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Multiple origins of high reciprocal symbiotic specificity at an intercontinental spatial scale among gelatinous lichens (Collemataceae, Lecanoromycetes)

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ABSTRACT

Because the number of fungal species (mycobionts) exceeds the number of algae and cyanobacteria (photobionts) found in lichens by more than two orders of magnitude, reciprocal one-to-one specificity between one fungal species and one photobiont across their entire distribution is not expected in this symbiotic system, and has not previously been observed. The specificity of the cyanobacterium *Nostoc* found in lichens was evaluated at a broad geographical scale within one of the main families of lichen-forming fungi (Collemataceae) that associate exclusively with this photobiont. A phylogenetic study was conducted using *rbc*LXS sequences from *Nostoc* sampled from 79 thalli (representing 24 species within the Collemataceae), and 163 *Nostoc* sequences gathered from GenBank. Although most of the lichen-forming fungal species belonging to the Collemataceae exhibited the expected generalist pattern of association with multiple distinct lineages of *Nostoc*, five independent cases of one-to-one reciprocal specificity at the species level, including two that span intercontinental distributions, were discovered. Each of the five distinct monophyletic *Nostoc* groups, associated with these five highly specific mycobiont species, represent independent transitions from a generalist state during the evolution of both partners, which might be explained by transitions to asexual fungal reproduction, involving vertical photobiont transmission, and narrowing of ecological niches.

1. Introduction

Lichens are associations of a fungus (mycobiont) and at least one photoautotrophic symbiont (photobiont) resulting in a stable thallus of specific structure (Ahmadjian, 1993). One of the most challenging and controversial aspects of lichen biology has been the unveiling of the identity of the photobionts and the respective specificities of the partners (Beck et al., 2002; Rikkinen et al., 2002; Rikkinen, 2003). We refer to the term specificity as the number of partners that can be selected by one species (Joneson and Lutzoni, 2009). It is widely accepted that lichen mycobionts have a high degree of specificity towards photosynthetic partners (specialists), though rarely restricted to a single monophyletic haplotype group, whereas lichen photobionts have relatively lower specificity (generalists) (Beck et al., 2002; O'Brien, 2006). Concordantly, the mycobionts are believed to be more dependent on the photobiont than the reverse, because lichen mycobionts have never been found free-living (with one exception, see Wedin et al., 2004), whereas several photobiont lineages found in lichens (including *Nostoc*) have been reported to be free-living.

Specificity has been studied in several different groups of cyanolichens where the cyanobacterium of the genus *Nostoc* is the photosynthetic partner. *Nostoc* is frequently found free-living on various substrates, as well as in symbiotic associations with a diversity of hosts such as bryophytes, gymnosperms (cycads) and angiosperms (*Gunnera*) (Rai, 1990; Friedl and Büdel, 1996; Guevara et al., 2002). The degree of specificity of cyanolichen-forming fungi has been the subject of many studies, reporting mixed conclusions ranging from low to high fungal specificity for *Nostoc* haplotype groups (Paulsrud et al., 1998, 2000, 2001; Oksanen et al., 2002; Paulsrud and Lindblad, 2002; Rikkinen et al., 2002; Summerfield et al., 2002; Lohtander et al., 2003; Wirtz et al., 2003; O'Brien et al., 2005; Summerfield and Eaton-Rye, 2006; Myllys et al., 2007). Studies with an extensive sampling, including a large number of lichen species, have tended to find that most mycobiont species can associate with two or more different *Nostoc* haplotype groups (Wirtz et al., 2003; O'Brien et al., 2005; Summerfield and Eaton-Rye, 2006; Myllys et al., 2007; Elvebakk et al., 2008). However, even when specificity of the mycobiont has been reported as being high in some lichens

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species, reciprocally high specificity has not been reported for the associated photobiont.

It has also been suggested that photobiont specificity can be expressed at the community level, with different lineages of *Nostoc* being associated with different ecological guilds of mycobionts. Rikkinen et al. (2002) proposed two groups of fungal symbionts based on their photobiont selection: the "*Nephroma* guild", which mainly includes epiphytic lichens, and the "*Peltigera* guild", which mainly includes terricolous lichens. However, the sampling that led to this conclusion was also phylogenetically structured; where members of the "*Peltigera* guild" belonged to the *Peltigeraceae* (a family including primarily terricolous/muscicolous species), and members of the "*Nephroma* guild" belonged to a monophyletic group sister to the *Peltigeraceae* (Miadlikowska and Lutzoni, 2004), mostly the *Nephromataceae* (which includes primarily epiphytes), preventing the distinction between shared ecology and shared ancestry as the explanation for this observed pattern. Moreover, subsequent studies have found evidence for a gradual transition between guilds and some anomalies, where some *Nostoc* of epiphytic species are shared with terricolous species (Stenroos et al., 2006; Myllys et al., 2007; Elvebakk et al., 2008).

To better understand the specificity in cyanolichens, we studied the Collemataceae, a family of lichen-forming fungi associated exclusively with *Nostoc* species (Kirk et al., 2008), which includes both saxicolous/terricolous and epiphytic species. These lichens are distinguished by having a gelatinous thallus in which *Nostoc* cells are the most prominent component. Collemataceae includes approximately 250 species (Kirk et al., 2008), most having a wide distribution and very frequently colonizing soils, rocks and bark of trees, while others are rare species that can only be found in old-growth forests (Purvis and James, 1992; Aragón et al., 2005). It has been suggested that the abilities of these lichen-forming fungal species to grow in many different conditions are provided mainly by their photobiont (Adams, 2000).

The first goal of our study was to evaluate the degree of fungal specificity within this family across a broad spectrum of the geographical distribution of each species sampled, up to intercontinental scales when possible. Second, we wanted to determine if the observed variation in levels of specificity is correlated with ecological or phylogenetic structure, and mode of reproduction. To achieve these objectives, we used a molecular phylogenetic approach to investigate the genetic variability of *Nostoc* (based on their *rbc*LXS gene cluster [RuBisCo large and small subunits, and chaperone gene]) found in 79 Collemataceae thalli (representing 24 lichen-forming fungal species) collected in various geographic areas. The *rbc*LXS locus has been shown to provide an appropriate level of variation to study patterns of specificity in *Nostoc*, providing comparable results to multilocus datasets (O'Brien et al., 2005; Rajaniemi et al., 2005; Stenroos et al., 2006; Myllys et al., 2007). The 16S rDNA and $tRNA^{Leu}$ (intron) sequences are often used as markers for this same purpose, however, they are of limited usefulness as a single marker for studying specificity in cyanolichens. The 16S rDNA is highly conserved and often does not allow discrimination of bacterial strains at the subgeneric level (Fox et al., 1992; Ernst et al., 2003), while the $tRNA^{Leu}$ (intron) has a complex evolutionary history that may give misleading results about evolutionary relationships due to problematic DNA sequence alignments (Costa et al., 2002; Oksanen et al., 2004; Rikkinen, 2004; Stenroos et al., 2006).

2. Materials and methods

2.1. Taxon sampling

*Nostoc rbc*LXS sequences from 79 Collemataceae thalli (representing 24 lichen-forming fungal species) collected in Europe, North America and South America from different habitats were obtained. Details of the material, area of collection, location of voucher specimens and GenBank accession numbers are presented in Table 1. A total of 163 *rbc*LXS sequences of free-living and symbiotic *Nostoc* from GenBank were also added to the alignment. *Anabaena augstumalis*, *A. cylindrica*, *Fischerella muscicola* and *Trichormus variabilis* sequences from GenBank were used as outgroups based on earlier work by O'Brien et al. (2005) and Rajaniemi et al. (2005).

2.2. DNA extraction, amplification and sequencing

Total DNA was extracted from one or two different parts of the lichen thalli using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. For the 28 thalli that were sampled in two different parts, two DNA extracts were obtained. Dilutions (1:10, 1:30 and 1:50) of total DNA were used for PCR amplification. The *rbc*LXS partial region was amplified and sequenced by standard polymerase chain reaction (PCR) using the primer pair CW and CX (Rudi et al., 1998). The PCR amplification, PCR product purification, sequencing reactions and automated sequencing were performed according to the methodology of O'Brien et al. (2005).

2.3. Phylogenetic analyses

The nucleotide sequences were aligned with the program MAFFT v.5 (Katoh et al., 2005) and the alignments of the coding regions were manually adjusted with reference to the amino acid translations using MacClade 4.01 (Maddison and Maddison, 2001). Ambiguously aligned regions were excluded from phylogenetic analyses, including the entire spacer region between *rbc*L and *rbc*X, which varied in length from 18 to 372 bp. Seven data partitions were considered; each coding region (*rbc*L, *rbc*X) was partitioned by codon position and the spacer between *rbc*X and *rbc*S was treated as a single partition. Each partition was subjected to a specific step matrix using StMatrix 4.2 (Lutzoni and Zoller, Duke University, www.Lutzonilab.net/downloads/) and were analyzed simultaneously using weighted Maximum Parsimony as the optimization criterion with PAUP* 4.0b10 (Swofford, 2002). A heuristic search of 1000 random addition sequences (RAS) was conducted, with TBR branch-swapping, multrees option in effect and zero-length branches collapsed. Bootstrap analysis (Felsenstein, 1985) was used to estimate phylogenetic uncertainty on 1000 bootstrap data sets. Heuristic searches were performed on each bootstrap dataset as above, except that only two RAS per bootstrap replicate were used based on the high resolving power of the original data when 1000 RAS were implemented. Bayesian analysis was also performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Optimal models of nucleotide substitution were fit for each of the seven data partitions, using the Akaike Information Criterion (AIC) as implemented in MrModeltest 2.0 (Nylander, 2004). Markov chain Monte Carlo chain lengths for all analyses was 5×10^6 generations with trees sampled every 100 generations; the first 20,000 trees were discarded as "burn-in". For the remaining 30,000 trees, a majority rule consensus tree was assembled using the "sumt" option of MrBayes and posterior probabilities (PP) were calculated for each node. All analyses were run through the Bioportal web-based service platform for phylogenomic analysis at the University of Oslo (www.bioportal.uio.no).

3. Results

3.1. DNA sequencing

Only one *Nostoc* genotype was obtained from individual thalli, including the 28 that were sampled in two different parts and

Table 1
Voucher information and GenBank ascension numbers of *rbc*LXS DNA sequences for cyanobacterial strains of Collemataceae used in this study.

Source	Locality/Substrate/Herbarium voucher info	GenBank accession number
Collema auriforme 1	Spain, Jaén, saxicolous, MA-16249	EU877462
C. auriforme 2	Spain, Cantabria, saxicolous, MA-16250	EU877461
C. crispum 1	Spain, Cuenca, terricolous, MA-16251	EU877460
C. crispum 2[a]	Germany, Göttingen, terricolous, Duke-37900	DQ185273
C. fasciculare 1	Spain, Cuenca, epiphytic, MA-10297	EU877463
C. fasciculare 2	Spain, Toledo, epiphytic, MA-16252	EU877464
C. flaccidum 1[a]	Finland, Orimattila, epiphytic	DQ266040
C. flaccidum 2[a]	Finland, Artjärvi, epiphytic	DQ266039
C. flaccidum 3	Spain, Cantabria, epiphytic, MA-16253	EU877465
C. flaccidum 4	Spain, Cantabria, epiphytic, MA-16254	EU877466
C. flaccidum 5	USA, North Carolina, DUKE-38714	GQ184606
C. furfuraceum 1	USA, saxicolous, DUKE-38715	EU877472
C. furfuraceum 2	Portugal, Guarda, epiphytic, MA-16259	EU877471
C. furfuraceum 3	Spain, Jaén, saxicolous, MA-16257	EU877469
C. furfuraceum 4	Croatia, Peljesac, epiphytic, MA-16258	EU877470
C. nigrescens 1	Spain, La Rioja, epiphytic, MA-16261	EU877475
C. nigrescens 2	Spain, Jaén, epiphytic, MA-16262	EU877473
C. nigrescens 3	Spain, Guadalajara, epiphytic, MA-16263	EU877474
C. polycarpon 1	Spain, Albacete, saxicolous, MA-7428	EU877477
C. polycarpon 2	Spain, Cáceres, saxicolous, MA-16264	EU877476
C. subnigrescens 1	Croatia, Peljesac, epiphytic, MA-16265	EU877479
C. subnigrescens 2	Portugal, Redinha, epiphytic, MA-16266	EU877478
C. subnigrescens 3	Portugal, Marvao, epiphytic, MA-16267	EU877480
C. tenax 1	Spain, Cantabria, terricolous, MA-16268	EU877483
C. tenax 2	Spain, Cáceres, terricolous, MA-16269	EU877482
C. tenax 3	Spain, Madrid, terricolous, MA-12607	EU877481
C. undulatum 1	Spain, Vizcaya, saxicolous, MA-16255	EU877468
C. undulatum 2	Spain, Cantabria, saxicolous, MA-16256	EU877467
Leptogium austroamericanum 1	Argentina, Salta, epiphytic, MA-16271	EU877485
L. austroamericanum 2	Colombia, Magdalena, saxicolous, MA-16270	EU877484
L. azureum 1	Argentina, Salta, epiphytic, MA-16274	EU877488
L. azureum 2	Chile, Limarí, epiphytic, MA-16273	EU877487
L. azureum 3	Brazil, Rio de Janeiro, epiphytic, MA-16272	EU877486
L. brebissonii 1	Spain, Cáceres, epiphytic, MA-7654	EU877490
L. brebissonii 2	Spain, La Coruña, epiphytic, MA-16275	EU877489
L. corniculatum 1	Spain, Toledo, saxicolous, MA-16277	EU877491
L. corniculatum 2	Spain, Madrid, saxicolous, MA-16276	EU877492
L. corticola 1	Costa Rica, P. Arenas, epiphytic, DUKE-38726	EU877493
L. corticola 2	USA, North Carolina, epiphytic, MA-16278	EU877494
L. cyanescens 1	Spain, La Coruña, epiphytic, MA-16279	EU877497
L. cyanescens 2	USA, North Carolina, saxicolous, DUKE-39467	EU877496
L. cyanescens 3	Panamá, epiphytic, MA-14598	EU877495
L. furfuraceum 1	Croatia, Peljesac, epiphytic, MA-16284	EU877502
L. furfuraceum 2	Portugal, Marvao, epiphytic, MA-16285	EU877503
L. furfuraceum 3	Portugal, Guarda, epiphytic, MA-16286	EU877504
L. furfuraceum 4	Spain, Toledo, epiphytic, MA-16280	EU877498
L. furfuraceum 5	Spain, Albacete, epiphytic, MA-16281	EU877499
L. furfuraceum 6	Spain, Madrid, epiphytic, MA-16282	EU877500
L. furfuraceum 7	Spain, Jaén, epiphytic, MA-16283	EU877501
L. gelatinosum 1	Spain, Granada, epiphytic, MA-16021	EU877506
L. gelatinosum 2[a]	USA, Washington, DUKE-138054	DQ185289
L. gelatinosum 3	Spain, Madrid, saxicolous, MA-16022	EU877505
L. lichenoides 1	Sweden, Södermanland S-L24403	GQ184605
L. lichenoides 2	Spain, Albacete, epiphytic, MA-9429	EU877508
L. lichenoides 3	Spain, Cantabria, epiphytic, MA-16025	EU877507
L. lichenoides 4	Spain, Cantabria, epiphytic, MA-16023	EU877509
L. lichenoides 5	Portugal, Guarda, epiphytic, MA-16287	EU877510
L. lichenoides 6	Spain, Jaén, epiphytic, MA-16227	EU877511
L. magnussonii 1	Spain, Jaén, epiphytic, MA-16288	EU877514
L. magnussonii 2	Spain, Jaén, epiphytic, MA-16289	EU877515
L. magnussonii 3	Sweden, Södermanland, saxicolous, S-F52892	EU877512
L. magnussonii 4	Denmark, Bornholm, saxicolous, S-L33726	EU877513
L. magnussonii 5	Sweden, Södermanland, saxicolous, S l-27064	EU877517
L. magnussonii 6	Spain, La Rioja, epiphytic, MA-16290	EU877516
L. pseudofurfuraceum 1	Argentina, Salta, epiphytic, MA-16291	EU877521
L. pseudofurfuraceum 2	USA, Arizona, epiphytic, ASU-505643	EU877518
L. pseudofurfuraceum 3	USA, Arizona, epiphytic, ASU-515352	EU877520
L. pseudofurfuraceum 4	USA, Arizona, epiphytic, DUKE-43230	EU877519
L. pseudofurfuraceum 5	Argentina, Salta, epiphytic, MA-16293	EU877522
L. pulvinatum 1	Spain, Málaga, epiphytic, MA-16032	EU877524
L. pulvinatum 2	Spain, Cuenca, saxicolous, MA-16031	EU877523
L. saturninum 1[a]	Finland, Ristiina, epiphytic	DQ266038
L. saturninum 2[a]	Finland, Puumala, epiphytic	DQ266037
L. saturninum 3	France, Meres les vals, epiphytic, MA-16024	EU877526

(*continued on next page*)

Table 1 (*continued*)

Source	Locality/Substrate/Herbarium voucher info	GenBank accession number
L. saturninum 4	Spain, Albacete, epiphytic, MA-9431	EU877527
L. saturninum 5	USA, North Carolina, epiphytic, MA-16294	EU877528
L. saturninum 6	Canada, B. Columbia, epiphytic, DUKE-38736	EU877525
L. schraderi 1	Spain, Cuenca, saxicolous, MA-12861	EU877530
L. schraderi 2	Spain, Madrid, saxicolous, MA-16243	EU877529

[a] Sequences obtained from GenBank.

when each one of the three total DNA dilutions were used for PCR amplifications. These results indicated that a single cyanobacterial type was present in each thallus and that cyanobacteria external to lichen thalli (contamination) were not present at detectable levels. The sequences of *Nostoc* symbionts are denoted according to the lichen-forming host species from which they were obtained.

3.2. Phylogenetic analyses

The final *rbc*LX alignment consisted of 248 and 407 unambiguous nucleotide positions of *rbc*L and *rbc*X, respectively, of which 322 were variable and 289 parsimony informative. The resulting Bayesian phylogram (Fig. 1) contains 28 *Nostoc* sequences from specimens of *Collema* and 51 from *Leptogium* specimens. It also includes *Nostoc* symbionts from 127 specimens of other cyanolichens, nine *Nostoc* plant symbionts, two *Nostoc* symbionts of another taxonomically unrelated fungus (*Geosiphon pyriforme*, Glomeromycota) and 25 free-living *Nostoc* strains.

Most of the *Nostoc* included in this study formed two distinct clades (Clades I and II, Fig. 1). However, *Nostoc* sequences from specimens of *Stereocaulon exutum*, *S. fronduliferum* and *Protopannaria pezizoides* (GenBank sequences), together with a sequence of free-living *Nostoc*, form two clades outside Clades I and II (Fig. 1). The *Nostoc* clade I includes free-living *Nostoc* strains, whereas the *Nostoc* Clade II includes both free-living and symbiotic *Nostoc* strains, as reported in previous studies by O'Brien et al. (2005) and Svenning et al. (2005). Clade II comprises three well-supported subclades. Subclade 1 is formed by photobionts sequenced from two South American specimens representing two widely distributed *Leptogium* species (*L. azureum* and *L. cyanescens*), a North American specimen of *Peltigera didactyla*, a European specimen of *Fuscopannaria leucophaea* and five free-living *Nostoc* strains. Subclade 2 corresponds to the photobionts of *Leptogium lichenoides*, *Sticta hypochroa*, *S. gaudichaldia* and one specimen of *Peltigera malacea* and *Protopannaria pezizoides*. Subclade 3 includes *Nostoc* of terricolous/saxicolous, epiphytic and facultative epiphytic lichens of Collemataceae, and other lichen families, as well as nine plant symbionts (*Blasia pusilla*, *Cycas circinalis*, *Gunnera manicata* and *Stangeria paradoxa*) and *Geosiphon pyriforme*, along with free-living *Nostoc*. The phylogenetic relationships reported by Wirtz et al. (2003), O'Brien et al. (2005), Stenroos et al. (2006) and Myllys et al. (2007) are confirmed in this study, since closely related *Nostoc* strains found by them form well-supported clades within *Nostoc* Clade II.

Two different patterns of specificity are observed among Collemataceae species. The photobionts of most of the 24 lichen-forming fungal species sampled here can be found in multiple clades (Fig. 1) and/or in clades that include the photobionts of multiple mycobionts (generalists). However, there were five independent cases of one-to-one specificity where all sampled photobionts associated with a single fungal species form a monophyletic group exclusive of the photobionts of all other species examined: *Collema flaccidum*, *Leptogium furfuraceum*, *L. lichenoides*, *L. magnussonii* and *L. saturninum* (Fig. 1). The sampled distributions of two of these specialists (*C. flaccidum* and *L. saturninum*) span intercontinental scales (North America and Europe).

4. Discussion

Two different patterns of specificity are co-occurring between collemataceous fungi and *Nostoc* (Fig. 1). The first pattern is the most commonly reported situation for lichens (Rikkinen et al., 2002; O'Brien et al., 2005) where a monophyletic group of photobiont haplotypes is associated with multiple species of lichen-forming fungi, and where lichen-forming fungi associate with multiple photobiont haplotype groups, i.e., a reciprocal generalist pattern. This reciprocal generalism, which has been observed in other fungal symbioses such as green algae lichens, mycorrhizae and fungus-growing insects (Blaha et al., 2006; Cordeiro et al., 2005; Brachmann and Parniske, 2006; Guzow-Krzeminska, 2006; Piercey-Normore, 2006; Aanen et al., 2007; Porras-Alfaro and Bayman, 2007), applies to most lichens sampled for this study. However, a group of four *Leptogium* and one *Collema* species exhibit a second pattern of specificity where a monophyletic *Nostoc* haplotype group is associated with only one species of lichen-forming fungi, and this lichen-forming species is found only in association with this unique *Nostoc* group; representing a rare case of reciprocal (one-to-one) specificity by both symbiotic partners. Surprisingly, this extreme pattern of reciprocal specificity holds up even when specimens were sampled from different geographic regions at intercontinental scales (*Leptogium saturninum* and *Collema flaccidum*, Fig. 1 and Table 1). Two of the highly specific species, *C. flaccidum* and *L. saturninum*, were also found to be highly specific by Myllys et al. (2007), though our results clearly indicate that this pattern cannot be generalized to the entire Collemataceae as these authors suggested.

It has been suggested for lichens and other symbiotic relationships that reproductive strategies and ecological factors are the driving forces leading to symbiotic specificity (Pochon and Pawlowski, 2006; Yahr et al., 2006; Rikkinen and Virtanen, 2008; Schlick-Steiner et al., 2008; Elvebakk et al., 2008; Piercey-Normore, 2009). A strict co-dispersal of both symbionts (vertical transmission of the photobiont) is more likely to foster co-speciation while independent dispersal of partners (horizontal transmission of the photobiont) is expected to favour less specificity because relichenization at each generation is more likely to be successful if a broader range of photobiont strains is compatible and likely to be selected by the mycobiont (Beck et al., 2002; Romeike et al., 2002; Nelsen and Gargas, 2008). Conversely, the ability to associate with a diversity of photobionts may allow the colonization of a wide range of ecological conditions if the photobionts vary in their ecological requirements (Yahr et al., 2006; Nelsen and Gargas, 2008). It has been suggested that ecological variability has selected for low host specificity in Antarctic cyanolichens (Wirtz et al., 2003) and in other symbiotic associations, including coral–algal symbioses (Rowan and Knowlton, 1995; Pochon and Pawlowski, 2006), mycorrhizae (Porras-Alfaro and Bayman, 2007) and fungus-growing insects (Aanen et al., 2007; Schlick-Steiner et al., 2008).

The five lichen species exhibiting the unusually high level of reciprocal specificity in this study have in common a mostly asexual mode of reproduction where the vegetative propagules (isidia) contain both symbionts, allowing a vertical transmission of the photobiont from one fungal generation to the next. The sexual

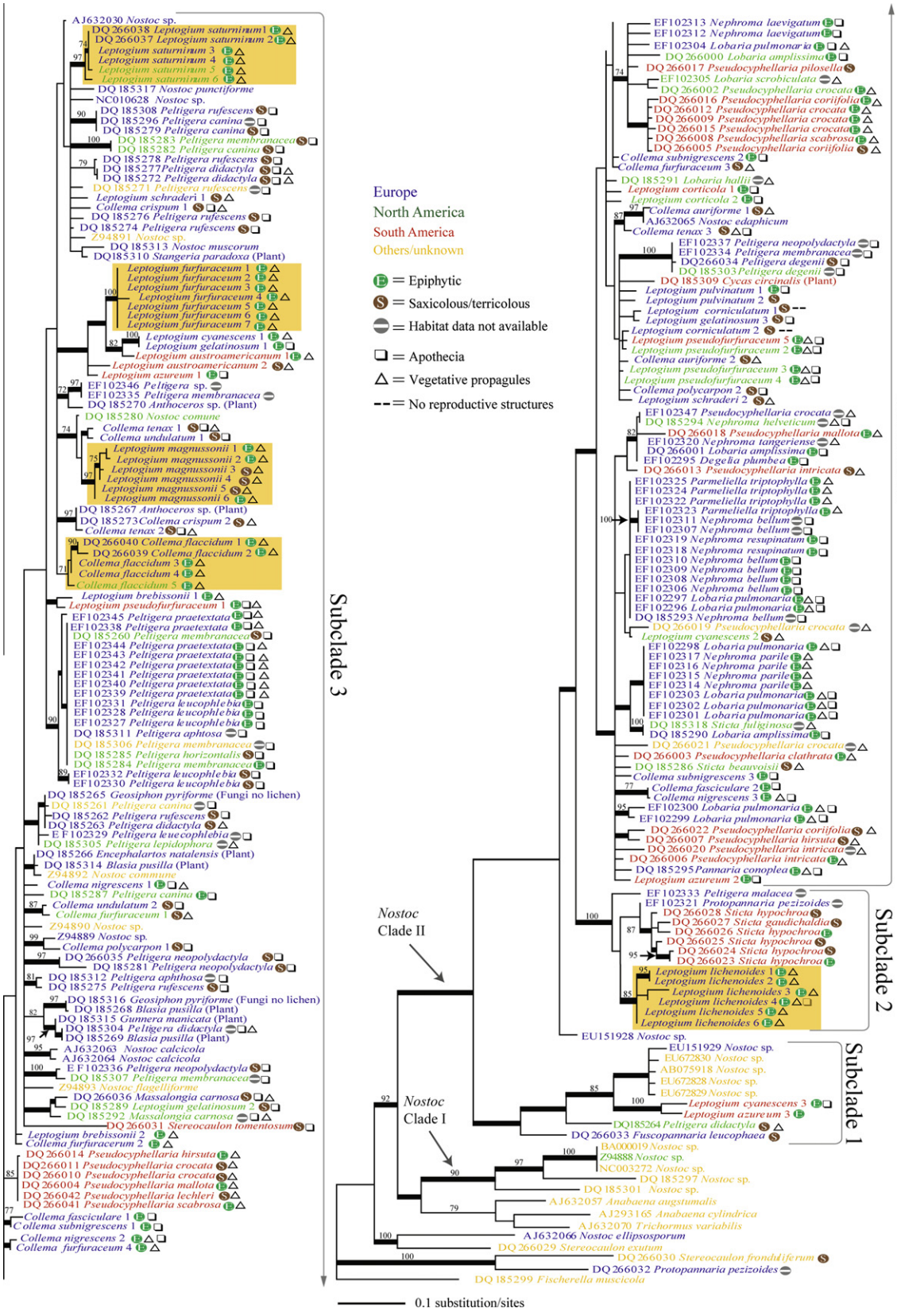

Fig. 1. Bayesian inference of phylogenetic relationships of the cyanobacterium *Nostoc* associated with lichen-forming fungi belonging to the family Collemataceae and allied symbiotic and free-living *Nostoc* spp. based on *rbc*LX sequences. Geographic provenances, substrates, and reproductive modes of the lichens from which *Nostoc* sequences were obtained are shown when applicable. Thickened branches represent posterior probabilities ⩾0.95 generated from the Bayesian analysis. Numbers above branches represent bootstrap support values (⩾70) derived from a wMP analysis. Names of the lichen-forming fungal host species, or plant host, are shown for *Nostoc* strains found in lichen thalli, or associated with plants. All other taxonomic names refer to free-living Cyanobacteria. Numbers preceding names indicate accession number of sequences obtained from GenBank. Monophyletic groups highlighted in orange represent five cases where one-to-one reciprocal specificity was detected so far.

reproductive structures (apothecia) of these five lichen-forming species, which impose horizontal transmission of the photobiont from generation to generation, are rarely observed. Although most of these species have wide distributions, they only occur in areas with high humidity, in association with very old trees (old-growth forests), and mainly in shady microhabitats (Purvis and James, 1992; Aragón et al., 2005; Otálora et al., 2008). Therefore, high co-specificity seems to be linked to vertical transmission of the photobiont and specialization to narrow environmental conditions.

Our results show that species that do not possess both traits do not exhibit this one-to-one pattern of specificity. Thus, species such as *C. auriforme*, *C. furfuraceum*, *C. crispum* and *L. cyanescens*, which are mainly dispersed by isidia, but are cosmopolitan, very frequent, and also occur in several ecological niches, are not specific in their choice of photobionts (Fig. 1). This suggests that symbiotic breakdown might be common after the dispersal of vegetative propagules and that partners having to reestablish their symbiotic association open opportunities for photobiont switching. In this context, these vegetative propagules can be seen not only as a dispersal mechanism for photobionts to be shared by various lichen-forming fungal species, but also as a dispersal mechanism of the mycobiont to associate with different photobionts. It is also possible that low-frequency sexual reproduction can effectively contribute to photobiont switching in cases of lichens with broad ecological spectra, contrary to lichens with very narrow and specific ecological requirements. Species considered in this study that usually produce apothecia (*Collema nigrescens*, *C. tenax*, *L. azureum*, *L. corticola*, *L. gelatinosum* and *L. pseudofurfuraceum*) show low degree of specificity even if they are growing in old-growth forests. Therefore, both favourable environmental conditions and vertical photobiont transmission may be necessary to maintain reciprocal specificity. This is consistent with the finding of low a specificity among sexual lichens from a wide range of habitats in the *Peltigeraceae*, *Nephromataceae* and *Lobariaceae* (O'Brien et al., 2005; Myllys et al., 2007).

Geographic patterns are not associated with the evolution of specificity, as was demonstrated previously (O'Brien et al., 2005; Stenroos et al., 2006; Elvebakk et al., 2008); lichens growing in different geographic regions can share symbiotic *Nostoc* from the same haplotype group. This fact is evident in the clade of *Nostoc* found in *L. saturninum* and *C. flaccidum* sampled in North America, as well as North and South of Europe (Fig. 1 and Table 1). Furthermore, *Nostoc* of diverse cyanolichens from both Hemispheres are grouped together within the sublcades 2 and 3 (Fig. 1) as demonstrated, for example, by the photobionts of European *Leptogium lichenoides*, *Peltigera malacea* and *Protopannaria pezizoides*, forming a monophyletic clade together with photobionts of *Sticta hypochroa* and *S. gaudichaldia* from Argentina, in subclade 2.

The high reciprocal specificity reported here is not likely to be a residual ancestral state, but rather the product of multiple independent events. First, the five cases of high reciprocal specificity occurred independently during the evolution of *Nostoc*. None of the one-to-one specificity cases belong to the same lineage within the *Nostoc* phylogeny (Fig. 1). Second, closely related lichen-forming fungal species such as *L. furfuraceum* and *L. pseudofurfuraceum* (Otálora et al., 2010) exhibit different patterns of specificity (Fig. 1). While *L. furfuraceum* exhibits the pattern of high reciprocal specificity, *L. pseudofurfuraceum* seems to be a generalist at least at an intercontinental scale (Fig. 1). Likewise, each one of the five highly specific lichen-forming fungal species is part of a distinct lineage, distributed across two of the four main monophyletic groups (groups A and C) within the Collemataceae phylogeny of Otálora et al. (2010).

Our results also include some evidence supporting the hypothesis of high reciprocal specificity being associated with specific lineages of *Nostoc*. For example, phylogroups within subclade 2 seem to be more prone to exhibit high reciprocal specificity. In addition to the one-to-one reciprocal specificity between *Leptogium lichenoides* and a *Nostoc* phylogroup belonging to subclade 2 of the *Nostoc* Clade II, *Sticta hypochroa*, *S. gaudichaldia*, *Protopannaria pezizoides* and *Peltigera malacea* are not associated with other *Nostoc* strains than their respective *Nostoc* phylogroup within subclade 2 (Fig. 1). This result opens the possibility that the evolution of certain lineages of *Nostoc* could require high co-specificity.

Consistent with previous findings (O'Brien et al., 2005; Stenroos et al., 2006; Elvebakk et al., 2008), our results suggest that substrate preferences do not structure photobiont range in Collemataceae lichens, as has been suggested for *Peltigera* and *Nephroma* (Rikkinen et al., 2002). Lichen photobionts colonizing different substrates are nested together in well-supported monophyletic groups within subclade 3, such as the "*L. magnussonii*" or "*Pseudocyphellaria* species" (*P. hirsuta*, *P. crocata*, *P. mallota*, *P. lechleri* and *P. scabrosa*) clades (Fig. 1), demonstrating that *Nostoc* strains belonging to the same haplotype group can be found in epiphytic, saxicolous and terricolous lichens.

Until now, photobionts have been found associated with many lichen-forming fungal species, in spite of the existence of some patterns of specificity at a very small spatial scale (Yahr et al., 2004; Ohmura et al., 2006; Stenroos et al., 2006; Myllys et al., 2007; Piercey-Normore, 2009). However, our study shows that two patterns are present in cyanolichens; the commonly reported generalist pattern and a rare reciprocal high co-specificity, where all examined specimens of a lichen species have been found to be associated with a single lineage of *Nostoc* throughout it's range, which has not been recovered from any other symbiotic association. Therefore, we suggest that the specificity among the gelatinous lichen-forming fungi and *Nostoc* seems to be governed by three simultaneous driving forces: (1) A low specialization of the symbiotic partners associated with sexual reproduction of the mycobiont (i.e., a single photobiont haplotype group is associated with many lichen-forming fungal species), (2) a rare high reciprocal co-specialization due to the dispersal of both symbionts through vegetative propagules associated with asexual reproduction of the mycobiont with very narrow ecological niches in old-growth forest communities or areas with high humidity, and (3) a rare high reciprocal specificity, that can be inherited by subsequent diverging/cospeciating lineages of *Nostoc*, and that is independent of the reproductive mode of the mycobiont (i.e., subclade 2, Clade II). These evolutionary processes leading to a dual pattern of rare high reciprocal co-specificity operating in concert with a more common low specificity among symbionts, might be also at work in symbiotic systems other than the Collemataceae, but was never detected.

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