Macroevolution of Specificity in Cyanolichens of the Genus Peltigera Section Polydactylon (Lecanoromycetes, Ascomycota)

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Abstract.—Patterns of specificity among symbiotic partners are key to a comprehensive understanding of the evolution of symbiotic systems. Specificity of mutualistic partners, within a widespread monophyletic group for which all species are sampled has rarely been explored. Here, we assess the level of specificity between the cosmopolitan lichen-forming fungus (mycobiont) from the genus Peltigera, section Polydactylon, and its cyanobacterial partner Nostoc (cyanobiont). The mycobiont and cyanobiont phylogenies are inferred from five nuclear loci and the rhlX region, respectively. These sequences were obtained from 206 lichen thalli, representing ca. 40 closely related Peltigera species sampled worldwide, doubling the number of known species in this group. We found a broad spectrum of specificity for both partners ranging from strict specialists to generalists. Overall, mycobionts are more specialized than cyanobionts by associating mostly with one or a few Nostoc phylogroups, whereas most cyanobionts associate frequently with several Peltigera species. Specialist mycobionts are older than generalists, supporting the hypothesis that specialization of mycobionts to one or few cyanobionts, is favored through time in geographic areas where species have been established for long periods of time. The relatively recent colonization of a new geographic area (Central and South America) by members of section Polydactylon is associated with a switch to a generalist pattern of association and an increased diversification rate by the fungal partner, suggesting that switches to generalism are rare events that are advantageous in new environments. We detected higher genetic diversity in generalist mycobionts. We also found that Peltigera species specialized on a single Nostoc phylogroup have narrower geographical distributions compared with generalist species. [Cyanobiont; lichen symbiosis; multilocus phylogeny; mutualistic interactions; mycobiont; photobiont; species delimitation.]
A broad spectrum of specificity has been reported for mutualistic symbioses, ranging from high reciprocal specificity wherein two species interact only with one another across their geographic ranges, to low reciprocal specificity wherein both species are generalists, each interacting with several partners, with intermediate cases of a specialist species forming a mutualistic association with a generalist species (Ollerton 2006; Otálora et al. 2010; O’Brien et al. 2013). Here, we use the term generalist to refer to a species interacting with a relatively high number of species globally, that is, throughout its geographic range. Generalism should favor the exploitation of different niches and reduce pressure from specific limited resources, whereas specialization potentially optimizes the benefits obtained from a specific partner (de Vienne et al. 2013). According to the theory of the geographic mosaic of coevolution (Thompson 2005), patterns of specificity may vary geographically as interactions between species often evolve in at least slightly different ways across their range. Ideally, the resulting patterns of long-term coevolutionary processes should be studied at a global geographic scale rather than being restricted to a few local populations representing only a small fraction of the geographic range of the interacting species (Thompson and Cunningham 2002; Thompson 2005).

The level of dependence on another species to develop and contribute substantially to the next generation (facultative, obligate, or specialized) is an intrinsic aspect of symbiotic interactions (Wolin 1985). For example, lichen-forming fungi (mycobionts) are usually obligate mutualists associated with one or several photosynthetic partners (Honegger 1998; Friedl and Büdel 2008). Cases where lichen-forming fungi occur in a free-living saprotrophic stage are extremely rare, for example, Stictis (Wedin et al. 2004), and are found in lineages where a loss of lichenization occurred, such as in the case of Stictidaceae within Lecanoromycetes (Lutzoni et al. 2001, see also Chen et al. 2015). Lichen photobionts appear less dependent on the mycobiont, as several are known to grow freely in nature (e.g., Trentepohlia and Nostoc spp.), and are often more easily isolated in vitro (and grow faster) than the fungal partner (Lutzoni and Miadlikowska 2009; McDonald et al. 2013). For example, Nostoc strains associated with Peltigera have been isolated in axenic cultures (Drew and Smith 1967), whereas this has never been achieved for Peltigera mycobionts. Lichen-forming fungi involved in symbioses with cyanobacteria (cyanolichens) have been the most recalcitrant to isolation in pure culture (McDonald et al. 2013).

The mode of transmission of symbionts from one generation to the next (vertically versus horizontally) can also have a major impact on evolutionary processes and specificity. In lichens both modes of transmission occur, either within a single species, or species can use mostly or solely one or the other mode of transmission. In general, horizontal transmission of the photobiont is the most common mode of transmission for lichens (e.g., Otálora et al. 2010; Dal Grande et al. 2012; Werth and Scheidegger 2012). Vertical transmission of the photobiont occurs through thallus fragments and specialized vegetative propagules (e.g., soredia, isidia, phyllidia) containing both the mycobiont and the photobiont. Horizontal transmission occurs when the mycobiont is reproducing sexually and resulting spores have to be sufficiently close to an appropriate photobiont to initiate the next generation of lichen thalli (Lutzoni and Miadlikowska 2009). Photobiont switches (a change of photosynthetic partner by the mycobiont) are common in lichen-forming fungi (Piercey-Normore and DePriest 2001; O’Brien et al. 2013; Magain and Sérusiaux 2014). They can cause changes in thallus morphology, enable colonization of new environments, and drive speciation (Fernández-Mendoza et al. 2011; Magain and Sérusiaux 2014).

Lichen mycobiont–photobiont patterns of associations are challenging to study partly because of unclear species delimitations among the interacting fungi, algae, and cyanobacteria. Lichen-forming fungi, like many other organisms with few morphological features (e.g., Medina et al. 2013), may include many cryptic species (e.g., Leavitt et al. 2011; Lumbsch and Leavitt 2011; Lücking et al. 2014), harbor morphological convergences (e.g., Magain and Sérusiaux 2012; Bendiksky and Timdal 2013; Otálora and Wedin 2013), or exhibit strong morphological plasticity reducing even more the availability of diagnostic traits (Pino-Bodas et al. 2011; Magain et al. 2012). Approximately 16,000 of all known fungal species are lichen-forming (Kirk et al. 2008). This number is likely to be an underestimate of the lichen species richness because a) many morphological lichen species occur across extensive geographic areas, and may in fact comprise several genetically isolated lineages, and b) various taxonomic groups remain understudied, and c) many areas of the world remain poorly explored in terms of their lichen flora (Lücking et al. 2014). For lichen photobionts the situation is worse due to their small unicellular or filamentous growth forms, compounded by phenotypic shifts between the symbiotic (in vivo) and cultured (in vitro) stages (Vandamme et al. 1996; Beltrami 2008; Flechtner et al. 2013; Fučíková et al. 2014). Moreover, as for prokaryotes in general, the evolutionary histories of cyanobacteria are often obscured by multiple horizontal gene transfers (Doolittle 1999; Oren 2004).

Associations between lichenized fungi and their cyanobacterial Nostoc partners have been the subjects of numerous studies. The current paradigm is that a single lichen thallus hosts a single strain (single genotype) of Nostoc (Paulsrud and Lindblad 1998), which usually has a wide geographic distribution, and can be found in association with a broad taxonomic range of mycobiont species across continents (Paulsrud et al. 2000). However, cases with more than one photobiont genotype occurring within an individual lichen thallus have also been reported (e.g., Casano et al. 2011). Nostoc strains associated with lichen-forming fungi are closely related to strains forming symbiotic associations with bryophytes and angiosperms, as well as to free-living strains (O’Brien et al. 2005). Based on
Here, we present the results of a macroevolutionary study of the lichen-forming genus *Peltigera* (Peltigerales, Lecanoromycetes) section *Polydactylon* and its *Nostoc* cyanobionts. This section (one of eight for the genus *Peltigera* [Miadlikowska and Lutzoni 2000]) is cosmopolitan and its members are especially abundant in boreal old growth and tropical mountain forests (Martínez et al. 2003). Currently, 19 species are recognized in section *Polydactylon* (e.g., Vitikainen 1994, 1998; Sérusiaux et al. 2009). Most of them reproduce sexually (i.e., apothecia are commonly observed) presumably requiring the reacquisition of a compatible *Nostoc* to establish the next generation of thalli (horizontal transmission of *Nostoc*). Specialized vegetative propagules (isidia, soredia, phyllidia) that enable a codispersal of both partners (vertical transmission of the photobiont) occur only in a few members of this section (e.g., *P. pacifica* Vitk.). However, all species may propagate via simple thallus fragmentation.

The circumscription of morphospecies of section *Polydactylon* has never been evaluated within a comprehensive phylogenetic framework. The extensive morphological variation reported for some species suggests that they may harbor multiple distinct species, which might not share a most recent common ancestor (Miadlikowska et al. 2003). Due to its cosmopolitan distribution at the section level, but more restricted and distinct distributions at the species level, its abundance in many parts of the world, and its association with a single type of photosynthetic symbiont (the cyanobacterium genus *Nostoc*), the monophyletic section *Polydactylon* is a good candidate for a worldwide study of the evolution of specificity.

The aims of this study were to: 1) confirm the delimitation of section *Polydactylon* and its phylogenetic placement within the genus *Peltigera* using multilocus data; 2) evaluate morphospecies within this section using a molecular phylogenetic approach (i.e., as evolving metapopulation lineages, sensu De Queiroz 1998), and species discovery methods based on multilocus data; 3) infer phylogenetic relationships among cyanobionts associated with members of section *Polydactylon* in a broad context of symbiotic and free-living *Nostoc* strains; and 4) explore the biogeographic patterns, specificity, and macroevolution of mycobiont–cyanobiont associations and the factors shaping these patterns.

**MATERIALS AND METHODS**

**Taxon Sampling**

Over 2000 specimens were loaned from herbaria (AMNH, B, BG, CGMS, CONN, DUKE, H, LG, MAF, MEXU, NSPM, NY, O, PTZ, QFA, UBC, UDBC, UGDA, UMEX, UPS) and various private collections, or collected during numerous field trips part of this study (Reunion Island in 2009; Norway, Canada: Quebec, USA: North Carolina and Alaska in 2011, Russia, Peru, and Brazil in 2012). Species from the seven remaining sections of the genus *Peltigera* (59 individuals representing sections Chloropeltigera, Peltidea, Horizontalae, Peltigera, Retjóveta, Phlebia, and Hydrothyrinae; Miadlikowska and Lutzoni 2000), as well as outgroup taxa from the order Peltigerales suborder Peltigereinae (five individuals; Miadlikowska and Lutzoni 2004), were also selected for this study (Supplementary Table S1, available on Dryad at http://dx.doi.org/10.5061/dryad.h6v7g).

**Molecular Data Acquisition**

We extracted DNA from approximately 950 representative, well-preserved, lichen specimens lacking any visible symptoms of fungal infection following two extraction protocols: Cubero et al. (1999) or modified Zolan and Pukkila (1986) using a 2% sodium dodecyl sulphate (SDS) as the extraction buffer. We amplified the internal transcribed spacer (ITS) of the nuclear ribosomal tandem repeat of the mycobiont from about 950 lichen thalli representing a broad geographic and morphological diversity of the group, using the ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) primers. A total of 233 of these specimens from section *Polydactylon* were included in our phylogenetic analyses (shown in Supplementary Table S1, available on Dryad). From these we selected 115 specimens, that each represented a unique ITS haplotype, or in some cases identical haplotypes collected from distant geographic regions (e.g., different continents). We amplified four additional loci, including the nuclear ribosomal large subunit (nLSU) using primers LR0R and LR7 (Vilgalys and Hester 1990), and three protein-coding genes: RNA polymerase II largest subunit (RPBI) using primers RPBI-AF (Stiller and Hall 1997) and RPBI- CR (Matheny et al. 2002), β-tubulin using the forward primer T1 (O’Donnell and Cigelnik 1997) or btl.34F (O’Brien et al. 2009) and the reverse primer BT2B (Glass and Donaldson 1995), and elongation factor 2 region 1 (EFT2.1) using primers EFT2.1_1F (Miadlikowska et al. 2014) and EFT2.1_3R (primer sequence: 5’-ATCCCTGTATACCAATGCAATGCC-3’). These four loci were also sequenced for 16 specimens from the remaining seven sections of *Peltigera*, as well as four representatives of closely related genera (outgroup) from the suborder Peltigineae. We included 34 additional specimens from other sections of *Peltigera* and one additional outgroup specimen for which sequences were available in GenBank (Supplementary Table S1, available on Dryad).

We selected a set of 208 specimens from section *Polydactylon* (including 206 for which we sequenced the ITS, and most of the 115 individuals for which multiple loci were sampled), to sequence the rbcL region (i.e., the last 82 amino acids of the RUBISCO large subunit [rbcL],
a putative chaperone gene \[\text{rbc}X\] and two intergenic spacers; Li and Tabita (1997) of their cyanobiont Nostoc using the CX and CW primers (Rudi et al. 1998). We also sequenced the rbcLX region for 26 specimens from other sections of the genus Peltigera. To this rbcLX dataset we added 9 rbcLX sequences available in GenBank for section Polydactylon, 80 sequences from cyanobionts of other sections of the genus Peltigera. We also added 203 GenBank sequences of rbcLX representing Nostoc associated with other genera of lichenized fungi or other organisms, Nostoc that are free-living, and three other closely related cyanobacterial genera (Supplementary Table S1, available on Dryad). This study included a total of 526 rbcLX sequences of cyanobacteria.

PCR conditions are provided in Madihlkovska et al. (2014) and literature cited therein. All PCR products were cleaned with ExoSAP (Affymetrix Inc., CA, USA) following the manufacturer’s protocol. Sequencing was carried out in 10 μL reactions using: 1 μL of primer (10 μM), 1 μL of purified PCR product, 0.75 μL of Big Dye (Big Dye Terminator Cycle sequencing kit, ABIPRISM version 3.1, Perkin–Elmer, Applied Bio-systems, Foster City, CA), 3.25 μL of Big Dye buffer, and 4 μL of double-distilled water. Automated reaction clean up and visualization was performed at the Duke Genome Sequencing and Analysis Core Facility of the Institute for Genome Sciences and Policies (for details see Gaya et al. 2012).

**Single Locus and Concatenated Datasets**

Sequences were edited using Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, MI) and subjected to BLAST searches (Wheeler et al. 2007) to confirm the fungal or cyanobacterial origin of each sequence fragment. Sequences were aligned manually using MacClade version 4.08 (Maddison and Maddison 2005). Ambiguously aligned regions (sensu Lutzoni et al. 2000) were delimited manually and excluded from phylogenetic analyses. Prior to data concatenation, single-locus phylogenies were generated for all five fungal loci (ITS, LSU, RPB1, \(\beta\)-tubulin, and EFT2.1) using RAxML-HPC2 version 7.2.8 (Stamatakis 2006; Stamatakis et al. 2008) as implemented on the CIPRES portal (Miller et al. 2010). Optimal tree and bootstrap searches were conducted with the rapid hill-climbing algorithm for 1000 replicates with the GTRGAMMA substitution model (Rodriguez et al. 1990). Protein-coding genes were partitioned following their codon positions and introns, whereas a partition with two subsets was defined for the ITS (5.8S and ITS1+ITS2). To detect topological incongruence among single-locus datasets, a reciprocal 70% ML bootstrap support criterion was implemented (Reeb et al. 2004; Mason-Cramer and Kellogg 1996). No significant conflict was detected among the single locus datasets, except for the placement of: 1) the \(P. \text{scabrosa} / P. \text{sp. 7 group (EFT2.1 versus all other loci), 2) P. \text{neopolypodium} 5 and P. \text{scabrosa} 3 (EFT2.1 versus ITS), and 3) P. \text{neopolypodium} 3 (ITS versus LSU and RPB1) (see the "Results" section). Because conflicting relationships did not involve major topological rearrangements, we combined the single locus datasets into three concatenated fungal matrices: Matrix 1, consisting of four loci (LSU, RPB1, EFT2.1, and \(\beta\)-tubulin) for 106 representatives (42 from section Polydactylon, 59 from the remaining sections of Peltigera, and 5 outgroup species), which was used to confirm the monophyly and placement of section Polydactylon within Peltigera (Table 1, Figs. 1 and 2); Matrix 2, consisting of five loci (ITS, LSU, RPB1, EFT2.1, and \(\beta\)-tubulin) for 119 representatives of Peltigera section Polydactylon (loci sequenced as part of this study for 115 specimens, and from Madihlkowska et al. [2014] for the remaining four); and Matrix 3, consisting of Matrix 2 with the addition of recoded characters derived from ambiguous regions of the ITS, LSU, and selected introns of three protein-coding genes, using PICS-ORD (Lücking et al. 2011). The latter computes pairwise distances as sequence identities or cost scores, ordinates the resulting distance matrix by means of Principal Coordinates Analysis, and encodes the principal coordinates as ordered integers for each delimited ambiguous region (Table 1 and Fig. 1).

Four additional single-locus matrices were generated: Matrix 4, consisting of 526 rbcLX sequences of cyanobacteria, which were collapsed down to 417 sequences using a 100% similarity criterion in Sequencher; Matrix 5, consisting of ITS sequences from 206 representatives of Peltigera section Polydactylon for which we sequenced the rbcLX region of the co-living Nostoc; Matrix 6, containing a subset of Matrix 4 restricted to the symbionts from section Polydactylon, consisting of 209 rbcLX sequences (206 from Peltigera section Polydactylon and three outgroup sequences); and Matrix 7, containing one representative of all ITS haplotypes detected in section Polydactylon (Table 1, Fig. 1, Supplementary Text B, available on Dryad). All alignments were deposited in TreeBase (19666). In Matrix 2 all five loci were available for 74 of the 119 individuals, whereas sequences from four, three, and two loci were available for 31, 12, and 2 individuals, respectively. For the rbcLX datasets (Matrices 4 and 6, Table 1) the two spacers were not alignable, and for this reason they were excluded from phylogenetic analyses.

### Phylogenetic Analyses

For maximum likelihood (ML) and Bayesian analyses on Matrices 1 and 2, data subsets were established using PartitionFinder (Lanfear et al. 2012). Thirteen initial subsets within Matrix 1 (LSU, \(\beta\)-tubulin 1st, 2nd, 3rd codon positions and introns, RPB1 1st, 2nd, 3rd codon positions and intron, EFT2.1 1st, 2nd, 3rd codon positions and intron, and 16 subsets within Matrix 2 (LSU, ITS1, ITS2, 5.8S, \(\beta\)-tubulin 1st, 2nd, 3rd codon positions and intron, RPB1 1st, 2nd, 3rd codon positions and intron, EFT2.1 1st, 2nd, 3rd codon positions and intron) were considered to estimate the optimal partitioning scheme for subsequent phylogenetic analyses. We used the greedy algorithm to explore the nucleotide
Table 1. Characterization of matrices used for phylogenetic analyses for both symbiotic partners

<table>
<thead>
<tr>
<th>Matrix number</th>
<th>Symbiont</th>
<th>Taxonomic breadth</th>
<th>Locus</th>
<th>Number of taxa</th>
<th>Number of char incl./ Total number of sites</th>
<th>Number of variable char.</th>
<th>Number of parsimony-inf. char.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mycobiont</td>
<td>Genus Peltigera</td>
<td>Concatenated: 4</td>
<td>106</td>
<td>3135 / 3276 (0.96)</td>
<td>975 (0.31)</td>
<td>778 (0.25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+ outgroup)</td>
<td>LSU</td>
<td>84 (0.80)</td>
<td>1218 / 1392 (0.88)</td>
<td>244 (0.20)</td>
<td>163 (0.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RPB1</td>
<td>98 (0.93)</td>
<td>638 / 716 (0.89)</td>
<td>276 (0.43)</td>
<td>235 (0.35)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EFT2.1</td>
<td>79 (0.74)</td>
<td>794 / 942 (0.83)</td>
<td>261 (0.33)</td>
<td>236 (0.28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ß-tubulin</td>
<td>80 (0.76)</td>
<td>495 / 686 (0.72)</td>
<td>194 (0.39)</td>
<td>162 (0.33)</td>
</tr>
<tr>
<td>2</td>
<td>Mycobiont</td>
<td>Section Polydactylon</td>
<td>Concatenated: 5</td>
<td>119</td>
<td>3803 / 4621 (0.82)</td>
<td>566 (0.15)</td>
<td>410 (0.11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITS</td>
<td>119 (1.00)</td>
<td>905 / 907 (0.96)</td>
<td>150 (0.30)</td>
<td>123 (0.24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LSU</td>
<td>118 (0.99)</td>
<td>1252 / 1374 (0.94)</td>
<td>78 (0.06)</td>
<td>61 (0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RPB1</td>
<td>109 (0.92)</td>
<td>676 / 716 (0.94)</td>
<td>87 (0.13)</td>
<td>61 (0.09)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EFT2.1</td>
<td>84 (0.71)</td>
<td>818 / 942 (0.87)</td>
<td>120 (0.15)</td>
<td>81 (0.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ß-tubulin</td>
<td>104 (0.87)</td>
<td>552 / 682 (0.81)</td>
<td>131 (0.24)</td>
<td>84 (0.15)</td>
</tr>
<tr>
<td>3</td>
<td>Mycobiont</td>
<td>Section Polydactylon</td>
<td>Concatenated: 6</td>
<td>120</td>
<td>3907 / 4769 (0.82)</td>
<td>690 (0.18)</td>
<td>526 (0.13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITS</td>
<td>120 (0.100)</td>
<td>475 / 907 (0.52)</td>
<td>135 (0.28)</td>
<td>108 (0.23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LSU</td>
<td>84 (0.80)</td>
<td>1231 / 1374 (0.90)</td>
<td>69 (0.06)</td>
<td>53 (0.04)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RPB1</td>
<td>98 (0.93)</td>
<td>676 / 716 (0.94)</td>
<td>89 (0.13)</td>
<td>61 (0.09)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EFT2.1</td>
<td>78 (0.74)</td>
<td>817 / 942 (0.87)</td>
<td>120 (0.15)</td>
<td>81 (0.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ß-tubulin</td>
<td>80 (0.76)</td>
<td>560 / 682 (0.82)</td>
<td>129 (0.23)</td>
<td>82 (0.15)</td>
</tr>
</tbody>
</table>

We performed Bayesian analyses on Matrices 1, 2, 4, and 6 and using MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001) as implemented on the CIPRES portal with the partition schemes described in Supplementary Table S2, available on Dryad. For Matrix 1 we used the default priors and completed 50 million generations, sampling every 500th generation. Matrices 2 was partitioned according to the codon-by-locus partition scheme (Table S1 and Supplementary Table S2, available on Dryad) and the best models for each subset of the partition were estimated using MrModelTest (Nylander 2004). We used the default priors and completed 40 million generations, sampling every 1000th generation. Matrices 4 and 6, the subsets were defined according to codon positions and the GTR+G model was implemented for all subsets. We ran the program for 29 million generations, using substitution models available in RAxML and MrBayes, as well as all the models available, under different selection criteria (AIC, AICc, and BIC) as implemented in PartitionFinder. Matrices 4 and 6 (Nostoc rbcL) were divided into three subsets according to codon positions. Subsets and substitution models selected for all matrices analyzed phylogenetically are included in Supplementary Table S2, available on Dryad.

The final RAxML searches for optimal trees and bootstrap analyses on Matrices 1, 2, 4, and 6, were implemented using the rapid hill-climbing algorithm for 1000 replicates with the GTR+GAMMA substitution model. RAxML analyses were performed on Matrix 3 using the same data partition as for Matrix 2 with the addition of one subset to accommodate recoded characters (PCS-ORD) used to capture phylogenetic signal from ambiguously aligned regions (Table 1).

Figure 1. Flowchart showing the matrices used for each analysis and the figures reporting the results. Top row: matrices, middle row: software used to perform analyses; bottom row: figures reporting specific analytical results.
default settings, sampling every 1000th generation. Two independent runs, each composed of four chains, were performed for each matrix. We assessed the convergence of chains using Tracer version 1.5 (Rambaut and Drummond 2007) and Are We There Yet (AWTY, Nylander et al. 2008) as implemented on the website http://king2.scs.fsu.edu/CEBProjects/awty/awty_start.php, last accessed 3 August 2016.

Matrix 2 was also analyzed with BEAST version 1.7.4 (Drummond and Rambaut 2007) to generate a chronogram (relative time). The optimal nucleotide substitution model was selected using MrModelTest independently for all exons across all loci, and for all introns (see Supplementary Table S2, available on Dryad). We ran BEAST with default priors, unlinking substitution models, but linking clock models (a lognormal relaxed clock) and tree models, for 50 million generations, sampling every 1000th generation. For the protein-coding genes, each codon position was treated as a separate subset and a lognormal distributed prior on the relative rate of the different codon positions was applied. We assessed the convergence of the analysis using Tracer and AWTY.
Species Discovery Methods
Species delimitation of Peltigera (mycobiont) was assessed with Structurama (Huelsenbeck et al. 2011) and bGMYC (Pons et al. 2006; Reid and Carstens 2012) (Fig. 1 and Supplementary Fig. S3, available on Dryad). For Structurama, we analyzed three sets of taxa derived from Matrix 2, representing each of the three major clades separately: the polydactyloid clade with 25 individuals, the dolichorhizoid clade with 70 individuals, and the scabrosoid clade with 24 individuals. We coded alleles for six loci: ITS1, ITS2, ß-tubulin, EFT2.1, LSU, and RPB1. For the polydactyloid clade dataset, we also coded alleles of the 5.8S. We applied different priors (see Supplementary Figs. S1 and S2, available on Dryad) to detect their effect on species delimitation. Analyses were run for 1 million generations, sampling every 1000th generation. The species delimitation. Analyses were run for 1 million generations for three different priors (gamma hyperprior shape parameter was set to vary 1000 generations, with threshold values of 2 and 100 and a starting point of 1,1,25. A species was circumscribed if the posterior probability for grouping a set of haplotypes together was higher than the posterior probability of any grouping containing one of these haplotypes. Discrepancies between Structurama and bGMYC were resolved following the most inclusive (broadly defined) species circumscriptions using monophyly as a grouping criterion.

Haplotype Network Reconstruction
We generated haplotype networks using TCS version 1.21 (Clement et al. 2000) based on those 206 mycobiont ITS haplotypes for which we sequenced the rbcLX of their co-living Nostoc symbiont (Matrix 5, Table 1 and Fig. 1). We divided the dataset into six subgroups based on sequence similarity (scabrosoid, polydactyloid without nana, 1, nana 1, scabrosella-sp. 7, occidentalis-sp. 6, and the remaining individuals from the dolichorhizoid group) so that none of the sites were ambiguously aligned, and therefore, all sites could be included in these six separate TCS analyses. Haplotypes were connected using a parsimony criterion with the 0.95 threshold value, and gaps considered as a 5th state. When different paths of equal number of changes occurred in the ITS haplotype networks, the path favoring indels rather than substitutions was selected because of the high frequency of short indels in the ITS spacers (Gaya et al. 2011).

Estimating Similarity among Nostoc and Peltigera Groups
Based on their Spatio-temporal Association Networks
We performed three analyses with UniFrac (Lozupone and Knight 2005; Lozupone et al. 2006) as implemented on the website (http://bmf2.colorado.edu/UniFrac). We compared 1) the phylogenetic breadth of Nostoc phylogroups associated with each mycobiont species using the Nostoc tree derived from Matrix 6 (Fig. 1); 2) Nostoc phylogroup composition in different biogeographic regions (as illustrated by the map in Fig. 3) using the Nostoc tree derived from Matrix 6; and 3) Peltigera species composition in different biogeographic regions (map in Fig. 3) using the ML tree of Peltigera section Polydactylon based on Matrix 2 (Fig. 3). Analyses 2 and 3 were performed twice, once with the biogeographic regions defined as depicted in Fig. 3, and a second time with finer geographic divisions: 1) the three northern regions (NA, WP, and EP) were further split into arctic-boreal and temperate subregions; 2) the neotropic (NT) region was divided into South and Central America; and 3) Australasia was subdivided by considering New Zealand and Papua New Guinea as distinct subregions. Mycobiont species represented by a single thallus or a single photobiont sequence (P. melanorrhiza, P. havaiensis, P. sp. 5, P. pulverulenta 3, P. nana 2, P. macr) were excluded from the analyses and the Oriental region was omitted from the coding scheme due to the limited number of specimens available from this region.
FIGURE 3. Phylogeny of the lichen-forming genus *Peltigera* section *Polydactylon* (mycobiont, i.e., fungal partner). Most likely (ML) tree using Matrix 2 (Table 1, Fig. 1), consisting of 119 OTUs representing 39 putative monophyletic species. The tree was rooted according to the topology presented in Fig. 2. Values associated with each internode represent ML bootstrap support (ML-BS; first value), Bayesian posterior probability (PP; middle value) and ML bootstrap support derived from the analyses on Matrix 3 (Fig. 1), which incorporated recoded ambiguously aligned characters (using PICS-ORD) excluded from the alignments (POML-BS; last value). Thick internal branches represent internodes with ML-BS $\geq 70\%$, POML-BS $\geq 70\%$, and PP $\geq 0.95$. Colored horizontal bars (color scheme according to the map on the top left corner) following each OTU or group of OTUs (delimited with square brackets) indicate the geographic origin of specimens (see Supplementary Text Part A, available on Dryad, for the delimitation of geographic regions) included in the phylogenetic analysis, as well as other available specimens (not included in Matrix 2) that have identical ITS sequences with OTUs included in the phylogenetic analysis. The proportion of each color inside these bars corresponds to the relative number of specimens from each region. Unique ITS haplotypes have a square instead of a horizontal bar. Vertical black bars delimit recognized or putative species. Two gray boxes in the dolichorhizoid clade indicate polytomies that were resolved when recoded characters (PICS-ORD) were added to Matrix 2 to form Matrix 3. The corresponding parts of the phylogeny resulting from the ML search based on Matrix 3 are shown in gray boxes with round corners. Abbreviations used in the map in the top left corner refer to the following regions: Afrotropics (AT), Australasia (AU), East Palearctic (EP), Neotropics (NT), Oriental (OR), Pacific North West (PNW), and West Palearctic (WP). Red dots refer to the placement of the minor conflicts detected in the single-locus topologies (see Materials and Methods “Single Locus and Concatenated Datasets” 1 Scale = nucleotide substitution/site.
**Ancestral State Inferences of Peltigera–Nostoc Mutualistic Interactions**

We inferred ancestral states for cyanobiont pools (subgroups) that include *Nostoc* phylogroups shared by *Peltigera* species. These pools correspond to networks formed by *Nostoc* phylogroups associated with the same *Peltigera* species. These networks unveiled three cyanobiont pools: *occidentalis*, *dolichorhiza*, and *scabrosella*. *Peltigera* species associated with *Nostoc* phylogroups of the *occidentalis* pool, for example, were never found associated with *Nostoc* phylogroups of the *scabrosella* pools. We hypothesize that each *Peltigera* species can only associate with *Nostoc* phylogroups from one of the three *Nostoc* pools. Therefore, we considered these three pools as potential character states for our ancestral state reconstruction analyses.

To infer ancestral states, we used SIMMAP version 1.5.2 (Bollback 2006), with default settings, on 2000 chronograms obtained from the BEAST analysis of Matrix 2. The number of chronograms was determined by a sensitivity analysis where we progressively increased the number of trees until the addition of more trees did not change the results in a way that would impact our conclusions. We also used BEAST directly to infer ancestral states by completing 10 million generations and sampling every 1000th generation when analyzing Matrix 2. Finally, we used BayesTraits version 1.0 (Pagel et al. 2004) on the same subset of 2000 trees derived from the BEAST analysis of Matrix 2. We constrained selected branches (ancestors) on certain states, and compared the harmonic mean of the iterations by calculating Bayes factors to determine which ancestral states, and compared the harmonic mean of the iterations by calculating Bayes factors to determine which ancestral states leads to the highest likelihood (Pagel and Meade 2004). We performed sensitivity analyses, increasing the number of trees and the number of iterations until the results were not influenced by the addition of more trees and/or iterations. We used the reversible jump function and a gamma hyperprior of mean and variance varying from 0 to 10 and completed 20 million iterations for each constrained state.

**Diversification Analyses of Peltigera Section Polydactylon**

We first conducted a phylogenetic analysis on a modified Matrix 2, that is, with a single representative per species, using *BEAST* (Heled and Drummond 2010). For each species, we selected one of the representatives with the highest number of loci available. We used a strict molecular clock and ran the analyses for 50 million generations, sampling every 1000th generation. We discarded 10% of the trees (burn-in) and generated a majority rule consensus tree based on the remaining 45,000 trees.

We performed BiSSE analyses (Binary State Speciation and Extinction; Maddison et al. 2007) as implemented in the R package BAMMtools (Rabosky 2014) on the same dataset. We ran it for 2,000,000 generations, sampling every 1000th generation. We let the program determine the best priors, then increased the number of trees until the addition of more trees did not change the results in a way that would impact our conclusions. We also used BAMM (Rabosky 2014) as implemented in the R package BAMMTools (Rabosky 2014) on the same tree. We let the program determine the best priors, then ran it for 2,000,000 generations, sampling every 1000th generation, using default settings.

**Results**

**Phylogeny of the Mycobiont Peltigera at the Genus and Section Levels**

The genus *Peltigera* and its eight sections as defined in Miadlikowska and Lutzoni (2000) are monophyletic and highly supported (Fig. 2). All well-supported relationships (Fig. 2) are congruent with the results shown in Miadlikowska et al. (2014) including three major clades within the genus *Peltigera* (Clades I–III).

Within section *Polydactylon*, we recognize three main and highly supported lineages, named polydactylid, dolichorhizoid and *scabrosa* clades (Fig. 3). The last two clades share a most recent common ancestor. Overall, relationships across section *Polydactylon* are highly supported. However, the dolichorhizoid clade includes a few species complexes that are not fully resolved. As predicted based on phenotypic diversity and broad geographic distributions, some “well-established” species, such as *P. neopolydactyla* and *P. scabrosa*, are polyphyletic. In contrast, some putative narrow endemics are conspecific with more widespread species (e.g., *P. dissecta* nested within *P. hymenina*). Many individuals are part of monophyletic entities outside all currently recognized species (P. spp. 1–11; Fig. 3).

Overall, our phylogeny of section *Polydactylon* suggests that its species richness might be at least twice as high as currently recognized. This is remarkable given that *Peltigera* species form large conspicuous foliose thalli that are regularly collected, and that multiple studies aimed at revising morphological species diversity (e.g., Holtan-Hartwig [1993] and Vitikainen [1994] for Europe). With a few minor exceptions (i.e., within species), no significant conflict was detected between the topologies generated by the phylogenetic analyses of Matrices 2 and 3 (i.e., without and with PIC5-ORD characters, respectively; Table 1). Most relationships were highly supported by both analyses and a few poorly supported
Internodes received complementary high support from one or the other analysis. The average bootstrap support for the topology without versus with PICS-ORD characters was 74.55% (73 internodes supported above 70%) and 82.15% support (81 internodes supported above 70%), respectively (Fig. 3). However, we also noticed a decrease in ML-BS values caused by the addition of PICS-ORD characters, especially at some deeper internodes. This pattern of higher values toward the tip of the tree and lower values for deeper internodes was also observed in previous studies when ambiguously aligned regions were recoded with INAASe or other methods (see Lutzoni et al. 2000 and Miadlikowska et al. 2003).

**Comparison of Species Discovery Methods**

When implemented on the entire section *Polydactylon*, bGMYC analyses performed well on the polydactyloid and scabrosoid clades (corroborated by morphological and geographical data) but did not on the dolichorhizoid clade. A bGMYC analysis restricted to the dolichorhizoid clade, which enabled the inclusion of more sites (which were ambiguously aligned and excluded from the analysis of the entire section), improved the results even if phylogenetic uncertainty associated with a rapid radiation detected in this clade (see the results of the diversification analyses below) led to low support values. The results of Structurama are highly dependent on the shape parameter used (see Supplementary Text Part C, available on Dryad, for a discussion on the sensitivity of the priors).

For section *Polydactylon* we expected a high number of undiscovered species. Although both methods agreed in the total number of fungal species in section *Polydactylon* (37 or 38 species assigned by Structurama, and 37 species by bGMYC) and the overall assignment of individuals to delimited species, several discrepancies (involving splitting versus lumping of species) distinguished the two approaches, especially in the dolichorhizoid clade (Supplementary Fig. S3, available on Dryad). The final estimations of Structurama for the dolichorhizoid clade were 18 species (gamma shape = 19); 11 species (gamma shape = 3) for the polydactyloid clade; and 8 species (gamma shape = 5) for the scabrosoid clade (Supplementary Figs. S1, S2, and S3, available on Dryad). bGMYC delimited 21 species within the dolichorhizoid clade, and 8 species in each of the remaining two clades (Supplementary Fig. S3, available on Dryad).

Our proposed species assignments within the dolichorhizoid clade (22 putative species), the polydactyloid (eight species) and scabrosoid clades (eight species; outermost dotted line delimitations in Supplementary Fig. S3, available on Dryad) were based on the consensus of both methods (when possible), monophyly, morphological traits (including from type specimens), and geographical distributions of the studied taxa. Only 14 species names are currently available for these 38 monophyletic putative species. The remaining 24 newly delimited species (awaiting formal description) represent predominantly allopatric or sympatric cryptic entities collected mostly from poorly explored regions of the world. The actual geographic ranges of species in section *Polydactylon* are more restricted than previously reported based on phenotypic traits alone (Supplementary Table S3, available on Dryad; Martínez et al. 2003).

**Phylogeny of the Nostoc Cyanobiont**

Our rbcLX phylogeny (Fig. 4) revealed *Nostoc* as a non-monophyletic group similar to previous studies (O’Brien et al. 2005; Svenning et al. 2005; O’Ilorá et al. 2010). We recovered *Nostoc* clades I and II, as well as the three subclades (1–3) of clade II, in agreement with O’Ilorá et al. (2010). However, significant support was obtained only for *Nostoc* clade I and subclade 2 of clade II (Fig. 4). Subclade 3 is composed of a large polytomy of numerous small, often well-supported and internally resolved subgroups. Moreover, the Bayesian analyses on the cyanobacterial *rbcLX* did not fully converge according to AWTY. Nearly all cyanobionts sampled from *Peltigera* belong to the broadly defined genus *Nostoc*, clade II, subclade 3. Only a few *Nostoc* strains found in *Peltigera* thalli belong to subclade 2 (Fig. 4).

In our phylogeny, cluster I from O’Brien et al. 2013 was not retrieved as monophyletic (not annotated in Fig. 4), but the remaining five clusters (II–VI) recognized by O’Brien et al. (2013) are represented. Here, we defined 14 new *Nostoc* phylogroups (VII–XX; Fig. 4) corresponding to well-supported clades of *Nostoc* associated with mycobionts from section *Polydactylon*. To the set of 30 unique *Nostoc* haplotypes (HT 1–30) defined by O’Brien et al. (2013) we added 17 (HT 31–47) new unique haplotypes associating with section *Polydactylon* and 15 haplotypes (HT 48–62) associating with other sections of *Peltigera* (Fig. 4).

**Patterns of Association between Peltigera Species and Nostoc Phylogroups**

Three main association patterns occurred within section *Polydactylon*: specialist mycobionts with non-specialist cyanobionts, non-specialists associating with non-specialists, and specialists associating with specialists (Figs. 5 and 6a). One-third of *Peltigera* species in section *Polydactylon* were associated with only one *Nostoc* phylogroup, being thus potentially strict specialists (Fig. 6b). Less than a quarter of *Nostoc* phylogroups are known to associate with only one *Peltigera* species (Fig. 6d), and we anticipate this proportion will shrink with additional sampling of *Peltigera* and members of the order Peltigerales in general. Overall, nearly three quarters of *Peltigera* species of section *Polydactylon* associate with one or two different *Nostoc* phylogroups, whereas more than half of their *Nostoc* phylogroups associate with more than two *Peltigera* species (Fig. 6b,d). Therefore, the main trend involves *Peltigera* species specializing on very few *Nostoc* phylogroups, whereas these cyanobionts tend to...
Figure 4. Phylogeny of *Nostoc* (cyanobiont). This is a 50% majority rule consensus tree of 26,100 trees that resulted from a Bayesian analysis of Matrix 4 (rbcLX dataset; Fig. 1) representing 417 unique *rbcL* haplotypes. The tree was rooted according to Otálora et al. (2010). Values associated with each internode represent ML bootstrap support (ML-BS; before slash) and Bayesian posterior probability support (PP; after slash). Thick internodes received ML-BS $\geq 70$ and PP $\geq 0.95$. Clades and subclades of *Nostoc* were defined according to Otálora et al. (2010). Newly sequenced *Nostoc* cyanobionts associated with mycobionts from section *Polydactylon* are shown in bold, whereas those from other sections of *Peltigera* are in gray bold. Geographic origin is provided after the name of each terminal OTU and for published sequences downloaded from GenBank.
Recognized phylogroups of *Nostoc* are represented by Roman numbers; phylogroups II–VI refer to O’Brien et al. (2013), whereas phylogroups VII–XX (defined here) represent significantly or moderately supported monophyletic groups encompassing *Nostoc* associated with representatives of section *Polydactylon*. Colors were attributed to each phylogroup (and four subclades within phylogroup XIX) except the ones defined by O’Brien et al. (2013), which do not contain any of the newly added *Nostoc* sequences from section *Polydactylon*. Geographic range (full squares), mycobiont affinity within the genus *Peltigera* (full circles) and associated mycobiont...
FIGURE 4. Continued

Asterids within section Polydactylon (full stars) are provided for each Nostoc phylogroup and selected clades. When associated with identical rbcL haplotypes, a terminal branch representing different mycobionts was replaced by a cone (horizontally oriented) comprising all collapsed individuals. Abbreviations for Pelitogia species are: aphit = P. aphitidis; bel = P. britannica; leuc = P. lutea; pen = P. neocanina; neck = P. neocorinthis; can = P. canina; ver = P. venosa; por = P. porinum; kri = P. Kristinii; fas = P. fuscopunctata; pra = P. praetextata; cin = P. cinnamomeus; pol = P. polydactylon; mem = P. membranaceus; hor = P. horizontalis; nec = P. neopolydactyla. Abbreviations for geographic regions are: BC = British Columbia, Ala = Alabama, Ore = Oregon, NZ = New Zealand, Pen = Pennsylvania, NC = North Carolina, AZ = Arizona, PNG = Papua New Guinea, Nu = Namivak. Scale = nucleotide substitution/site.
diversify their association across many Peltigera species. Only two species (P. hymenina and P. dolichorhiza) were found to associate with more than three Nostoc phylgroups, some of these phylgroups associating with more than three species of Peltigera (Fig. 6a). We found two cases of one-to-one specificity involving a specialist Peltigera (P. neopolydactyla and P. sp. 11) with a specialist Nostoc (phylgroups Xb and IX, respectively).

We further built networks of Nostoc phylgroups (Nostoc pools) that are shared by Peltigera species (Fig. 7b). UniFrac analyses clustered Peltigera species into five groups based on the Nostoc phylgroups with which they associate (Fig. 7a). The fusion of these Nostoc networks and UniFrac clusters formed the Nostoc pools and their internal cores shown in Fig. 7b. One subset of Peltigera species associates with Nostoc from the scabrosella pool (except P. neopolydactyla, which associates with a unique Nostoc phylgroup), two subsets (1 and 2) associate with the occidentalis pool, and two subsets (dolichorhiza and polydactylon cores) associate with the dolichorhiza pool (Fig. 7a–c). Nostoc phylgroups found with P. spp. 8 and 9 (two Asian species, Fig. 5) could not be assigned to any pool, most likely due to our low sampling from Asia.

**Macroevolution of Peltigera–Nostoc Association Networks**

Both ancestral state reconstruction methods converged on the same ancestral states for the nodes of interest for this study (Fig. 7d). However, the degree of confidence varied considerably. In most cases, the greatest level of confidence resulted from SIMMAP analyses. The Bayes factors obtained with BayesTraits cannot be directly compared with the other results, but when testing the character state reconstructed as ancestral by other methods against the other states, BayesTraits always generated a significant positive value favoring the reconstructed state. Based on SIMMAP analyses, the most recent common ancestor of the section Polydactylon was associated with Nostoc from the occidentalis pool (Fig. 7d), which is specialized on a cosmopolitan Nostoc (phylogroup V, Supplementary Fig. S4, available on Dryad). Similarly, all Peltigera species spread over 10,000 km or more associate with at least two Nostoc phylgroups, except P. polydactylon, which is specialized on a cosmopolitan Nostoc (phylogroup V, Supplementary Fig. S4, available on Dryad). The best model selected by MEDUSA and by BAMM (PP = 0.53 with BAMM) detected one major acceleration in diversification rate (within the dolichorhizoid clade, see the asterisk on Fig. 7d), which is associated with a southward colonization from the boreal biome to temperate and tropical biomes, and to the Southern Hemisphere in general, as well as a major switch from the occidentalis to the dolichorhiza pool of cyanobacteria (see Figs. 5 and 7).

**BiSSE Analyses**

BiSSE analyses revealed a similar extinction rate for specialist and non-specialist Peltigera species (2.96 × 10^2, 1.44 × 10^2, respectively), but the speciation rates were much higher for non-specialists, compared with specialists (4.2 × 10^2, 6.36 × 10^-10, respectively). Similarly, we detected a higher rate of transition from non-specialists to specialists than vice versa (1.72 × 10^2, 9.91 × 10^-9, respectively). A model with constrained equal rates was significantly rejected by AIC.

**Specificity, Range, Genetic Diversity, and Age of Peltigera Species**

The average distance between the farthest apart localities where two conspecific fungal specimens were sampled was 7854 km for non-specialists, and 3473 km for specialists (the difference is significant according to a Welch’s unpaired t-test, P-value = 0.019). The average latitudinal range for non-specialists is 25.14° and 8.7° for specialists (Welch’s P-value = 0.005).

All Peltigera species distributed across latitudinal ranges spanning at least 20° associate with at least two different Nostoc phylgroups, except P. polydactylon, which is specialized on a cosmopolitan Nostoc (phylogroup V, Supplementary Fig. S4, available on Dryad). Similarly, all Peltigera species spread over 10,000 km or more associate with at least two Nostoc phylgroups. This includes species that are generalists across their range and species that specialize on a different phylgroup in a different bioclimatic zone.

The average number of ITS haplotypes within non-specialist Peltigera species (7.4) is twice that of specialists (3.2), a difference that is significant (Welch’s test P-value = 0.0135; Fig. 8c). Specialist Peltigera species are on average twice as old as non-specialists, that is, 6.9 versus 3.2 million years.

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FIGURE 5. Continued

compared with the next adjacent haplotype(s). The sizes of the circles are proportional to the number of Peltigera mycobionts with identical ITS sequences. The colors within circles of the fungal haplotype networks correspond to phylogroups of Nostoc (as defined in Fig. 4, and represented in the legend provided here) in association with each individual mycobiont (i.e., sampled from the same thallus). White circles, or fractions of circles, indicate Nostoc haplotypes that were placed outside of all defined phylogroups (Fig. 4). Their haplotype numbers are provided, preceded by HT.
3.3 relative units of time, respectively (Welch's $P$-value = 0.034; Fig. 8d).

**DISCUSSION**

*Discovery of Previously Unknown Biodiversity*

Our study has unveiled previously unknown biodiversity for both the fungal and cyanobacterial partners of *Peltigera* section *Polydactylon*. With this study, we have circumscribed 38 *Peltigera* species, whereas only 14 species names could be attributed to these lineages (Vitikainen 1994, 1998; Sérusiaux et al. 2009). Unsuspected biodiversity composed mainly of cryptic species is frequently encountered in phylogenetic studies of lichen-forming fungi (see e.g., Lumbsch et al. 2011), but rarely at this scale, where we nearly tripled the known number of species (but see Lücking et al. 2014). Similarly, our study unveiled 25 *Nostoc* phylogroups associating with these 38 *Peltigera*
Figure 7. *Peltigera–Nostoc* association networks and evolution for *Peltigera* section *Polydactylon*. a) UniFrac clustering of *Peltigera* species based on the respective set of *Nostoc* phylogroups with which they associate. Color shades on the tree define clusters of *Peltigera* species sharing at least one *Nostoc* phylogroups as shown in panel b. b) Delimitation of *Nostoc* pools within clusters of *Peltigera* species. Colored circles with Roman numbers represent *Nostoc* phylogroups (as defined in Fig. 4 and shown in legends of Figs. 5 and 6) where each line represents a *Peltigera* species that was found associated with the two connected *Nostoc* phylogroups. Colored background inside the *Nostoc* pools indicate the sets of *Nostoc* phylogroups associated clusters (cores) as defined and colored in panel a. Unique *Nostoc* phylogroups are shown outside of the three delimited *Nostoc* pools. c) PCA from the UniFrac analysis. Each dot represents a *Peltigera* species and their proximity reflects similarity in the respective set of *Nostoc* phylogroups with which they associate. Colored circles correspond to *Nostoc* pools and their cores as defined in panels a and b. d) Reconstruction of the ancestral pool of *Nostoc* associated with *Peltigera* species depicted in the chronogram presented in Fig. 5. Three main pools of *Nostoc*, as delimited in panel b, were coded. Internal branches are colored according to the pool of *Nostoc* reconstructed as the ancestral state. Pie charts associated with nodes summarize the results from three different analyses. The first pie charts represent posterior probabilities generated with SIMMAP, the second pie charts represent posterior probabilities generated with BEAST, the third values represent the Bayes factor for the ancestral state color above that number compared with the state color below that number, from the BayesTraits analysis. The colors correspond to the state (*Nostoc* pool) that was reconstructed as ancestral with significant value. The asterisk shows the branch where a drastic increase in diversification rate was detected using M E D U S A and BAMM.
species, and an additional 17 unique Nostoc haplotypes (outside of phylogroups). Although only a few hundred lichen photobionts are recognized so far (Friedl and Büdel 2008) compared with the high species richness of described lichen mycobionts (estimated at 16,000 species; Kirk et al. 2008) our study reveals that their diversity may not be much lower than that of the mycobionts, and hence that lichenized fungi have a much broader range of partners to choose from.

Of the 25 Nostoc phylogroups found in association with species of section Polydactylon, 22 are exclusively associated with the genus Peltigera, and 18 are restricted to section Polydactylon. However, for many parts of the world (Papua New Guinea, New Zealand, or South America for instance) most of the available sequences were generated as part of this study for which only Peltigera populations were targeted. In a few cases, a Nostoc phylogroup associated with Peltigera species can also be associated with lichen-forming species representing different lichen genera (e.g., phylogroup XII) or even be associated with plants (e.g., phylogroup XIA).
Without this thorough phylogenetic reconstruction and species delimitation, the potential for specificity between symbionts would have been underestimated and widespread generalism might have been inferred across the section. This more balanced biodiversity between a monophyletic group of lichen-forming fungi and their cyanobionts at a worldwide scale provided an unprecedented eco-phylogenetic framework to revisit hypotheses of symbiotic associations, and to gain a more comprehensive understanding of the patterns and evolution of specificity in symbiotic systems.

Patterns of Association and Specificity between Peltigera Species of Section Polydactylon and their Nostoc Cyanobionts

Associations of myco- and photobionts within section Polydactylon span the whole spectrum of specificity, wherein either partner can be a strict specialist or a generalist. These associations and levels of specialization are not random. They are structured geographically, ecologically, and phylogenetically. The specialization of Peltigera species to one Nostoc phylogroup can be seen as an adaptive process toward optimized fitness to a set of local biotic and abiotic environmental factors. The association of some Peltigera species with more than one Nostoc phylogroup can be driven by natural selection resulting in adaptations to different bioclimatic zones (geographical range expansion). For example, P. neopolypadactyla 1 and P. occidentalis are local specialists (they associate with distinct Nostoc phylogroups in different regions) by partnering with phylogroup VIIa in the boreal region across all continents but with phylogroup VIIb in temperate USA or the very rare phylogroup VIIc in Arizona (Fig. 5). Peltigera neopolypadactyla 2 associates with phylogroup XIII throughout the boreal zone but with phylogroup X in temperate Asia (Fig. 5). Fernández-Mendoza et al. (2011) found a similar pattern of association for the lichen-forming species Cetraria acuta and its Nostoc photobiont. Generalists interacting with generalists should promote large geographic ranges and broad niche spectra. This type of interaction would be advantageous for lichen-forming species and Nostoc phylogroups colonizing new geographic areas or habitats. Most South American Peltigera species from section Polydactylon are generalists, associating with several Nostoc phylogroups (Fig. 5). Moreover, they associate with most (9 of 14) of the unique Nostoc rbcL haplotypes (i.e., single haplotypes outside of delimited phylogroups; Fig. 4) that we found as part of this study (Fig. 6).

For the two cases of reciprocal one-to-one specificity involving phylogroups IX and Xb paired with P. sp. 11 and P. neopolypadactyla 5, respectively (Figs. 5 and 6), Nostoc specialists are very rare within section Polydactylon. By default, cases of high specificity are favored by low sampling, which means that overall we are overestimating high specificity for the photobiont because we only sampled Nostoc associated with Peltigera. We do not have convincing cases of specialist cyanobionts associated with generalist Peltigera species because they involve Nostoc haplotypes that have been sampled only once. Specialization by Nostoc phylogroups toward one Peltigera species may exist, but these Peltigera species are also specialists. A specialist fungus associated with one or two generalist cyanobionts may represent an optimal equilibrium between high specificity resulting in the adaptation of the symbiotic thalli to a local set of environmental factors and low specificity to maximize the probability of finding an appropriate photobiont through horizontal transmission. This trend of higher specialization by the mycobiont compared with its cyanobiont, already detected in Peltigera, to some extent, by O’Brien et al. (2005, 2013) and Myliys et al. (2007), might relate to the unequal level of dependency, where in general, the mycobiont is more dependent on the cyanobiont than vice-versa (Lutzoni and Miaudlikowska 2009), and to the mode of transmission of the cyanobiont from one generation of lichen thalli to the next, which is mostly horizontal in section Polydactylon (the mycobiont needs to re-establish an association with a photobiont at the onset of every generation). Under such circumstances, it is likely that natural selection would lean toward Peltigera species associating with generalist Nostoc phylogroups, which should be more abundant in nature. It is assumed that vertical transmission of the photobiont (through vegetative propagules that contain both partners of the lichen thallus) would facilitate a one-to-one reciprocal specialization, and promote coevolution and/or cospeciation of the partners. Oálora et al. (2010) reported five species of Collena and Leptogium (Collemataceae, Peltigerales) exhibiting a one-to-one specificity with their cyanobacterial partners (whereas most mycobiont species in this family are generalists). These fungal specialist species reproduce mainly by vegetative propagules, and grow exclusively on old trees in very humid conditions, which is concordant with the expectation of vertical transmission and narrow ecological amplitudes for strict specialists (Oálora et al. 2010). Similarly, when comparing the sexually reproducing species Degelia atlantica with the sexually reproducing D. plumbea, Oálora et al. (2013) reported that the genetic diversity was lower in both partners in the asexual species (this species exhibiting a narrower ecological niche) compared with the sexually reproducing species. For the two cases of such extreme specialization of both partners found in this study (P. neopolypadactyla 5 and P. sp. 11 with phylogroup Xb and IX, respectively) none of these species have specialized vegetative propagules and there is no reason to believe that these species would reproduce mostly by thallus fragmentation. Therefore, the high reciprocal specificity detected here is likely to be determined genetically, for example, by highly specific interspecies signaling.
as reported for P. malacea and its Nostoc phylogroup (O’Brien et al. 2013), rather than the result of vertical transmission.

Joneson et al. (2010) reported that extracellular communication between lichen symbionts can occur without cellular contact, and the authors identified a variety of fungal genes that are involved in self and non-self recognition. Lectins secreted by the fungal partner can play an important role in recognizing compatible photobiont cells (Galun and Kardish 1995), and in their communications with both green algae (Legaz et al. 2004) and cyanobacteria (Vivas et al. 2010). Therefore, the pairing of Peltigera species with cyanobionts is complex, involving genetic processes, and the observed patterns of specificity are not simply the result of the presence/absence or dominance of certain Nostoc phylogroups in a given locality, as illustrated by sympatric populations of Peltigera species that are specialized on different phylogroups in the boreal zone (P. neopydactyla 1, P. occidentalis, P. scabrosa 1, and P. scabrosa 4 with phylogroup VIIa, P. neopydactyla 2 with phylogroup XIII, P. polydactyla with phylogroup V, and P. scabrosella with phylogroup Xla).

**Biogeographic and Climatic Factors Shaping Peltigera–Nostoc Associations**

Because Peltigera species appear more dependent on their cyanobiont than vice versa, we wondered if the geographical range on Nostoc determines the distribution range of Peltigera species. As for most organisms, climate is an important factor shaping Nostoc distributions, as Nostoc phylogroups tend to be restricted to a single bioclimatic zone (Figs. 4 and 5 and Supplementary Fig. S4, available on Dryad). Most Nostoc phylogroups have extensive longitudinal ranges, but rather narrow latitudinal spectra (Supplementary Fig. S4a, available on Dryad). Thus, like for green algal photobionts (Fernández-Mendoza et al. 2011; Peksa Škaloud 2011) climate plays a major role in shaping the distribution of cyanobacterial photobionts. Phylogroup V is unusual in being present across a wide range of latitudes and longitudes (Fig. 4 and Supplementary Fig. S4, available on Dryad). In general, the geographic ranges of Nostoc phylogroups reflect their level of specialization, with generalist Nostoc phylogroups spanning more extensive geographical distributions than specialist Nostoc.

This pattern is mirrored by the *Peltigera* species of section *Polydactyla*: the range of fungal species associated with more than one Nostoc phylogroup is broader than that of specialists that are associated with only one phylogroup (Fig. 8a,b). As expected, most *Peltigera* species restricted to the boreal areas were restricted to boreal-only Nostoc phylogroups (P. scabrosa 1, 2, 4, P. neopydactyla 4, P. scabrosella-P. sp. 7 associated with phylogroups VIIa, Xla, IV, VIlld), whereas boreal species with distributions extending to the temperate zone (P. neopydactyla 1, 2, P. occidentalis) switch to a temperate Nostoc phylogroup (VIIb, VIIc, X Figs. 4 and 5 and Supplementary Fig. S4, available on Dryad). Other broadly distributed species such as *P. hymenina* and *P. polydactyla* are associated with Nostoc phylogroups that have ranges covering more than one bioclimatic zone (phylogroup XVI for *P. hymenina*, and phylogroup V for *P. polydactyla*). Therefore, being a generalist, a local specialist in multiple biogeographical regions, or a strict specialist on a widespread phylogroup, are three viable strategies that can result in a broad geographical range for *Peltigera* species. Studies on other taxa in Peltigerales (Collemataceae and the genus *Degelia*, Otálora et al. 2010, 2013), also suggested that specialist species have more restricted niches and distributions compared with generalists. However, asexual reproduction was also an important factor in these studies, whereas in section *Polydactyla* similar patterns were detected even with specialist species reproducing sexually.

However, the bioclimatic range of the Nostoc phylogroup, and consequently the availability of an appropriate Nostoc partner, is not the fact restricting the distribution of *Peltigera* species. For example, *Peltigera* species associated with the nearly cosmopolitan Nostoc phylogroup V (P. polydactyla, P. sp. 10, P. dolichorhiza, P. sp. 2b and 3) have more limited distributions than their Nostoc partner. This is true for several *Peltigera* species, even within a bioclimatic zone, like *P. pacifica* (restricted to Pacific Northwest), which associates with a cyanobiont (phylogroup XIII) that is present throughout the circumboreal belt (Fig. 5, Supplementary Fig. S4b, available on Dryad). Overall, the distribution of Nostoc phylogroups exceeded the distribution of *Peltigera* species in the section *Polydactyla*, both in terms of geographic distance and latitudinal range. This pattern emerged even though we underestimated the distribution of Nostoc phylogroups by restricting our study to species from section *Polydactyla*.

**Symbiotic Switches across Space and Time**

Most *Peltigera* species associated with Nostoc from the *scabrosella* and *occidentalis* pools (Fig. 7) are sympatric (for instance in boreal forests), and phylogroups from these two pools were frequently sampled in the same localities. Yet, no Nostoc phylogroups from distinct pools were found associating with the same species of *Peltigera*, despite the boreal regions being our most intensively sampled biome. This suggests that we can rule out the hypothesis that *Peltigera* mycobionts can associate with all Nostoc phylogroups present in a specific locality.

In the *dolichorhiza* pool, two common species, *P. hymenina* and *P. polydactyla*, were never found with the same Nostoc phylogroup, despite being partially sympatric. Therefore, specificity occurs even inside these Nostoc pools (Figs. 4, 5, and 7b). Associations are also sometimes geographically and phylogenetically structured, that is, in some cases *Peltigera* species associate with several closely related Nostoc phylogroups as exemplified by P. neopydactyla 1 and P. occidentalis...
associated with Nostoc phylogroups VII–c (occidentalis pool) and species from the South American group associated with phylogroups XIX–d (dolichorhiza core) (Fig. 7b).

The scabrosoid clade is the result of the earliest split within section Polydactylon, and the dominance of boreal species in this clade strongly suggests that they originated in boreal forests in association with Nostoc from the occidentalis pool (Figs. 5 and 7). So far, the majority of Nostoc phylogroups from the occidentalis pool was found only with species from section Polydactylon. Contrary to the occidentalis pool, several phylogroups from the dolichorhiza and scabrosella pools are associated with mycobionts from other sections of Peltigera. The origin and radiation of the South American group (within the dolichorizoid clade) was correlated with a switch from the occidentalis to the dolichorhiza Nostoc pool (Fig. 7d). Peltigera sp. 6 also shifted from the occidentalis to the dolichorhiza pool, as part of an independent colonization of South America by this lineage. A switch from the occidentalis to the dolichorhiza pool also occurred along the branch leading to P. melanorhiza, a species restricted to the Azores. Switches of Nostoc pools are therefore frequently linked with a change of geographic distribution, but not necessarily, as exemplified by a switch from the occidentalis to the scabrosella pool in the scabrosoid clade (Figs. 5 and 7).

Contribution of Specificity to the Diversification of Peltigera

The much lower transition rate from specialists to generalists, versus generalists to specialists, suggests that specialization is frequent and could occur through time in many lineages, whereas transitions to generalism would be rare events. Otálora et al. (2010) concluded that in Collemataceae, extreme cases of one-to-one reciprocal specialization between Nostoc and these lichen-forming fungi were derived states, evolving several times independently from generalist ancestors. We found higher speciation rates for generalists. We also found a rapid radiation in the clade including the group of generalist species almost exclusively present in the Neotropics (Figs. 5 and 7). The only member in this clade that specializes on a single phylogroup, P. pacifica, has a very narrow distribution (Pacific Northwest) perhaps due to the fact that the species rarely produces apothecia and disperses mainly asexually by vegetative propagules (involving a vertical transmission of Nostoc). The other two species resulting from an early split within this clade, P. nespolidactyla 1 and 2, are local specialists associating with one Nostoc phylogroup locally, while species in the South American group are generalists (Fig. 5).

Whether this radiation is driven by the generalist profile of the species in this group, by the dispersion to a new geographical area, by the colonization of a tropical environment, or by a combination of these is not known. The recency of this radiation could mean that as these species spread to South America, they were exposed to new habitats with novel Nostoc phylogroups. Under this evolutionary context, the ability to associate with many different Nostoc phylogroups would be advantageous, favoring generalism. We hypothesize that transitions to generalism are rare events, linked with a spread to new biogeographical areas, which can result in higher speciation rates. The ages of Peltigera species (i.e., the amount of time since they diverged from their most recent common ancestor) could also be linked to their levels of specificity, as we found that specialist species were older than generalists. Indeed, the fact that generalists are only found on short branches, representing young species, suggests that species do not stay generalist for long periods of time, and specialization might be required for the species to persist, explaining the high rates of transition from generalist to specialist and the fact that specialist species are older in general.

In newly invaded geographical areas (such as in the Neotropics for the dolichorizoid clade), the dominant association pattern involves generalist Peltigera associating with a mixture of generalists and rare specialist Nostoc strains (Fig. 6a). This might represent the first stage of a process of specialization of the mycobiont subjected to new selective pressures. The next stage could be the differential specialization across the geographical range as observed in P. neopolidactyla 1 and P. nespolidactyla 2, eventually leading to the specialization to a single cyanobiont in a given bioclimatic zone, as exemplified in this group by P. pacifica.

Peltigera–Nostoc Associations in Light of Theories on Mutualism

Law and Lewis (1983) suggested that the “inhabitant” (corresponding to the photobiont for most lichens), should be under selective pressure to reproduce asexually, and have a lower rate of evolution compared with the exaptant (corresponding to the mycobiont in lichen symbiosis) or closely related free-living taxa. It is generally assumed that only the fungal partner reproduces sexually in lichens (Büdel and Scheiddeger 2008). We found common instances of a low level of genetic variation or no variation at all within a single Nostoc phylogroup associated with a single species of Peltigera, or occasionally with closely related species (for instance P. scabrosa 4 and P. scabrosa 1), which can share the same Nostoc haplotypes (Figs. 4 and 5). Identical Nostoc haplotypes were frequently identified across a large geographic scale (Fig. 4 and Supplementary Fig. S4, available on Dryad). These Nostoc haplotypes were identical even in the extremely variable spacer region (unalignable across the Nostoc strains included in this study). This broad distribution of highly similar Nostoc strains (based on rclX sequences) can be a signature of low evolutionary rates in Nostoc involved in lichen symbioses, as well as low rates of nucleotide substitutions due to purifying selection for an
optimal association with specific lichen-forming species. However, this pattern of highly similar Nostoc strains covering large areas could also be explained by efficient long-distance dispersal mechanisms for certain Nostoc phylogroups.

Correspondingly, because cyanobionts are predominantly transmitted horizontally in Peltigera, the presence of the same Nostoc haplotype within and among different species of Peltigera can be explained by a parallel acquisition of the same cyanobiont, rather than coevolution of a fast-evolving exhabitant with a slow-evolving Nostoc partner. Recent studies (see Sachs et al. 2011) on a large variety of microbial symbionts demonstrated that the Law and Lewis paradigm was too simplistic. Indeed, the mutualistic framework set by Law and Lewis (1983) involving a positive frequency-dependent selection, evolutionary stasis, and high asexuality of one symbiont is consistent in some symbiotic associations, but highly incoherent in others (Sachs et al. 2011), and therefore, there is probably a continuum of different stages between an arms race in parasite–host systems and the Law and Lewis paradigm.

The Red King hypothesis (Bergstrom and Lachmann 2003) states that in mutualistic interactions, while both partners need to find a viable equilibrium to maintain the symbiosis, the slower partner wins the race because, by reaching the equilibrium more slowly, it can invest less and benefit more from the symbiosis. Specialists may be under strong selection to meet the requirements of the high specificity for their symbiotic partner and hence are genetically less diverse. In early-diverged species from section Polydactylon (P. sp. 11, P. sp. 9, P. scabrosa 1, P. scabrosa 4, P. neopolydactyla 5, P. neopolydactyla 6) both partners are highly stable (a single or very few similar ITS haplotypes per Peltigera species associated with one or a few Nostoc haplotypes). However, this would require a predominant role of clonal reproduction by the specialist fungi to explain the observed pattern because the ITS spacers are not under strong purifying selection.

Therefore, our results support the hypotheses of both Law and Lewis (1983) and Bergstrom and Lachmann (2003) by demonstrating that both symbionts can benefit if involved in a mutualistic relationship for a long time. This long-term specialization results in a low rate of evolution leading to the reduced genetic diversity and slow diversification of both partners, because the most frequent or best-adapted haplotype is positively selected (Law and Lewis 1983) to maintain the optimal benefits in the symbiosis.

Because we do not see a high level of specialization for the photobiont, it is very likely that in the process of lichenization, the mycobiont is capturing the photobiont (shared by other species), rather than the photobiont infecting the mycobiont. The fact that the lichen-forming fungus (Peltigera) is highly dependent on Nostoc (never found free-living) but not vice-versa also supports the fungal capture of Nostoc. As a consequence, the mycobiont might be adapting to the photobiont more than Nostoc adapting to Peltigera. Slow evolution of the cyanobiont (embedded in long-lived thalli) can be explained by a reduced selective pressure from the environment and a high selection from the mycobiont to maintain the relationship with the optimal cyanobacterial partner. A strong specialization of the mycobiont toward a single cyanobiont may limit its ability to switch to a different Nostoc partner and might explain why strict Peltigera specialists cannot expand to new regions (and have narrower geographic ranges compared with generalists).

We hypothesize two categories of fungal species regarding their symbiotic status: 1) a “suboptimal” status where partners that associated recently, and/or experienced new environmental conditions, would be evolving faster, driven by positive selection leading to an optimal association, and where associations with many different cyanobionts would be advantageous during this selective process, and 2) an “optimal” status, where the mycobiont is specialized to interact with one or two Nostoc partners, for example, and the association has reached an optimal equilibrium under specific environmental conditions. This is a case where a Peltigera species–Nostoc phylogroup pair is drastically more successful in a given area, compared with the same mycobiont species associated with other Nostoc phylogroups.

With time, local specialists might become strict specialists. For example, P. neopolydactyla 1 (Fig. 5) could speciate to form two species, one in a temperate zone in North America specializing on phylogroup VIIb, and a second species in boreal region, specializing on phylogroup VIIa. Similarly, populations of P. neopolydactyla 2 might split into two taxa, one in its temperate range in Asia, in association with phylogroup X, and another in its boreal range in association with phylogroup XIII (Fig. 5). In the case of P. neopolydactyla 2, it seems that specimens from Yunnan associating with Nostoc phylogroup X are already genetically distinct from the boreal populations associating with phylogroup XIII (they do not share ITS haplotypes, and form two monophyletic lineages; Fig. 5) whereas in P. neopolydactyla 1, there is no such clear distinction among populations as the same haplotype was found in association with Nostoc phylogroup VIIb and VIIb. The ability to switch cyanobionts can facilitate the expansion of mycobionts to new environments where the former cyanobiont is not available, to avoid competition for cyanobions from the co-existing mycobionts, or to choose a better-adapted cyanobiont in a changing environment. These observations are in agreement with the geographic mosaic of coevolution theory where a species may adapt and become specialized on another species differentially within different geographic regions (Thompson 2005). However, switches from specialization to generalism are also possible, and might represent rare events and transition states, when dispersion to new habitats and the presence of new Nostoc phylogroups make it advantageous to associate with several partners.
SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.h6v7g.

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