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Dissociation and horizontal transmission of codispersing lichen symbionts in the genus *Lepraria* (Lecanorales: Stereocaulaceae)

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Summary

• Lichenized fungi of the genus *Lepraria* lack ascomata and conidiomata, and symbionts codisperse by soredia. Here, it is determined whether algal symbionts associated with *Lepraria* are monophyletic, and whether fungal and algal phylogenies are congruent, both of which are indicative of a long-term, continuous association between symbionts.

• The internal transcribed spacer (ITS) and part of the actin type I locus were sequenced from algae associated with *Lepraria*, and the fungal ITS and mitochondrial small subunit (mtSSU) were sequenced from fungal symbionts. Phylogenetic analyses tested for monophyly of algal symbionts and congruence between algal and fungal phylogenies.

• Algae associated with *Lepraria* were not monophyletic, and identical algae associated with different *Lepraria* individuals and species. Algal and fungal phylogenies were not congruent, suggesting a lack of strict codiversification.

• This study suggests that associations between symbionts are not strictly maintained over evolutionary time. The ability to switch partners may provide benefits similar to genetic recombination, which may have helped this lineage persist.

Key words: *Asterochloris*, codiversification, dispersal, *Lepraria*, photobiont, re-lichenization, symbiont-switch.

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Introduction

The mode in which symbionts are transmitted is considered one of the most important steps towards coevolution (Maynard-Smith, 1991). Vertical transmission maintains associations between symbionts over generations through codispersal, while horizontal transmission forces offspring to acquire new symbionts, thereby permitting opportunities for symbiont exchange. Co-diversification is well known among vertically transmitted bacterial-animal symbioses (Peek *et al.*, 1998; Clark *et al.*, 2000; Funk *et al.*, 2000; Lo *et al.*, 2003; Degnan *et al.*, 2004) and vertically transmitted fungal-animal symbioses (Chapela *et al.*, 1994; Hinkle *et al.*, 1994). Strict vertical transmission is expected to stabilize relationships between lineages, linking them over evolutionary time. Maintenance of fixed associations, with subsequent evolution, should lead to the formation of monophyletic groups, which associate solely with the symbiont and its relatives. In contrast, the breakdown and loosening of relationships may lead to the symbionts and their relatives associating with nonmonophyletic assemblages. Furthermore, the continued association between symbiont lineages, with subsequent

evolution, might be predicted to lead to parallel cladogenesis (concordant evolutionary histories) between symbiont lineages (Futuyma, 1998).

Lichen symbioses are believed to be coevolved (Ahmadjian, 1987). However, recent studies have revealed only low amounts of codiversification between fungal and green algal lichen symbionts (Kroken & Taylor, 2000; Dahlkild *et al.*, 2001; Piercey-Normore & DePriest, 2001; Zoller & Lutzoni, 2003). Furthermore, many fungal taxa associate with eukaryotic photobionts which are not monophyletic, and are shared with other lichen-forming fungi, demonstrating that tight, exclusive relationships between symbionts are not maintained over time (Kroken & Taylor, 2000; Dahlkild *et al.*, 2001; Helms *et al.*, 2001; Piercey-Normore & DePriest, 2001; Romeike *et al.*, 2002; Opanowicz & Grube, 2004; Piercey-Normore, 2004; Yahr *et al.*, 2004; Cordeiro *et al.*, 2005; Blaha *et al.*, 2006; Guzow-Krzeminska, 2006; Ohmura *et al.*, 2006; Piercey-Normore, 2006; Yahr *et al.*, 2006; Hauck *et al.*, 2007). With the exception of the vegetatively reproducing *Cladonia perforata* (Yahr *et al.*, 2004), which was found to associate with a narrow range of algae that were shared among other species, all studies have investigated species and genera in which the fungus regularly or occasionally reproduces with meiotically or mitotically derived fungal propagules, which disperse the fungus independently of the photobiont. When this occurs, the relationship between the fungal and algal partners is decoupled. Upon germination, fungal spores must somehow obtain a suitable photobiont, which may be free-living (Etges & Ott, 2001; Sanders & Lücking, 2002; Sanders, 2005; Hedenås *et al.*, 2007), or obtained through the theft from, or parasitism of, another lichen (Friedl, 1987; Ott, 1987a,b; Stenroos, 1990; Rambold & Triebel, 1992; Ott *et al.*, 1995; Gaßmann & Ott, 2000; Lücking & Grube, 2002). Both these strategies have been used to explain the re-lichenization of lichen-forming fungal species lacking a shared mode of dispersal (Beck *et al.*, 1998, 2002).

Dispersal by means of soredia or thallus fragmentation may circumvent symbiont decoupling, by carrying both symbionts together and maintaining links between lineages over generations. A small number of lichenized genera disperse solely by thallus fragmentation or the production of soredia. The fungal genus *Lepraria* (*c.* 40 spp.) has never been found to produce meiotically derived ascospores or mitotically derived conidia. *Lepraria* is vegetatively reproducing, and symbionts are presumed to be vertically transmitted by means of soredia (propagules containing fungal and algal cells) or thallus fragmentation. This lineage provides an optimal system to examine whether relationships between symbionts remain intact over evolutionary time in the absence of fungal haplospore production, and the obligate reassociation step between generations. Here we assess the effectiveness of vertical transmission of fungal–algal associations in the genus *Lepraria* over evolutionary time by testing the hypotheses that algal symbionts of the lichenized fungal genus *Lepraria* form a monophyletic group, and that fungal and algal phylogenies are congruent, both of which suggest a tight, long-term perpetuation of the symbiosis between fungal and algal lineages. We then compare the patterns seen in this study with those from other codispersed symbioses.

Materials and Methods

Taxon selection

Thirty-four *Lepraria* collections from a wide geographic and phylogenetic range were analyzed, along with additional non *Lepraria* collections (Supplementary material, Table S1). *Lepraria* has been shown to be polyphyletic (Ekman & Tønsberg, 2002), and we did not include *Lepraria* species found outside of the core group of *Lepraria* (*Lepraria* s. str.). Two other genera, *Siphula* and *Leprocaulon*, would have been ideal for this study based on their reproductive mode and species number. However, *Siphula* has also been shown to be polyphyletic (Platt & Spatafora, 2000; Stenroos *et al.*, 2002; Grube & Kantvilas, 2006), and the monophyly of *Leprocaulon* has yet to be confirmed. Specimens have been deposited in the Wisconsin State Herbarium (WIS) or were obtained from the Botanisches Museum & Botanischer Garten Berlin-Dahlem (B). The 110 new sequences generated in this study were supplemented with *Asterochloris* algal sequences from GenBank. All samples and sequences used in this study, with GenBank accession numbers, are shown in Table S1.

Molecular methods

DNA was extracted from lichen thalli, using the method of Grube *et al.* (1995) or the Qiagen DNeasy Mini Extraction Kit (Qiagen, Valencia, CA, USA), following the modifications of Crespo *et al.* (2001). An area of approx. 0.5 cm × 0.5 cm was used for DNA extraction from the *Lepraria* thalli, while a 0.5–1.0 cm-long portion of *Stereocaulon* thalli was used for DNA extraction. The algal rDNA internal transcribed spacer (ITS) and a portion of the actin type I locus (one exon and two partial introns) were PCR-amplified as in Nelsen & Gargas (2006) using the primers a-nu-ssu-1752-5′ (Nelsen & Gargas, 2006) and ITS4T (Kroken & Taylor, 2000) for the ITS, and a-nu-act1-0645-5′- and a-nu-act1-0818-3′-19 (Nelsen & Gargas, 2006) for the actin type 1 locus. Kroken & Taylor (2001) initially recovered two actin genes (type I and type II) from *Trebouxia*, and subsequently designed type I specific primers. In the present study, we used primers designed to preferentially amplify the actin type I locus (instead of the actin type II locus). While *Asterochloris* most likely has actin type II, we did not attempt to amplify it, and BLAST searches (Altschul *et al.*, 1997) of amplified fragments confirmed that we were indeed amplifying the actin type I locus (and not actin type II). Additionally, the fungal ITS was PCR amplified using the primers ITS1F (Gardes & Bruns, 1993) and ITS4A (DL

Taylor in Kroken & Taylor, 2001), while a portion of the fungal mitochondrial small subunit (mtSSU) was PCR-amplified using the mrSSU1 (Zoller *et al.*, 1999) and MSU7 (Zhou & Stanosz, 2001) primers.

The PCR reactions (total volume 12.55–13.05 μl) followed that of Nelsen & Gargas (2006), and contained 1.3–1.8 μl of diluted DNA (DNA was diluted 1 : 10 or 5 : 100 as listed in Grube *et al.* (1995) and Crespo *et al.* (2001), respectively), 0.38–0.39 μM of each primer, 4 μl water and 6.25 μl of Red Mix Plus (Gene Choice, Inc., Frederick, MD, USA), which consisted of 150 mM Tris-HCl, 40 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs and 0.05 units 4 μl^{-1} *Taq* DNA polymerase. Because DNA was not quantified, we have chosen to include the volume of diluted DNA used in the PCR reactions rather than the mass.

The PCR and cycle-sequencing reactions were performed in a Stratagene Robocycler (La Jolla, CA, USA), Techne Flexigene thermal cycler (Burlington, NJ, USA) or MJ Research PTC 200 thermal cycler (Waltham, MA, USA). The PCR amplification of the algal ITS began with an initial denaturation of 95°C for 5–7 min, and was followed by 10 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min, and then 25–35 cycles of 95°C for 1 min, 53–56°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 7 min. Identical conditions were used for the amplification of the actin locus, except that an annealing temperature of 60°C was used for the last 25–35 cycles. The PCR amplification of the fungal ITS followed Nelsen *et al.* (2007), and began with an initial denaturation of 95°C for 5 min, followed by 10 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min. This was followed by 35 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 1 min and terminated with a final extension of 72°C for 7 min. The PCR conditions for the mtSSU began with an initial denaturation of 95°C for 5–7 min, followed by 35 cycles of 95°C for 1 min, 56°C for 1 min and 62°C for 1 min and a final extension of 72°C for 7 min. The PCR products were then run in a 1% agarose gel, which was stained with ethidium bromide and visualized under UV light.

The PCR samples were cleaned with ExoSAP-IT (USB, Cleveland, OH, USA) following the manufacturer's protocols, and samples were sequenced as in Nelsen & Gargas (2006). Algal actin and fungal mtSSU samples were sequenced with the PCR primers, and algal and fungal ITS samples were sequenced with the ITS1 and ITS4 primers (White *et al.*, 1990). Cycle sequencing cocktails contained 1 μl PCR product, 0.33 μl 1 μM primer, 1 μl Big Dye Version 3.1 (Applied Biosystems, Foster City, CA, USA), 2–2.5 μl Big Dye Buffer and 7.18 μl water, and were run for 20–25 cycles at 96°C for 30 s, 46°C for 20 s and 60°C for 4 min (this protocol follows the manufacturer's instructions). Samples were cleaned with Sephadex G-50 fine (Amersham Biosciences, Uppsala, Sweden) in Centri-Sep columns (Princeton Separations, Inc., Adelphia, NJ, USA), using the protocol described on the University of Wisconsin Biotechnology Center's website (https://dna.biotech.wisc.edu/documents/Non-bead_cleanups.htm), or with magDTR dye terminator removal resin (Edge Biosystems, Galthersburg, MD, USA), following the manufacturer's instructions. Samples were sequenced at the University of Wisconsin Biotechnology Center (Madison, WI, USA). Chromatograms were read and sequences assembled in Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

Phylogenetic analyses – general

Sequences from Table S1 were manually aligned using Se-Al vs 2.0a11 (Rambaut, 1996), and two ambiguous regions in the second intron of the algal actin locus were excluded (21 bp: positions 392–412 relative to the actin sequence from the algal symbiont of *L. lobificans* 154 (DQ229898); 56 bp: positions 581–634 relative to the actin sequence from the algal symbiont of *L. lobificans* 154 (DQ229898)), while one region in the fungal ITS1 was excluded (45 bp: positions 101–147 relative to the fungal ITS sequence from *S. tomentosum* (EU008634)). The sequenced actin fragments contained two partial introns (maximum length of first intron = 212 bp; maximum length of second intron = 311 bp), and one exon (125 bp). The sequenced fragments varied in length, which was mostly the result of poor sequence quality at the 3′ and 5′ ends. Algal sequences were collapsed into 33 unique sequences using TCS v1.18 (Clement *et al.*, 2000).

Phylogenetic analyses – data and topological congruence

To determine if data partitions (algal ITS and actin; fungal ITS and mtSSU) were congruent, the 33 sequence algal dataset was reduced to include only algae with corresponding ITS and actin sequences, resulting in an alignment of 19 algae. Similarly, the 25 individual *Lepraria* fungal dataset was reduced to include 20 individuals with corresponding ITS and mtSSU sequences. The incongruence length difference (ILD) test (Farris *et al.*, 1994), which tests the null hypothesis of data congruence, was performed on the algal and fungal datasets in PAUP* 4.0b10 (Swofford, 2002), using a heuristic search of 1000 replicates with tree-bisection-reconnection (TBR) branch swapping and random taxon addition with 100 random addition replicates. In the ITS and mtSSU ILD test, a limit was imposed in which no more than 100 trees per replicate greater than length 1 were held at each step.

To determine if topologies between data partitions were congruent, heuristic searches were performed for the algal ITS and actin data partitions (using the 19 alga dataset), as well as the fungal ITS and mtSSU data partitions (using the 20 individual dataset) under the maximum parsimony (MP) criterion, using the same settings described for the algal ILD test. For each data partition, a strict consensus tree was constructed

from the most-parsimonious trees from each search. The consensus topology was then enforced as a constraint on the other data partition from that symbiont group, and a heuristic search was again performed (using the same settings). Trees obtained from the unconstrained and constrained searches were then compared by means of Templeton tests (Templeton, 1983).

Bayesian analyses were conducted on the individual and combined datasets for each symbiont group in MrBayes 3.0b4 (Huelsenbeck & Ronquist, 2001). The Akaike Information Criterion (AIC), as implemented in Mr ModelTest 2.2 (Nylander, 2004), was used to select the best-fit substitution model for the ITS, actin and combined data partitions for the algal group, and the ITS, mtSSU and combined data partitions for the fungal group. A Bayesian analysis for each partition was then run for 1 000 000 generations, at a temperature of 0.08 (algal ITS), 0.15 (algal actin and combined), 0.12 (fungal ITS and combined) or 0.09 (fungal mtSSU), using four chains and sampling every 100th tree. Initial trees were discarded for burnin. A majority-rule consensus tree was then constructed using all sampled postburnin trees in PAUP* 4.0b10 (Swofford, 2002). To test for congruence between partitions, the consensus topology obtained from the combined dataset for each set of symbionts was searched for in the 95% credibility interval of trees obtained from individual partitions (Buckley *et al.*, 2002). However, instead of using a 50% majority-rule consensus topology from the combined analysis, we used a 70% majority-rule consensus tree, to eliminate clades with very weak support. The presence of this topology in the credibility intervals was then taken as evidence for no significant incongruence between partitions (Buckley *et al.*, 2002).

If criteria for congruence were not met, the ITS datasets were analyzed individually and with the additional locus (actin or mtSSU).

Phylogenetic analyses – symbiont monophyly

A MP analysis was conducted on the 33 sequence algal dataset in PAUP*4.0b10, using the same settings as those described above, except that a limit was imposed which allowed for no more than 100 trees greater than length 1 to be saved in each replicate. Following this, 500 bootstrap replicates (Felsenstein, 1985) were performed using the same settings. A Bayesian analysis was performed on the same dataset using the methods described above-however, the analysis was run for 5 000 000 generations instead of 1 000 000, and the temperature was set to 0.08. Posterior probabilities were then mapped on to the most likely tree obtained from the Bayesian search.

We tested the hypothesis that sequences obtained from algal symbionts associated with *Lepraria* form a monophyletic group. This was accomplished by enforcing this hypothesis as a constraint in a MP analysis, and using an identical search to that described earlier. Unconstrained and constrained trees were then compared by means of Templeton tests (Templeton, 1983). This hypothesis was also tested in the Bayesian analysis by searching for this topology in the set of postburnin trees. The proportion of trees consistent with this hypothesis was taken as the probability of this hypothesis being correct.

We were careful not to include any *Lepraria* species shown to occur outside of *Lepraria* s. str.; however, we included species not sequenced in previous studies. Therefore, in the event that some of these taxa do not belong to *Lepraria* s. str., we excluded (from the 33 sequence dataset) any algae associated with *Lepraria* which did not have corresponding fungal sequences (taxa with fungal sequences have been shown to belong to *Lepraria* s. str. (M. P. Nelsen & A. Gargas, unpublished)). We then used the methods described earlier to test for the monophyly of algal symbionts associated with *Lepraria* (in this reduced dataset).

Actin sequences were missing for many taxa, and although this is not necessarily problematic (Wiens, 2006), we chose also to test for symbiont monophyly in the 19 sequence algal dataset to rule out any possibility of a negative effect from the incomplete dataset. Tests for monophyly followed those listed earlier.

Phylogenetic analyses – congruence between symbiont data and topologies

Fungal and algal sequences were generated from 25 *Lepraria* lichen thalli (Table S1). We determined if fungal and algal data and topologies were congruent. A single alignment containing sequence data from both symbionts (all four loci) was created, and ambiguous regions were deleted (similar to those described earlier). We then tested for data congruence using the ILD test as implement in PAUP*4.0b10, using the same settings described under data partition congruence, except that a limit was imposed whereby no more than 100 trees greater than length 1 were held at each replicate.

Maximum parsimony analyses were conducted on the fungal dataset, as well as the algal dataset, in PAUP*4.0b10, using the criteria described above, with no more than 100 trees greater than length 1 being held at each replicate. A strict consensus of MP trees was created for each symbiont group (fungi and algae). An identical heuristic search was then performed, with the strict consensus topology from the fungi enforced as a constraint on the algal dataset, and vice-versa. Trees from the constrained and unconstrained searches were then compared by means of Templeton tests.

Bayesian analyses were performed on each dataset (fungi and algae) as described under 'symbiont monophyly', with the temperature set to 0.1 and 0.12 for algal and fungal analyses, respectively. A 50% majority-rule consensus tree was created from all postburnin trees obtained from each symbiont. The hypothesis of topological congruence between fungi and algae was tested by searching for the consensus topology from the fungi in the set of postburnin trees for the algal dataset (and

vice-versa); the proportion of algal trees consistent with the fungal topology (and vice-versa) was taken as the probability of this hypothesis being correct.

To visualize fungal–algal associations, we condensed the algal (actin and ITS) and fungal (ITS and mtSSU) sequences into unique sequences. The algal symbiont from *S. botryosum* was added to the algal alignment and used as the outgroup, while *S. botryosum*, *S. subcoralloides* and *S. tomentosum* were added to the fungal alignment and used as the outgroup. Bayesian analyses were performed on the algal and fungal datasets as described under 'symbiont monophyly' and the temperature was set to 0.2 and 0.12 for the algal and fungal analyses, respectively. Trees were then compared to one another, and lines drawn connecting fungal and algal partners from the ingroup.

Results

Data and topological congruence among algal loci

The combined alignment of 19 algae consisted of 1168 characters, 331 of which were variable and 218 parsimony-informative. The ITS alignment had a length of 551 characters, of which 52 were variable and 29 parsimony-informative, while the actin alignment consisted of 617 characters, 279 of which were variable and 189 parsimony-informative. The MP analyses recovered 40 trees with a length of 75 steps for the ITS dataset, four trees with a length of 438 steps for the actin dataset, and 23 trees with a length of 517 steps for the combined dataset. Bootstrap analyses recovered five branches with support greater than or equal to 70 in the ITS analysis, while eight branches with bootstrap support greater than or equal to 70 were recovered in the actin and combined analyses. For the Bayesian analyses, the GTR + I + G model was recovered as the best-fit model for the ITS dataset, and the GTR + G model for the actin and combined datasets. Four branches with posterior probabilities (PP) greater than or equal to 0.95 were recovered in the ITS analysis, while nine branches with this degree of support were found in the actin and combined analyses.

The ILD test revealed no significant incongruence between algal data partitions ($P = 0.547$), and the Templeton test revealed no significant incongruence between algal ITS and actin topologies (ITS data with actin topology: $P = 0.2568$; actin data with ITS topology: $P = 0.2393$). Finally, the topology produced by the combined dataset in the Bayesian analysis was found in the 95% credible set of trees from the ITS and actin analyses, suggesting an absence of significant incongruence. Consequently, datasets were combined for analyses used to test the hypothesis of symbiont monophyly.

Data and topological congruence among fungal loci

The combined alignment of 20 fungal individuals consisted of 1358 characters, 166 of which were variable and 90 parsimony-informative. The ITS alignment had a length of 487 characters, of which 134 were variable and 75 parsimony-informative, while the mtSSU alignment consisted of 871 characters, 32 of which were variable and 15 parsimony-informative. The MP analyses recovered seven trees with a length of 181 steps for the ITS dataset, 1818 trees with a length of 35 steps for the mtSSU dataset, and 10 trees with a length of 216 steps for the combined dataset. For the Bayesian analyses, the GTR + G model was recovered as the best-fit model for the ITS dataset, the HKY + I model for the mtSSU, and the GTR + I + G model for the combined dataset.

The ILD test revealed no significant incongruence between fungal data partitions ($P = 1.000$), and the Templeton test revealed no significant incongruence between fungal ITS and mtSSU topologies (constrained and unconstrained topologies were of equal lengths for both data partitions). Finally, the topology produced by the combined dataset in the Bayesian analysis was found in the 95% credible set of trees from the ITS, but not from the mtSSU. The 50% majority-rule consensus trees from the ITS and combined datasets did not appear to be in conflict with that from the mtSSU, but the mtSSU tree had lower phylogenetic resolution, which may be the reason the exact topology obtained from the combined dataset was not found in the 95% credible set of trees from the mtSSU. This may be related to the small amount of phylogenetic information found in the mtSSU at this phylogenetic scale, relative to the ITS. We decided to combine the two loci, since the topologies were not in conflict in the Bayesian analysis and the Templeton tests failed to reject incongruence. However, in the event that the incongruence detected in the Bayesian analysis was significant, we also analyzed the fungal ITS dataset separately when comparing the fungal and algal topologies.

Algal symbiont monophyly

The alignment of 33 algae had a length of 1175 characters (ITS, 558; actin, 617), with 354 variable characters (ITS, 75; actin, 279), 224 of which were parsimony-informative (ITS, 35; actin, 189). The unconstrained MP analysis recovered 3027 trees with a length of 553 steps, while the constrained search recovered 9100 trees with a length of 605 steps. In the Bayesian analysis, the GTR + I + G model was found to be the best-fit for this dataset. Topologies produced from the MP and Bayesian analyses were largely congruent, with the exception of poorly supported branches.

All *Lepraria* individuals were found to associate with *Asterochloris* species as their photobiont. Several well-supported clades were recovered (Fig. 1), and despite poor support towards the base of the tree, photobionts associated with *Lepraria* were clearly not monophyletic. *Lepraria* photobionts were found in several clades, and a topology consistent with symbiont monophyly was rejected in both parsimony (Templeton test, $P \leq 0.0001$) and Bayesian ($P = 0.00$) analyses.

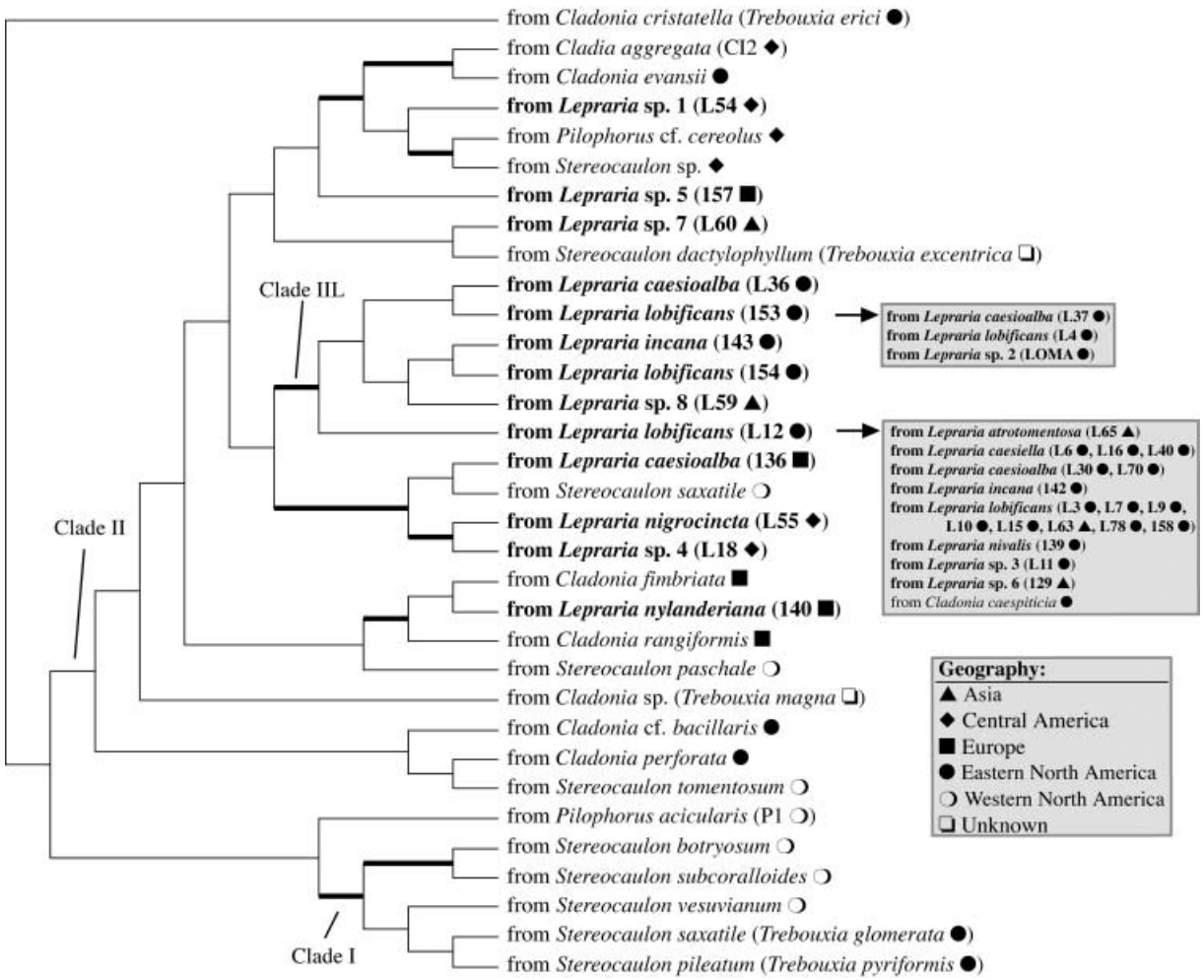

Fig. 1 The most-likely tree derived by Bayesian analysis of internal transcribed spacer (ITS) and actin sequences from *Asterochloris* algae. Branches with posterior probabilities ≥ 0.95 and maximum-parsimony bootstrap support of 70% or greater are in bold. The tree was rooted with an algal symbiont from *Cladonia cristatella* (*Trebouxia erici*), and algal sequences derived from *Lepraria* individuals are highlighted in bold face. Symbols following DNA number (listed in Supplementary material, Table S1) or species name denote the geographical origin of the specimen (see insert). Two algal sequences (from *Lepraria lobificans* (153) and from *L. lobificans* (L12)) were found in algae which associated with several fungal individuals and species; the additional fungal individuals and taxa which associated with these algae are shown in gray boxes to the right of the sequence used in the analysis. Clades I and II *sensu* Piercey-Normore & DePriest (2001) are shown.

When *Lepraria* symbionts without corresponding fungal sequences were excluded from the 33 alga dataset (results not shown), monophyly was again rejected in both the parsimony (Templeton test, $P \leq 0.0001$) and Bayesian ($P = 0.00$) analyses. The constrained MP analysis of the 19 alga dataset recovered 69 trees with a length of 561 steps (results of unconstrained search are listed above), and symbiont monophyly was also rejected in both the parsimony (Templeton test, $P \leq 0.0001$) and Bayesian ($P = 0.00$) analyses.

No *Lepraria* algal symbionts were found to occur in clade I (*sensu* Piercey-Normore & DePriest, 2001) of the most likely tree produced by the Bayesian analysis of the 33 algal dataset (Fig. 1). Instead, algal symbionts associated with *Lepraria* appeared to be restricted to the weakly supported clade II (*sensu* Piercey-Normore & DePriest, 2001). A number of *Lepraria* species associate with photobionts in a very strongly supported clade (clade IIL), which appears to occur nearly exclusively with *Lepraria*, with the exception of a single symbiont from *Cladonia caespiticia*. This clade consists of algal symbionts isolated from several species from a broad geographic range (eastern North America, Europe and China). All *Lepraria* individuals from eastern North America were found to associate with algae from this clade. Identical algal ITS and actin sequences (actin sequences were approximately five times as variable as the ITS sequences) were obtained from eastern North America and China. Additionally, two algae in this clade (from *Lepraria lobificans* (153 and L12)) in Fig. 1 were of special interest as they were recovered from a diverse range of *Lepraria* species (Fig. 1) and even *C. caespiticia*. *Lepraria* individuals from Costa Rica (*Lepraria nigrocincta*

(L55), *Lepraria* sp. 1 (L54) and *Lepraria* sp. 4 (L18)) associated with algae across clade II, with algal symbionts from *Lepraria* sp. 4 (L18) and *L. nigrocincta* (L55) forming a monophyletic group. *Lepraria* individuals from China (*Lepraria atrotomentosa* (L65), *L. lobificans* (L63), *Lepraria* sp. 6 (129), *Lepraria* sp. 7 (L60) and *Lepraria* sp. 8 (L59)) associated with algae found mostly in clade IIL, with one individual (*Lepraria* sp. 7 (L60)) occurring with an alga sister to a symbiont from *Stereocaulon dactylophyllum* (*Trebouxia excentrica*).

Topological congruence between fungal and algal phylogenies

Because of potential incongruence between the fungal ITS and mtSSU data partitions, algal/fungal congruence analyses were performed on both the combined fungal datasets, as well as the ITS alone. The fungal alignment (which contained individuals which had fungal ITS or fungal ITS and mtSSU sequences) consisted of 1366 characters (ITS, 487; mtSSU, 871), with 187 variable sites (ITS, 119; mtSSU, 30), 107 of which were parsimony-informative (ITS, 70; mtSSU, 15), while the algal alignment (which contained only individuals for which fungal sequences were obtained) consisted of 1157 characters (ITS, 548; actin, 609), with 174 variable sites (ITS, 26; actin, 148), 67 of which were parsimony-informative (ITS, 15; actin, 52).

The unconstrained MP analyses recovered 10 000 MP trees with a length of 208 steps for the algal dataset. The fungal ITS dataset resulted in 97 MP trees with a length of 250 steps, and the combined fungal dataset produced 86 trees with a length of 285 steps. When analyses were constrained, 3500 trees with a length of 303 steps were recovered for the algal dataset with the strict consensus from the combined fungal dataset imposed as a constraint, and 5700 trees with a length of 266 steps were obtained when constrained with the strict consensus from the fungal ITS dataset. When the fungal datasets were constrained with the strict consensus topology from the algae, 36 trees with a length of 333 steps were recovered for the combined fungal data and 48 trees with a length of 292 steps were produced by the fungal ITS dataset.

In the Bayesian analyses, the HKY + I model was selected as the best-fit for the algal dataset, while the GTR + I + G and GTR + G models were selected as the best fit for the combined and ITS fungal datasets, respectively. Majority-rule consensus topologies from Bayesian analyses were largely congruent with those from MP analyses. Consensus topologies from the combined and ITS datasets were identical, although posterior probabilities varied. Topological congruence between symbionts was rejected in MP analyses by means of Templeton tests for both the algal dataset with the combined and ITS fungal topologies ($P \leq 0.0001$), and the combined and ITS fungal datasets with the algal topology ($P \leq 0.0001$). Topological congruence was also rejected in the Bayesian analyses ($P = 0.00$ for all combinations).

When outgroups were included, the fungal alignment consisted of 25 sequences, with a length of 1394 sites (254 variable, 171 parsimony-informative), while the algal alignment consisted of 10 sequences, with a length of 1169 sites (251 variable, 79 parsimony-informative). The GTR + I + G and GTR + G models were found to be the best fit for the fungal and algal datasets, respectively. Figure 2 illustrates the most likely trees obtained from the Bayesian analyses of the combined algal and combined fungal datasets, with lines connecting fungal and algal symbionts.

Discussion

Asterochloris algae

Lepraria individuals associated with *Asterochloris* algae, consistent with Hildreth & Ahmadjian (1981) and Nelsen & Gargas (2006). Several *Trebouxia* species (*T. erici*, *T. excentrica*, *T. glomerata*, *T. irregularis*, *T. italiana*, *T. magna* and *T. pyriformis*) will be transferred to *Asterochloris* (Rambold *et al.*, 1998), but these taxonomic changes have not yet been formally made. These species differ from *Trebouxia* s. str. in several morphological characteristics (summarized in Helms, 2003), and molecular data confirms their separation (Friedl & Zeltner, 1994; Friedl & Rokitta, 1997; Piercey-Normore & DePriest, 2001). We include the *Trebouxia* species listed above in our definition of *Asterochloris*. *Asterochloris* frequently associates with Cladoniaceae and Stereocaulaceae (Rambold *et al.*, 1998; Persoh *et al.*, 2004; Miadlikowska *et al.*, 2006), but also with taxa outside these families (*Anzina* (Tschermak-Woess, 1980; Piercey-Normore & DePriest, 2001); *Diploschistes* (Friedl, 1987) and Porpidiaceae (Rambold *et al.*, 1998)). *Lepraria* individuals from eastern North America associated with algae from clade IIL (Fig. 1), and more *Lepraria* individuals and species from eastern North America should be examined to verify this trend. Figures 1 and 2 illustrate that algae with identical sequences (L12 and 153) occurred with several *Lepraria* species and another family (*C. caespiticia*, Cladoniaceae), which may be the result of distantly related fungi providing similar microhabitats for the same algae (Beck *et al.*, 2002).

Lack of pairwise codiversification

Codiversification is known from a number of vertically transmitted symbioses: ants, and the fungi they raise and farm (Chapela *et al.*, 1994; Hinkle *et al.*, 1994), endosymbiotic bacteria in clams (Peek *et al.*, 1998), and intracellular, endosymbiotic bacteria in cockroaches, termites and aphids (Clark *et al.*, 2000; Funk *et al.*, 2000; Lo *et al.*, 2003). However, Herre *et al.* (1999) discuss how the mode in which symbionts are transmitted (horizontal vs vertical) in ecological time does not necessarily translate to an evolutionary trend. Certain vertically and horizontally transmitted fungal endophytes

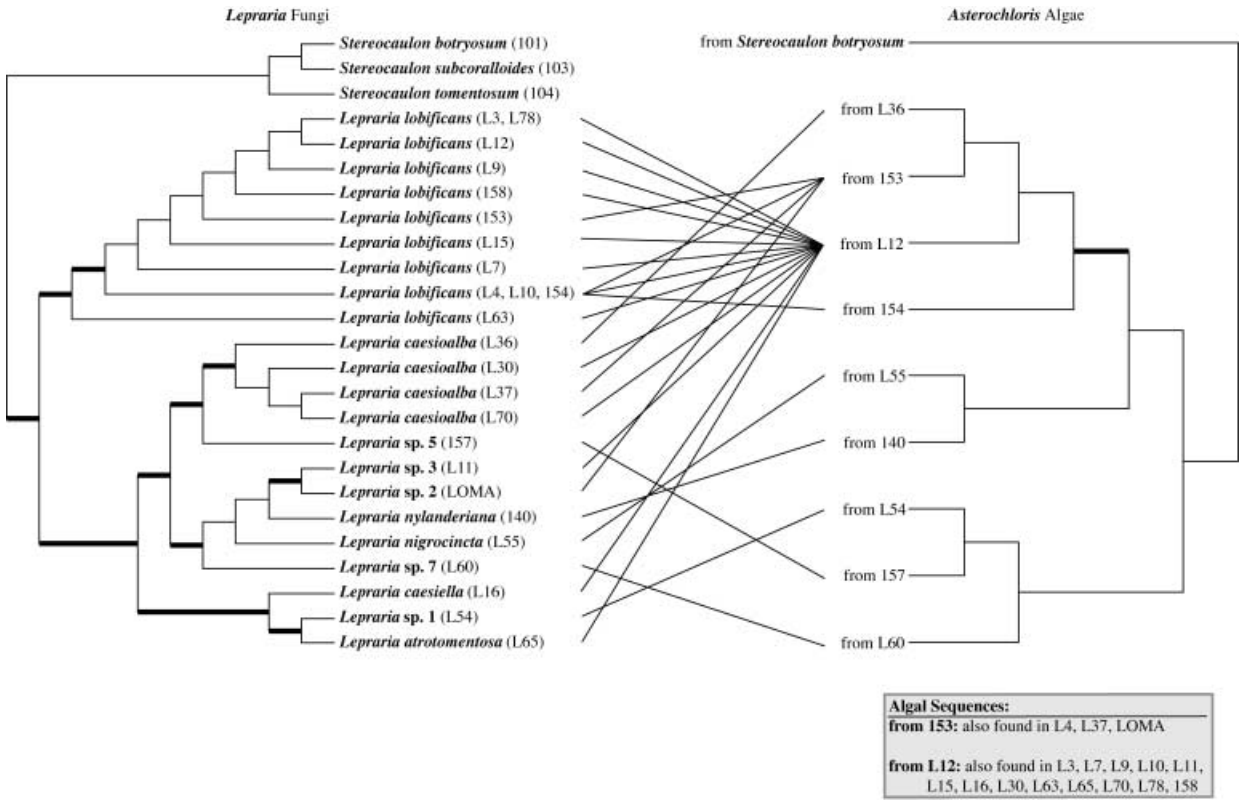

Fig. 2 A comparison between fungal–algal associations. The most-likely trees derived by Bayesian analysis of *Lepraria* fungal internal transcribed spacer (ITS) and mitochondrial small subunit (mtSSU) sequences and *Asterochloris* algal ITS and actin sequences are shown, with lines connecting fungal–algal associations. Branches with posterior probabilities ≥ 0.95 are in bold. The *Lepraria* tree is rooted with *Stereocaulon botryosum*, *S. subcoralloides* and *S. tomentosum*, and the *Asterochloris* tree with the algal symbiont from *S. botryosum*.

show evidence of codiversification (Schardl *et al.*, 1997), while other vertically transmitted fungal endophytes do not (Clay & Schardl, 2002). Additionally, in the fig-fig wasp symbiosis (which is horizontally transmitted in ecological time), mutualistic pollinating fig wasps have cospeciated with their fig hosts, while parasitic, nonpollinating fig wasps have not (Weiblen & Bush, 2002). A comparison of phylogenies from the maternally inherited, endosymbiotic, intracellular bacterial genus *Wolbachia* and its aphid hosts suggests that while symbionts are mostly transmitted vertically in ecological time, there is occasional switching (Werren & O'Neil, 1997; Herre *et al.*, 1999), with phylogenies between symbionts becoming less congruent as switching increases. The *Lepraria-Asterochloris* symbiosis appears somewhat similar to the *Wolbachia*-aphid symbiosis in that, while symbionts are generally presumed to be transmitted vertically in ecological time, they occasionally switch. Phylogenies of *Lepraria* fungi and *Asterochloris* algae do not show strong evidence of codiversification (Fig. 2), which is in agreement with previous studies of fungal-green algal lichens (Kroken & Taylor, 2000; Dahlkild *et al.*, 2001; Piercey-Normore & DePriest, 2001; Zoller & Lutzoni, 2003) and fungal-cyanobacterial lichens (Wirtz *et al.*, 2003; O'Brien *et al.*, 2005; Stenroos *et al.*, 2006; Myllys *et al.*, 2007) that produce independent spores.

Dissociation and symbiont-switching

Symbiont-switching could occur at several stages in the life cycle. Upon germination, soredia can fuse to form a single thallus (Jahns, 1972; Schuster *et al.*, 1985; Honegger, 1992), suggesting that the thallus may be composed of more than one fungal or algal individual. Hyphae from one fungus could potentially attach and penetrate algal cells it is not currently attached to. *Lepraria* species are known to grow intermixed, increasing the possibility of symbiont switching. Molecular studies of other lichen taxa have recovered multiple fungal (Murtaugh *et al.*, 2000; Robertson & Piercey-Normore, 2007) and algal (Bhattacharya *et al.*, 1996; Helms *et al.*, 2001; Romeike *et al.*, 2002; Guzow-Krzeminska, 2006; Piercey-Normore, 2006) genotypes or sequences from a single thallus; however, genetic heterogeneity within a thallus is not necessarily evidence for multiple individuals (Simon *et al.*, 2005; Robertson & Piercey-Normore, 2007). Additionally, some lichens are known to switch algae as they mature (Friedl, 1987), and the ability to harbour two distinct lichen photobionts has been discussed as an adaptation to changing environmental conditions such as shifts in light regimes (Piercey-Normore, 2006). We did not find multiple algae or fungi in a thallus, but feel it could potentially occur, and recommend that this be investigated

further in *Lepraria*. Intrahost algal heterogeneity is known among corals (Rowan & Knowlton, 1995; Little *et al.*, 2004), which associate with a wide range of algae during the juvenile stage, and later switch the dominant algal genotype (Little *et al.*, 2004).

Switching may also occur through the independent dispersal and re-lichenization of a symbiont, which initiates a new association between lineages. *Lepraria* does not produce specialized single-symbiont spores (such as ascospores or conidia), but *Lepraria* species produce a thick medullary layer composed solely of hyphae. If a portion of the medullary layer were able to disperse and acquire algae, it would provide another means by which switching could occur. Additionally, some *Lepraria* taxa have long fungal hyphae projecting from soredia, which could, perhaps, acquire new photobionts. These ideas, however, require further investigation. Independent dispersal may also occur as a result of symbiont mortality. Photobiont mortality presumably forces the mycobiont to re-lichenize or ultimately perish. Mycobionts have been found to survive for at least 1 yr in an unlichenized state (Etges & Ott, 2001), but it is generally believed they must re-lichenize in a short period of time, or associate with or parasitize incompatible algae until they meet their preferred partner (Honegger, 1992). In contrast, mycobiont mortality may not necessarily lead to the correlated death of its photobiont, as the occurrence of eukaryotic photobionts in the unlichenized state is well documented (Tschermak-Woess, 1978; Mukhtar *et al.*, 1994; Sanders & Lücking, 2002; Sanders, 2005; Hedenås *et al.*, 2007).

Advantages of dissociation and symbiont-switching

Codispersal has advantages, such as avoiding the perilous symbiont reassociation step in the life cycle, but it may also have disadvantages common to clonal lineages lacking recombination (Piercey-Normore, 2005). The inability to undergo sexual recombination is thought to lead to an accumulation of deleterious mutations in populations (Muller's Ratchet), and the failure to generate new genetic combinations capable of surviving selective pressures and a changing environment (Muller, 1964; Maynard-Smith, 1986). If a symbiotic association is unsuccessful, or if the symbionts encounter a new selective pressure that they are unable to survive, the maintenance of this association has a negative effect. The ability to switch partners could allow lineages such as *Lepraria* to colonize a new environment with different light or humidity regimes, as well as survive new selective pressures. Maintaining symbiotic associations may provide short-term advantages and allow rapid exploitation and colonization, but switching could permit a fine-tuning of the symbiosis, allowing these lineages to persist in a changing environment and survive over a longer timescale (as discussed in Buschbom & Mueller, 2006).

The advantages of associating with more than one partner are known from coral-algal symbioses. Algal symbionts affect the overall physiology of the coral symbiosis, with some algal symbionts leading to increased growth rates (Little *et al.*, 2004). Additionally, algae associated with corals display ecological preferences with respect to depth, and it may be more advantageous for the same coral to associate with different algae over an ecological gradient (Rowan & Knowlton, 1995). Similarly, Yahr *et al.* (2006) illustrated differences in the frequencies of associations between fungal and algal lineages with habitat/environmental variation in the lichen *Cladonia subtenuis*.

Switching may also be beneficial for escaping parasites or conferring resistance to parasites. Many lichen grazers and parasites are known (including parasites and grazers of *Lepraria* (Kümmerling *et al.*, 1993; Etayo & Diederich, 1998; Cole & Hawksworth, 2001; Czarnota & Kukwa, 2001; Lawrey & Diederich, 2003; Kukwa & Diederich, 2005; Prinzing, 2005)), and secondary metabolites appear to play a role in deterring them (Lawrey, 1980; Lawrey & Diederich, 2003; Benesperi & Tretiach, 2004; Gauslaa, 2004; Nimis & Skert, 2006). From the algal perspective, switching to a new fungal symbiont may be beneficial if the new symbiont produces secondary metabolites that prevent or deter grazing or infection and attack by lichenicolous fungi. It seems natural to ponder whether lichens with certain photobionts may be more susceptible or resistant to various parasites and grazers.

The inability to switch partners might be especially hazardous for clonal organisms. For the reasons discussed above, asexual lineages are thought to be evolutionary dead-ends (Poelt, 1970; Tehler, 1982) or short-lived over an evolutionary timescale (Maynard-Smith, 1986). However, cryptic recombination, previously unknown teleomorphic states and somatic recombination have been discovered in a number of seemingly asexual taxa (Pontecorvo, 1956; Tinline & MacNeill, 1969; Burt *et al.*, 1996; Paoletti *et al.*, 2005), and if future work detects recombination in *Lepraria*, it would provide another means by which new associations between fungal and algal genotypes can be formed. Even if recombination is detected, one might still expect the frequency with which new fungal–algal associations are forged to be lower in codispersing symbioses than in symbioses that typically disperse independently.

Conclusions

Lepraria fungi do not produce ascospores or conidia and are codispersed with their algal symbionts, yet the associations between *Lepraria* fungi and *Asterochloris* algae are not maintained over evolutionary timescales. Instead, a surprising amount of dissociation and re-lichenization seems to have occurred, suggesting that these relationships are unstable and break down over time. Symbiont-switching may potentially provide advantages similar to that of recombination, shuffling fungal and algal relationships, which may possibly help with the colonization of new habitats and surviving changing environmental conditions, parasitism and predation.

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Supplementary material

The following supplementary material is available for this article online:

Table S1 List of taxa used in this study, with collection information and GenBank accession numbers.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2007.02241.x
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