of Graphidaceae (Ascomycota, Lecanoromycetes, Ostropales) including 428 species. MycoKeys 6: 55–94. doi: 10.3897/mycokeys.6.3482.app1

Appendix 2

Full, best-scoring maximum-likelihood tree of the combined 3-gene dataset of all OTUs, showing detailed bootstrap support values. Genus-level clades are highlighted in blue and orange and unresolved or orphaned species-level clades are in black. (doi: 10.3897/mycokeys.6.3482.app2) File format: Adobe Portable Document Format (pdf).

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REVIEW ARTICLE

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Carbon-Water-Nitrogen relationships between lichens and the atmosphere: Tools to understand metabolism and ecosystem change

Cristina Máguas[1], Pedro Pinho[1], Cristina Branquinho[1], Britta Hartard[1,2], Michael Lakatos[2]

1 *FCUL, Centre for Environmental Biology, University of Lisbon, Campo Grande, 1749-016, Lisbon, Portugal*
2 *Dept. of Ecology, University of Kaiserslautern, P.O. Box 3049, 67653 Kaiserslautern, Germany*

Corresponding author: *Cristina Máguas* (cmhanson@fc.ul.pt)

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Abstract

Due to the close linking between the biosphere and atmosphere, there are clear impacts of changes in climate, atmospheric deposition of nutrients/pollutants and land use (Global Changes) on the terrestrial biosphere. Lichens, with a direct dependence on atmospheric conditions, are much more affected by their immediate microclimate than by the ecosystem's prevailing macroclimate. In contrast to higher plants, poikilohydric organisms have different mechanisms of water and CO_2 exchange. The application of stable isotopes to the understanding of the mechanisms that are fundamental to lichen gas exchange and water uptake is a promising tool for the evaluation of lichen response to environmental changes. Indeed, lichens have been shown to be influenced by a large number of natural and anthropogenic environmental factors, serving as ecological indicators. Thus, we may use these organisms to model the impact of key global change drivers, such as nitrogen deposition and biodiversity changes, at local scale. Particularly useful is the application of the Lichen Diversity Value (LDV) in order to evaluate the impact of global drivers. Moreover, it has been shown that these indices, associated with main photobiont types, green-algae (LDVch) or cyanobacteria (LDVcyh), and/or nitrophilous versus oligotrophic species, were good candidates as ecological indicators. Besides mapping with high spatial resolution the effects of climate alterations, lichen functional groups could also be used as an early-warning system in order to detect the first effects of climate change in ecosystems before sudden shifts occur on other components that may be less sensitive. Clearly, lichens possess the adequate traits to be used as powerful indicators of complex interactions between atmosphere and biosphere, and thus can generate potentially interesting models for global change drivers.

Key words
Climate change, Ecology, Photobionts, Physiology

Why do we need to understand biosphere-atmosphere interactions?

The biosphere has a significant impact on the composition of the atmosphere, *and the interaction between biosphere and atmosphere affects all living organisms, including humans.* Changes in climate, atmospheric deposition of nutrients/pollutants and land use (Global Changes) have observable impacts on the terrestrial biosphere (i.e. IPCC 2007; Rockstrom et al. 2009; Scheffer et al. 2009). It is relevant to quantify global change effects on ecosystem C/N stocks and exchange processes between the terrestrial biosphere (Canadell et al. 2000), the atmosphere and hydrosphere with a specific focus on C and N trace gases. In the near future it is crucial to understand the complex feedback mechanisms between the biosphere (e.g. biodiversity patterns) and atmosphere, under changing environmental conditions (IGBP 2007; Steffen et al. 2004).

Given the close linking between the biosphere and atmosphere, the exchange of carbon (CO_2) and water vapor between biosphere and atmosphere, and the deposition of nutrients and heavy metals to the plant or ground surface are very important areas of research. Research in this area involves measurements of the exchange of gases (i.e. CO_2 and water vapor) using eddy covariance (and other) flux techniques, spectroscopic and tracer release methods (for trace gases such as N_2O) , as well as satellite data and modeling approaches (i.e. Canadell et al. 2000). Thus, studying water and carbon fluxes can generally contribute to a better understanding of ecosystem functioning and biodiversity response to global change. Stable isotopes are currently used to investigate biosphere/atmosphere exchange processes and mechanisms at different spatial (from plant to ecosystem) and temporal (from short-to long-term responses) scales (Bowling et al. 2008; Bruggemann et al. 2011; Dawson et al. 2002; Unger et al. 2010, 2012).

Approximately 8% of the earth's land surface is covered by vegetation types dominated by lichens (Büdel et al. 2000), especially in environments with limited nutrition or water supply (Lange et al. 1992; Belnap et al. 2001). Any poikilohydric ground cover mediates the exchange of evaporative water between the pedosphere and atmosphere. Lichens potentially use a wide range of water sources such as soil water, precipitation, dew, fog or, in the case of some mosses and green algal lichens, even water vapor (e.g. Lange et al. 1988). Hence, these organisms are much more affected by their immediate microclimate than by the ecosystem's prevailing macroclimate. In contrast to higher plants, poikilohydric organisms have different mechanisms of water and CO_2 exchange. Thus, studying lichens as a globally important ground cover component and a physiological model of poikilohydric organisms may provide the scientific community with new insights of pedosphere-atmosphere exchange processes.

The importance of "*fingerprinting*" to study biogenic fluxes between lichens and the atmosphere

All environments with a scarcity of nutrients or water supply are dominated by poikilohydric cryptogams such as bryophytes (mosses and liverworts) and lichens. Over 33% of the Earth's surface is covered by semiarid and arid lands dominated by biological soil crusts composed mainly of cyanobacteria and lichens (Belnap et al. 2001). Even in forest ecosystems, lichens can sometimes constitute up to half of the above ground biomass. Accordingly, in these ecosystems poikilohydric organisms have a large effect on water and CO_2 fluxes. Poikilohydry determines the physiology and ecology of cryptogams, the water status of which is completely dependent on the environment, reaching equilibrium with the atmosphere (e.g. Green and Lange 1994). Moreover, there is increasing evidence that morphology and structure of the thallus of these organisms may be very important in determining CO_2 uptake rates and gas exchange in general (Máguas and Brugnoli 1996; Máguas et al. 1997; Lakatos et al. 2007; Hartard et al. 2008; Larsson et al. 2012).

The use of stable isotopes is a powerful research tool in environmental sciences. Its combination with: i) concentration measurements (providing "Keeling-type" plots; Keeling 1960) and ii) flux measurements, allow separation of net CO_2 exchange into photosynthetic and soil respiration components, and the evapotranspiration flux into soil evaporation and leaf transpiration (Yakir and Wang 1996). These and similar approaches help to define various fluxes in phanerogam dominated-ecosystems (Yakir and Sternberg 2000), and we may also use these techniques to investigate the origin of biogenic fluxes through a "molecular fingerprinting" approach. Carbon and oxygen isotopic composition are of major importance in evaluating physiological processes driving CO_2 and water exchange because they provide information on fractionation processes as well as characterize plant metabolism and interactions with the ecosystem (i.e. Dawson et al. 2002).

Due to their poikilohydric nature and the lack of stomatal control, lichens do not show the typical stable isotope compositions ($d^{13}C$) of higher plants. The $d^{13}C$ value of their organic matter (OM) vary between -12 and -23 ‰, and such a high $d^{13}C$ heterogeneity is thought to be due to discriminating factors such as the CO_2 diffusion resistance and CO_2 source, further than photosynthetic RuBisCO (Ribulose-1,5-bisphosphate carboxylase oxygenase) fractionation (Máguas and Brugnoli 1996; Máguas et al. 1997; Lakatos et al. 2007). Indeed, $d^{13}C$ values of lichens result from a combination of species-specific differences in resistances to inward CO_2 fluxes (Máguas and Brugnoli 1996; Máguas et al. 1997), CO2 source signature from the substratum (e.g. soil and bark) (Lakatos et al. 2007) and the CO_2-fixation mechanism of their photobiont (Máguas et al. 1993, 1995; Smith and Griffiths 1996, 1998; Smith et al. 1998) (Fig. 1). Recent studies confirm that two main categories of lichen are identifiable (Lakatos et al. 2007), depending on the existence or absence of a CCM (CO_2-concentrating mechanism) in the primary photobiont, producing a significant

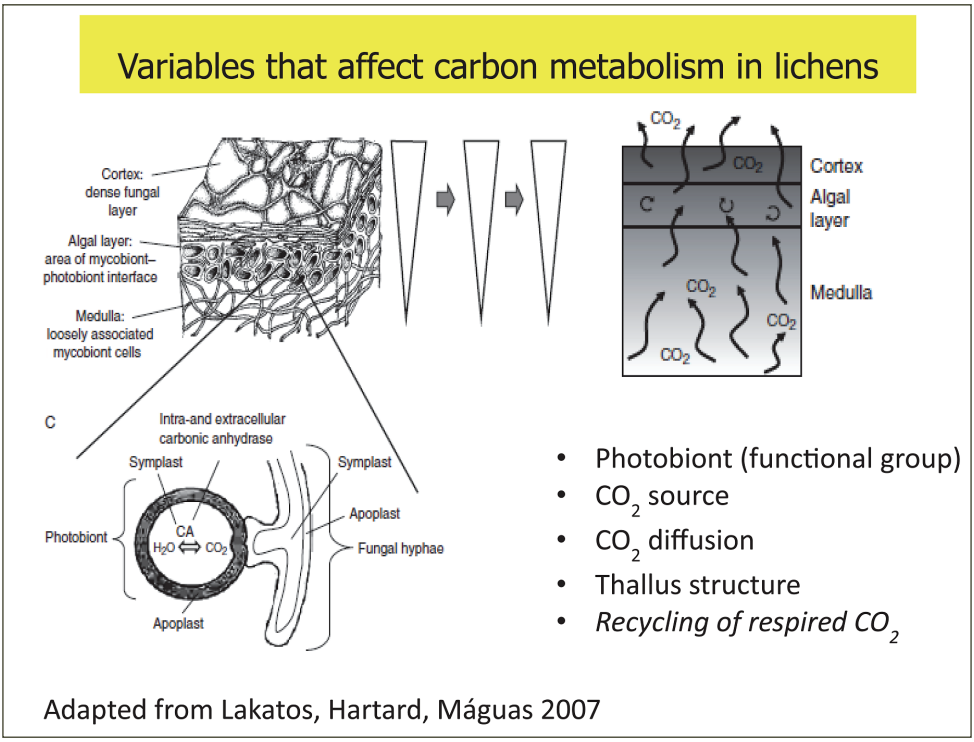

Figure 1. Schematic illustration of a cross-section through a lichen thallus with distinctive cortex, algal layer and medulla and the main factors contributing to thallus CO_2 exchange and the consequence for carbon $d^{13}C$ fractionation that affects organic matter in lichens.

difference of on average 10 ‰ on $d^{13}C$ values between the two groups (Fig.1). Cyanobacterial or green algal photobionts with CO_2-concentrating mechanisms increase the internal carbon pool near the carboxylation site of RuBisCO (Palmqvist et al. 1994a, b). As a consequence of the increased substrate availability, the rate of carboxylation is increased while that of photorespiration is decreased. Moreover, the CCMs tempers the effect of CO_2 diffusion within the thallus, e.g. at high water contents or super saturation (Máguas et al. 1997). Thus, $d^{13}C$ values of CCMs-containing lichens vary between -16 ‰ and -26 ‰ with a mean around -22‰. In tripartite lichens with two photobionts, (a primary green algal photobiont without a CCM and a secondary cyanobacterial photobiont in specialized thallus structures called cephalodia) the organic $\delta^{13}C$ values are determined by the green algal partner (Máguas et al. 1995; Green et al. 2002), since the main function of the cyanobionts is N fixation. Thus, OM of these tripartite lichens is characterized by $d^{13}C$ similar to C3-plants (mean of 18 species -32 ± 1.6 ‰; n = 43) since their green algae, such as *Myrmecia, Dictyochloropsis* (Smith and Griffiths 1996) and *Coccomyxa* (Palmqvist et al. 1994b), lack both pyrenoids and CCMs. Similar values can be found in lichens with only one photobiont: e.g. the CCM-lacking green algal genus *Trentepohlia* (Lakatos et al. 2006), which is the pre-

dominant photobiont in the tropics and subtropics (Sipman and Harris 1986). Thus, the presence or absence of a CCM is the principal explanation for the difference in $d^{13}C$ values between two different functional groups.

Over the surface of a single thallus CO_2 diffusion resistances can be modified by morphological structures such as the conglutinated cortical layers, thallus thickness and density and concomitant structural changes during water absorption. Hence, thinner thallus regions such as margins or tips, which are often loosely constructed and displaying lower CO_2 diffusion resistances, should also lead to lower $d^{13}C$ discrimination (depleted $d^{13}C$). However, the observed high heterogeneity of $d^{13}C$ of margins *versus* center parts within different growth forms and photobiont groups (from 0.25 to 2.5 ‰) does not give a clear indication of the expected discrimination factor associated with morphology and thallus structure, and no correlation with respect to growth form has been assessed (Lakatos et al. 2007).

Besides the major photosynthetic fractionations due to the transport and fixation of CO_2, one crucial factor influencing $\delta^{13}C$ is the origin of the carbon source used by lichens in a specific microenvironment. Several factors may contribute to this: i) depending on where they are located lichens can be attached to a substratum where the most direct CO_2 source is not atmospheric CO_2; and ii) different lichen species or different individual thalli might fix respired CO_2 from different ecosystem components . For example, it is well established that in macrohabitats such as closed forests, the source of ambient CO_2 gradually changes with height from the forest floor to the canopy (the vertical CO_2 profile) (e.g. Sternberg 1989; Buchmann et al. 1997). Moreover, CO_2 derived from different respiring substrata (e.g. soil, tree bark, leaf) has a $d^{13}C$ signal that is more depleted than that of ambient air.

As mentioned earlier, poikilohydric cryptogams can have a major influence on water and CO_2 fluxes in an ecosystem but their mechanisms of water exchange differ to those of higher plants. Their water status, for example, varies passively with surrounding environmental conditions, and they have neither a continuous influx of water nor stomata to control water deficit. Hence, during evaporation no isotopic steady state can be achieved and the $d^{18}O$ composition of both the thallus water and the evaporated water is expected to show progressive enrichment similar to the Rayleigh distillation process. As the water also transduces its oxygen isotopic signal to CO_2 *via* hydration of dissolved CO_2 (e.g. Amundson et al. 1998; Tans 1998; Stern, et al. 1999), respired CO_2 should also reflect the oxygen isotopic composition of the thallus water. Thus, the isotopic composition of the thallus water of lichens growing in their natural habitat appears to depend mainly on two separate factors: the isotopic signal of the predominantly available water source and the water potential difference between the thallus and the surrounding air (Hartard et al. 2008, 2009). From this, it follows that $d^{18}O$ values of lichens which are already in 'steady state', i.e. in continuous equilibrium with its surroundings, reflect the isotopic signal of the atmospheric vapor. In contrast lichens close to physical equilibration with their surroundings will approach isotopic equilibration with the surrounding isotopically lighter water vapor.

As a model organism, the globally distributed lichen *Cladina arbuscula* was studied under laboratory conditions as well as in the field. During a desiccation experiment, $\delta^{18}O$ values of thallus water and respired CO_2 became enriched by ~ 7‰ and followed an enrichment pattern similar to that of higher plants. However, the observed degree of enrichment was lower in comparison to higher plants due to (i) the lichen's inherent lower evaporative resistances and (ii) a stronger effect of the more depleted surrounding water vapor (Hartard et al. 2008, 2009).In the same species, when growing in its natural habitat, $\delta^{18}O$ values of thallus water principally proved to be highly depleted and strongly depended on the absorption of water vapor. Moreover, the results indicated that lichen cushions substantially reduce soil evaporation rates which may enhance their distinctive isotopic contribution to ecosystem water fluxes (Hartard et al. 2008, 2009). Therefore, the data indicate a strong influence of poikilohydric ground cover on soil evaporative fluxes, especially during drier periods without rain. During these periods, lichens predominantly utilize the more depleted air moisture and, hence, also evaporate more depleted water vapor into the atmosphere.

The use of lichens as ecological-indicators of biogenic fluxes between biosphere and the atmosphere

Lichens, which rely largely on the atmosphere for water and nutrient supply, can be used as suitable indicators of environmental changes in terrestrial ecosystems (Will-Wolf et al. 2006, Bergamini et al. 2005). However there are several concerns, such as to the type of measures that can be made with lichen communities and the spatial-temporal scales that may be studied. Recently, a standardized methodology has been established for collecting lichen diversity and abundance data in the field; this is the European Method (Asta et al. 2002). This methodology is based on a 50x10cm sampling grid, divided into 5 squares, that is placed on the four main aspects of tree trunks (Fig. 2). All lichen species occurring in the grid are identified and the number of squares occupied by each species that occurs on each tree is recorded as its frequency. From these data one can calculate two main variables: total species richness and total Lichen Diversity Value (LDV) (Fig. 2). The LDV is the sum of the frequency of all species on each tree, divided by the number of trees sampled. Geostatistical models are used to predict the spatial structure of the data, and ultimately interpolate the variable values in ensample locations using kriging (Fortin and Dale 2005). This process is based on variography analysis (Mitchell et al. 2000).

An alternative to total diversity is to use of functional traits, such as lichen photobiont type, eutrophication and water stress tolerance, which is strongly involved in species responses to several environmental factors. Indeed, in a study conducted in a Mediterranean *Quercus faginea* subsp *broteroi* forest, the lichen functional diversity was highly significantly related to potential solar radiation, an integrated measure of long-term microclimate. The green-algal lichens were positively related to potential solar radiation whereas cyanolichens were negatively related (Pinho et al. 2010). It this study it has been proved that lichen communities can be used to: i) model long-term microclimatic conditions with high spatial resolution and ii) model the disturbance caused

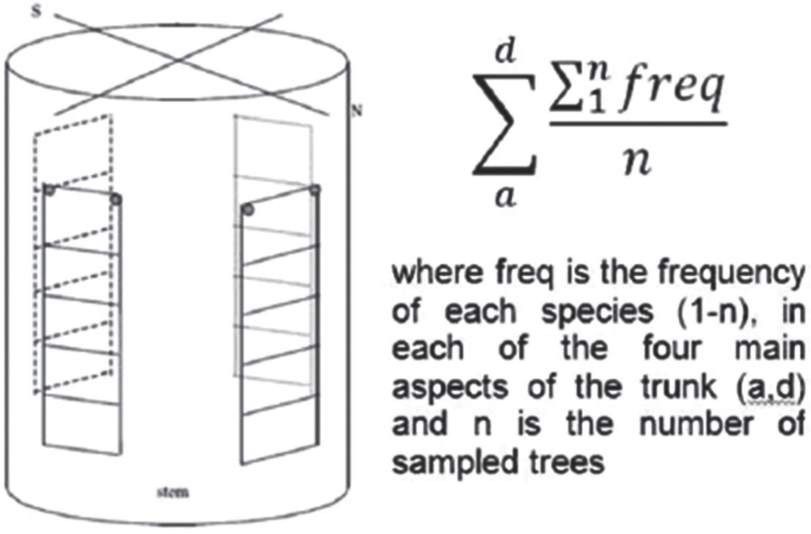

Figure 2. Distribution of the sampling grid in a tree trunk, original picture (Asta et al. 2002)

by neighborhood areas, even in natural parks with low-intensity human activities, for conservation purposes (Pinho et al. 2011, 2012a, b).

Lichens can also be classified according to their preferences or tolerance to eutrophication (Nimis and Martellos 2008). Several authors have pointed out that nitrogen from agricultural origin is playing an increasingly important role in changing lichen communities (Hultengren et al. 2004; Purvis et al. 2003). The results observed in an ecosystem oak woodland in southern Portugal were in agreement with the suggestion that the alteration induced by humans in the N cycle is among the most important drivers associated with global changes (Rockstrom et al. 2009). In this work, the dispersion and the distance of influence of eutrophication/NH_3 in lichens was found to be of short-range. However, the areas affected by it were found to be widespread in the territory, and always associated to agricultural sources (Pinho et al. 2011, 2012b).

Conclusions

Since lichens are directly influenced by microclimatic conditions, such as light, water, temperature and CO_2 concentration, the isotopic composition of their OM integrates environmental factors acting on their specific microhabitat over a range of weather conditions, as well as a variety of land-uses over long periods of time. The isotopic composition of OM is determined by an economic equilibration between carbon source and sink, which are mainly photosynthesis and respiration. Although the mycobiont dominates the OM pool, the carbon acquisition of the lichen depends

on the water content, light intensity and CO_2 fixation of the photobiont. Carbon isotope discrimination processes of ^{13}C can thus be related to CO_2 acquisition modes, CO_2 diffusion and CO_2 sources. To summarize, in several microhabitats, respired $d^{13}C$-depleted CO_2 serves as the carbon source for photosynthesizing lichens, thus biasing their characteristic isotopic signatures, which otherwise are determined by physiological processes. Thus, lichens can be used as tracers to point out the prevailing CO_2-sources in microhabitats. Therefore lichen OM will also indicate, especially in the younger thallus parts, an alteration in ambient ^{13}C-CO_2 caused by changes of urban-rural and land-use boundaries.

Moreover, the well-identified poikilohydric natures of these organisms make them sensitive tracers of water vapor fluxes. Indeed, application of the stable oxygen isotope ratio ($\delta^{18}O$) to gain insights into the yet unknown fractionation processes of terrestrial poikilohydric organisms showed that thallus water isotopic composition is additionally influenced by the prevailing environmental conditions to which they are exposed to. Thus, we may use these results to assess the effect of a substantial lichen ground cover on water exchange processes between the soil and atmosphere. Numerous nutrient poor habitats in extreme climates are dominated by poikilohydric organisms. Hence, globally, the effects of these organisms on overall water fluxes may even be more remarkable.

Lichen communities can thus be used to study important interactions between atmosphere and biosphere. Accordingly, the concomitant application of geostatistical models and *lichen functional diversity* (*green algal and cyanobacterial LDV indexes*) are a suitable way to use lichen communities as good indicators of complex interactions between atmospheric nutrients deposition (i.e. atmospheric NH_3) and the biosphere, as well as microclimatic changes due to forestry and land-use practices.

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