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# Strong fungal specificity and selectivity for algal symbionts in Florida scrub *Cladonia* lichens

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**Abstract**

**Symbiosis is a major theme in the history of life and can be an important force driving evolution. However, across symbioses, it is difficult to tease apart the mechanisms that structure the interactions among potential partners. We used genetic similarity and frequency-based methods to qualitatively and quantitatively examine the patterns of association among several co-occurring *Cladonia* lichen fungi and their algal photobionts in six disjunct Florida scrub sites. The patterns of association were described by the degree of specificity, i.e. the phylogenetic range of associated partners, and of selectivity, i.e. the frequency of association among partners. Six fungal species associated with only one algal internal transcribed spacer clade, with the remaining two fungi being associated with two algal clades. In all cases, the fungi associated in unequal frequencies with the observed algal photobiont genotypes within those clades — suggesting that both specificity and selectivity were higher than expected. Fungal species can be grouped into three significantly different specificity classes: photobiont specialists, intermediates and generalists. In contrast to the pronounced specificity for photobionts among fungal species, the different Florida scrub sites do not harbour distinct photobiont pools, and differential photobiont availability cannot explain the patterning of lichen associations at this spatial scale. Therefore, we conclude that fungal specificity and selectivity for algal photobionts are major factors in determining the local composition of symbiotic partnerships.**

*Keywords*: *Cladonia*, Florida scrub, photobiont, selectivity, specificity, symbiosis

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## Introduction

Symbiotic organisms, those living in close association, are ubiquitous. However, the scales (e.g. taxonomic, temporal and spatial) of their interactions and the mechanisms that control them vary, resulting in different patterns of association across symbioses (Paracer & Ahmadjian 2000). Patterns of association can be described in terms of specificity, the possible taxonomic range of acceptable partners, and selectivity, the frequency of association between compatible partners (Rambold *et al*. 1998); these two are not necessarily correlated. For example, low specificity illustrated by a host associating with several unrelated lineages of symbionts may be coincident with high selectivity, present in the form of the 'preferential' association with only one of those lineages. The examination of such patterns in a statistical and historical framework can offer clues to the processes that produce these patterns and that shape symbiont evolution (Page & Charleston 1998). For example, historical patterns such as cospeciation (Hafner & Nadler 1990; Currie *et al*. 2003) and taxonomic specificity (Pellmyr & Leebens-Mack 1999; Bidartondo *et al*. 2002) may arise as a result of adaptive coevolution (Thompson 1994), limited recognition capabilities (Miklashevichs *et al*. 2001), or strict codispersal (vertical transmission). Alternatively, non-historical patterns of association between symbionts may be caused by stochastic or local-scale interactions such as probabilistic association based on the availability of partners, fitness-based specialization for a subset of available partners, or frequent codispersal (Fox & Morrow 1981; Thompson 1999).

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Lichens are persistent associations between filamentous fungi and photosynthetic algae or cyanobacteria, in which these photosynthetic symbionts, called photobionts, live confined within the fungal structures (Nash 1996). The patterns of association between the partners in lichens are not well understood at either the fine taxonomic or the fine geographical scale. Classifications based on morphology have not been able to represent the amount of genetic diversity present in some green algal lineages (Kroken & Taylor 2000; Piercey-Normore & DePriest 2001), yet most of our understanding of symbiotic associations in lichens comes from empirical and literature surveys using these classification schemes (e.g. Ahmadjian 1993; Rambold *et al*. 1998). Several studies have specifically sought to understand the evolutionary processes underlying green-algal lichen associations across large taxonomic groups (Kroken & Taylor 2000; Helms *et al*. 2001; Piercey-Normore & DePriest 2001), where overall patterns of phylogenetic concordance were rejected for the studied groups. In addition, several recent field studies in cyanolichens (lichen fungi in association with cyanobacteria) have been conducted at smaller geographical (Wirtz *et al*. 2003) and taxonomic scales (Paulsrud *et al*. 1998, 2000, 2001). However, frequency-based population sampling has not yet been conducted in green-algal lichens to detect patterns within species at these finer scales.

Evolutionary processes were examined by Piercey-Normore & DePriest (2001) over long timescales in the lichen fungal genus *Cladonia* and its green-algal photobionts in the Asterochloris group of the genus *Trebouxia* (*sensu* Friedl in Rambold *et al*. 1998). In this study, cospeciation was rejected in favour of frequent algal switching, because few Asterochloris genotypes were shared among many unrelated species of *Cladonia* or among other fungal genera. Ecological mechanisms as drivers of symbiotic associations have also been tested in several extreme habitats where photobiont diversity might be limited by strong selective pressures. However, such explanations were ruled out in green-algal lichens for both heavy-metal-rich (Beck 1999) and marine rocks (Watanabe *et al*. 1997).

One of the most important mechanisms hypothesized to control symbiotic associations is the mode of symbiont transmission. Lichen symbionts are transmitted through both sexual and asexual forms of reproduction. Lichen fungi reproduce either sexually via spores, requiring *de novo* association with photobionts in each generation, or asexually with vegetative propagules containing both fungal tissue and photobiont cells. Honegger (1992) proposed the following model for the formation of symbiotic lichen structures. In the early development of both sexual and asexual lichens there is an undifferentiated thallus stage in which fungal–algal contacts are formed. During this phase, codispersed photobionts may be replaced by other photobionts that are available in the environment or even 'robbed' from other nearby lichen associations (Friedl 1987; Ott 1987). The frequency of such algal substitution or 'switching' in nature is unknown but this strategy may provide a certain level of flexibility in the early stages of symbiosis. Culturing of lichen fungi is feasible and has shown that fungi may even survive parasitically on incompatible or nonsymbiotic algae (Ahmadjian & Jacobs 1981), a strategy that may permit lichen fungi to exist in nature before contacting compatible algae. These studies have made important contributions to the study of lichen symbioses and provide a starting point for asking what mechanisms are responsible for structuring lichen associations in nature.

In the present study, we focus on local-scale, naturally occurring patterns of association in a distinct lichen community, both within and among geographically defined sites and among fungal species. In six Florida rosemary scrub sites, we studied a lichen community comprised of eight sympatric lichen fungi in the genus *Cladonia* and their green-algal photobionts. Community-level studies allowed us to survey the complement of all the *Cladonia* photobiont partners available among sites, while multiple photobiont samples per fungal species allowed us to quantify the photobiont variability associated with single species within and among sites. We describe the diversity of lichen photobionts in a single habitat type, Florida scrub, and assess specificity, the observed phylogenetic range of acceptable and available symbiotic partners, both qualitatively using clade-level measures and quantitatively using genetic diversity statistics. We test two hypotheses about photobionts in lichen associations: (i) photobiont availability determines the photobiont identity in lichens and (ii) fungal specificity and/or selectivity determines the photobiont identity in lichens. We present evidence to suggest that the pattern of photobiont associations is strongly linked to fungal species (fungal-species-level specificity). In addition, we find that photobiont pools — the diversity of available photobiont types in a site — are consistent among most Florida scrub sites, ruling out photobiont availability as an explanation for the pattern of lichen associations at this scale. These data can be used to explore the evolutionary mechanisms that generate fungal specificity and selectivity.

## Materials and methods

### *Ecological context*

We studied lichen communities that were typical of Florida rosemary scrub, a vegetation type characterized by open-structured low oak (*Quercus myrtifolia*, *Q. chapmanii*, *Q. inopina* and *Q. geminata*) and rosemary (*Ceratiola ericoides*) scrub. Florida scrub occurs on well-drained xeric sandy soils with very low fertility (Abrahamson *et al*. 1984) and is limited to three major disjunct regions: the central ridges of

the Florida peninsula, the Atlantic Coastal Ridge (ACR) and the North Gulf Coast (NGC) although isolated patches of scrub also exist. In these communities, lichens grow in mixed-species mats between shrubs on bare sand and increase in density following infrequent fires (Hawkes & Menges 1996).

### *Study species*

To sample the lichen associations in these sites, we used fungal species as our sampling units. A consistent suite of *Cladonia* species occurs in the open sand gaps of most rosemary scrub sites including *C. leporina* Fr., *C. pachycladodes* Robbins, *C. prostrata* A. Evans, *C. subtenuis* Abbayes, *C. evansii* Abbayes, *C. subsetacea* A. Evans, *C. perforata* A. Evans and *C. dimorphoclada* Robbins. These fungal species represent six of the 11 major lineages in the genus *Cladonia* (Stenroos *et al*. 2002). Each of these species differs in geographical distribution, although only *C. subtenuis*, *C. leporina* and *C. dimorphoclada* occur outside the southeast coastal plain. Based on the absence of spore-bearing structures, a few of the species are assumed to lack sexual reproduction, although none of these species bears specialized vegetative reproductive structures such as soredia. In all species, reproduction via vegetative fragments containing both partners is probably common. The poikilohydric nature of these terrestrial lichens means that trampling by animals including humans can result in branches being broken and dispersed when humidity is low, providing for a potentially important source of clonal reproduction.

### *Sampling strategy*

Lichen associations, including *Cladonia* fungi and their Asterochloris-group photobionts, were collected from six sites between January 1999 and January 2001. These six sites were distributed among the three regions, with four populations along the central ridges (LWRSF, CS, ABS-N and ABS-S), and one each in ACR and NGC. All sites were located in rosemary scrub and the limit of each population was determined by the limit of the habitat patch. All the sites were approximately 1 ha, except NGC, which covered an area of approximately 100 ha. For each *Cladonia* species present, we maximized distance between specimens, which consisted of a single thallus, to decrease the likelihood of sampling vegetative progeny. In each site, we sampled between 19 and 60 thalli, representing all *Cladonia* species present. Not all fungal species were present at each site. Over all the sites, we sampled between nine and 38 thalli per *Cladonia* species, with multiple samples of each species collected from each site (2–10 samples, average 4.7), and vouchers were deposited at Duke University. Collections from which photobionts were sampled are listed in the supplementary material.

### *Molecular methods*

Total genomic DNA, containing both fungal and algal photobiont DNA, was extracted from a single branch of each specimen (*c*. 10–25 mg) using the protocol of Piercey-Normore & DePriest (2001). Dilutions totalling between 2 and 5 ng of total genomic extracts per reaction were used as a polymerase chain reaction (PCR) template. Forward primers specific for either fungi (1780-5′F: 5′-CTG CGG AAG GAT CAT TAA TGA G-3′) or algae (1780-5′A: 5′-CTG CGG AAG GAT CAT TGA TTC-3′) (Piercey-Normore & DePriest 2001) and the universal reverse primer ITS4 (White *et al*. 1990) were used at 10 μM each to amplify the nuclear ribosomal internal transcribed spacer (ITS). Each PCR also included 0.1 mM dNTPs, and 2.5 units Klentaq with the manufacturer's buffer (AB Peptides). Fungal DNA was amplified using a 94 °C 3-min denaturation step followed by 27 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, with a 3-second auto-extension. The length of fungal ITS varied between 570 and 586 base pairs (bp) depending on species. Algal ITS (568 bp) was amplified using a 94 °C 3-min denaturation step followed by 35 cycles of 94 °C for 1 min, 50 °C for 30 seconds and 72 °C for 1 min. PCR products were purified using Millipore spin columns or Qiagen's QiaQuick kit. The PCR primers 1780–5′F or 1780-5′A and ITS4 were also used as sequencing primers using BigDye dye–terminator chemistry (Perkin Elmer) with a 10-ng PCR template and 2 μM primers. Sequences were determined on an ABI Prism 3700, edited and assembled using SEQUENCHER v3.1, exported into PAUP4.0*b10 (Swofford 2000) and aligned manually.

We sampled a total of 226 thalli and produced a total of 226 algal ITS sequences. We could confirm the fungal source using corresponding fungal ITS for 206 of these algal sequences. For the remaining 20 sequences, either we could not amplify the ITS or the sequences were of poor quality, so we trimmed our data set to the 206 samples for which we had both fungal and algal sequences. Approximately 10% of samples were chosen at random to resequence for data-checking. All of these samples were consistent with our original sequences.

Sequence alignments were produced separately for fungal and photobiont data sets. For the fungal ITS data set, all sequences were compared to a database of *Cladonia* ITS to verify field identifications. For the algal ITS, we used representatives of each of the distinct genotypes (supplementary material) and submitted representatives of newly produced sequences to GenBank, sequences AY712688–AY712711. For both fungal and algal aligned sequences, likelihood ratio tests were used to identify the best-fit model of sequence evolution using MODELTEST v3.06 (Posada & Crandall 1998). Bayesian phylogenetic analysis was conducted using MRBAYES vs. 3.0 (Huelsenbeck 2000). Model parameters were estimated in each analysis;

analyses began using random starting trees for 200 000 generations sampled every 100 for six complete runs. We examined plots of likelihood for each run to determine the number of generations required to reach a stationary state (burn-in). After discarding the trees collected during burn-in, we assembled all saved trees to calculate posterior probability as branch support. From this set of trees, we chose the tree with the lowest log-likelihood for illustration purposes. In addition, we assessed branch support by parsimony bootstrapping with 100 replicate heuristic searches using PAUP*4.0b10 (Swofford 2000).

A phylogeny of representative ITS sequences (GenBank accession numbers AY753583–AY753590) for each fungal species was estimated using Bayesian methods as above and an exhaustive search under maximum parsimony with default settings in PAUP. Gapped and ambiguous characters were removed, leaving an aligned length of 527 characters, of which 53 were parsimony informative.

We tested photobiont genetic differentiation using pairwise $F_{ST}$ (algal photobiont genetic structure among partitions) and the mean pairwise number of sequence differences, $\pi$ (Tajima 1993). Using the entire sample of photobiont sequences, we calculated pairwise $F_{ST}$ and compared it to the null hypothesis of random association using permutation tests in ARLEQUIN (Schneider *et al*. 2000), where *P*-values are the proportion of permutations leading to $F_{ST}$ values larger than those observed. These tests were used to examine two hypotheses of genetic structure of photobionts: that photobiont genetic structure is partitioned (i) according to site or (ii) according to fungal species. Values of $F_{ST}$ above 0.07 are generally thought to show substantial structure (Wright 1951). We also calculated the mean number of pairwise differences, $\pi$, to describe the diversity of partitions according to fungal partner. Sequence diversity of photobionts for each fungal species is treated as the measure of specificity for that fungus. Statistical *t*-tests were used to compare mean diversity measures and *P*-values were adjusted for multiple tests using the Bonferroni correction. The Bonferroni correction adjusts the threshold for significance in proportion with the number of tests performed to ensure that tests are conservative; in our case, when making 28 pairwise tests, *P*-values must be below 0.0017 to meet the 95% confidence limit. Three categories of photobiont specificity were designated based on significant differences in photobiont diversity among fungal species.

## Results

### *Photobiont diversity*

In our sampling of photobionts associated with *Cladonia* species of the Florida scrub, photobiont ITS diversity reflected only part of the phylogenetic diversity known for Asterochloris-group photobionts in *Cladonia* world-wide. Among the 206 Florida photobiont samples, we found 25 distinct ITS genotypes (Fig. 1). Although this number represents two-thirds of the number of genotypes known world-wide, the Florida genotypes represent only part of the total phylogenetic diversity. The genotypes were grouped phylogenetically into only three lineages, Clades I, IIa and IIb (Fig. 1), each of which contained many closely related genotypes. Clade I (identical to Clade I in Piercey-Normore & DePriest 2001) consisted of 16 genotypes with low sequence diversity (mean pairwise sequence difference, $\pi = 3.142 \pm 1.924$), only three types of which were found among the Florida samples ($\pi = 1.333 \pm 1.370$). In contrast, both Clades IIa and IIb (both of which are encompassed by Clade II of Piercey-Normore & DePriest 2001) contained more distinct genotypes and higher sequence diversity ($\pi = 6.667 \pm 3.737$, and $3.091 \pm 1.960$, respectively), and were largely restricted to Florida samples. Only Clade IIb was not supported as monophyletic in Bayesian analysis; Clades I and IIa were supported with more than 95% posterior probability and 88% bootstrap support. Each major clade contained at least one very common genotype (e.g. A, E, J, Q and U), and several derived types found only once or infrequently.

### *Photobiont patterns of association*

To address whether local photobiont availability determines patterns of association, we asked how photobiont pools and symbiotic associations differ among sampled scrub sites in Florida. Among all sites except NGC, which lacked Clade I genotypes (Fig. 2), the diversity and frequency of photobiont genotypes was similar and lacked geographical structure in photobiont diversity ($F_{ST} = 0.018$). In exact tests of $F_{ST}$ by site, one-third of comparisons showed significant differences — those between NGC and all other sites, and between ABS-N and ABS-S (Table 1). Genotype R was found only in these two sites, and was the most abundant genotype in ABS-N, where it supplanted the typically ubiquitous types Q and U. Several photobiont genotypes were found in only a single site, either within or among fungal species (genotypes C, K, O, W and X). However, these few site-restricted genotypes did not overwhelm the general pattern of homogeneous photobiont pools among most LWR and ACR sites (Table 2).

### *Fungal specificity for photobionts*

To address whether fungal specificity for photobionts determines patterns of association, we asked how faithfully fungal species predicts its associated photobiont genotypes and clades. For each fungal species, we used two correlated measures of photobiont specificity, (i) clade-level diversity,

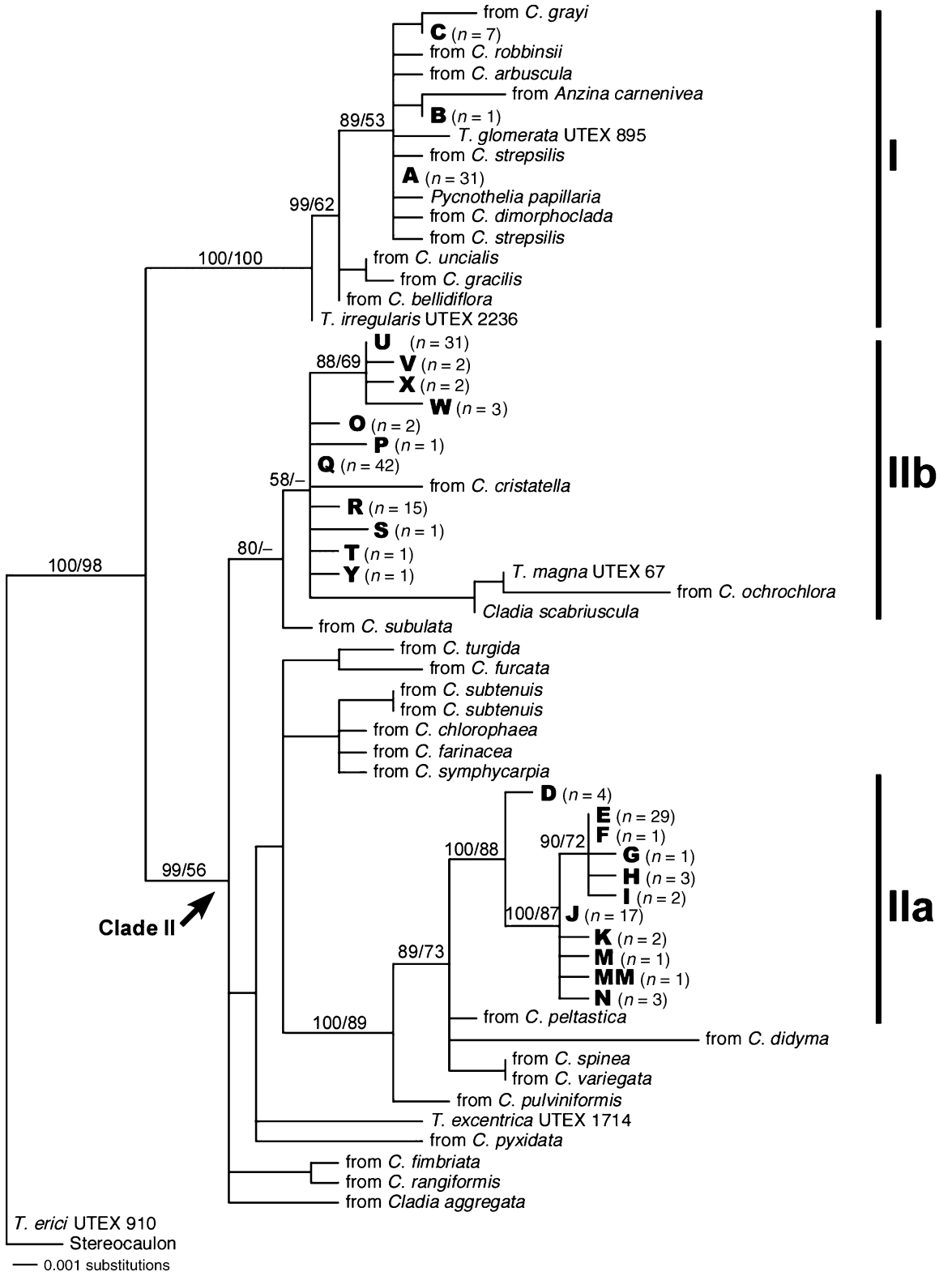


**Fig. 1** Maximum likelihood phylogeny of algal photobiont ITS genotypes from *Cladonia* and from cultures. Distinct Florida genotypes are denoted by bold letters, and clades with Florida photobiont genotypes are labelled I, IIa and IIb. Clade IIb has no statistical support and is named provisionally. Several genotypes without branch lengths, e.g. F, J, K, V, indicate differences due to short insertions (1–4 nucleotides) not considered in likelihood calculations. Branch support is shown as posterior probabilities followed by parsimony bootstrap values for the main branches. Dashes indicate bootstrap values less than 50%. Number of thalli sampled with each lettered genotype from this study are indicated after each letter as '*n* = '.

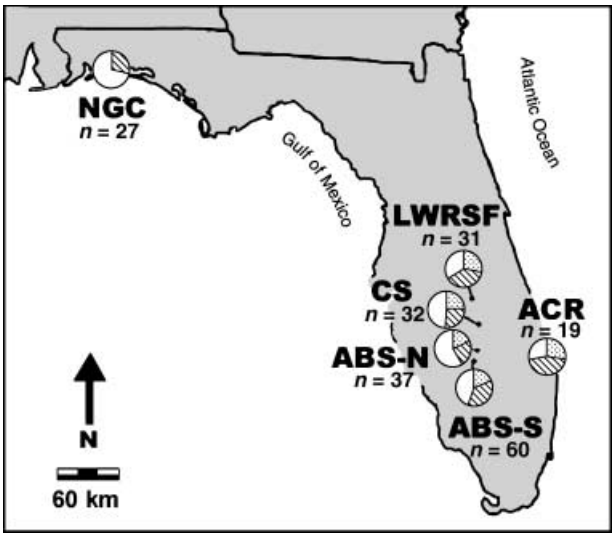


**Fig. 2** Map of sampled sites in Florida showing the frequencies of three photobiont clade types among all fungal species. Individual genotypes are classified as members of either Clade I (dark shading), Clade IIa (hatched), or Clade IIb (no shading). Each site has the same suite of eight *Cladonia* species except NGC, which lacks *C. dimorphoclada* and *C. subsetacea*. All sites are approximately 1 ha in size, except NGC, which is approximately 100 ha. Sample size is shown below each site name.

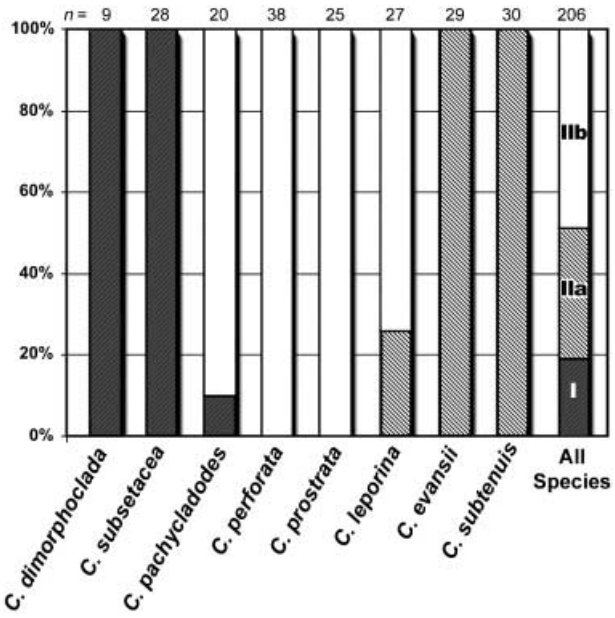


**Fig. 3** Per cent frequencies of photobiont clades for each of the eight *Cladonia* species and for all samples among all sites. Sample sizes are shown above the bars. Among the eight fungi, species within two groups do not differ significantly in photobiont pools (see Table 2), *C. dimorphoclada* and *C. subsetacea*, and *C. leporina*, *C. pachycladodes*, *C. perforata* and *C. prostrata*.

**Table 1** Comparisons of photobiont genetic differentiation by site

| | NGC | LWRSF | CS | ABS-N | ABS-S | ACR |
|---|---|---|---|---|---|---|
| NGC | 0.000 | | | | | |
| LWRSF | 0.069 | 0.000 | | | | |
| CS | **0.112** | −0.010 | 0.000 | | | |
| ABS-N | **0.077** | 0.012 | 0.049 | 0.000 | | |
| ABS-S | **0.071** | −0.007 | −0.007 | **0.044** | 0.000 | |
| ACR | 0.094 | −0.017 | −0.028 | 0.060 | −0.024 | 0.000 |

ARLEQUIN was used to test the structure of genetic variation across algal samples, using a Tamura & Nei model to calculate the distance matrix. Values are $F_{ST}$, or among-partition photobiont genetic variation, with bold entries significantly different from zero.

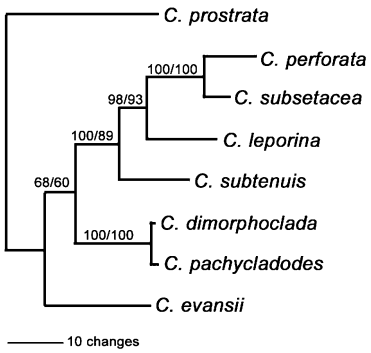


**Fig. 4** Maximum parsimony phylogeny of eight *Cladonia* study species. Branch support is shown by Bayesian posterior probability followed by parsimony bootstrap values.

the number of clades with which a species was associated in the sample, and (ii) sequence diversity, the mean number of pairwise differences among photobiont genotypes (π). High specificity is indicated by low clade and sequence diversity of photobionts. The degree of specificity varies among our sample of eight fungal species, with each species associating differentially with each of the three photobiont clades (Fig. 3, Table 2). Six of the sampled species (*C. dimorphoclada*, *C. evansii*, *C. prostrata*, *C. subtenuis*, *C. subsetacea*, and the endangered species *C. perforata*) each associated with a single clade of photobionts; the other two (*C. leporina* and *C. pachycladodes*) each associated with two clades (Fig. 3). Only two pairs of fungal species appeared to be closely related, *C. perforata* + *C. subsetacea*, and *C. pachycladodes* + *C. dimorphoclada* (Fig. 4). *Cladonia perforata* associated with Clade IIb, while *C. subsetacea* associated with Clade I, and even the sister species *C. dimorphoclada* and *C. pachycladodes* associated with different photobionts, Clade I only vs. Clade I and IIa, respectively.

We used π to classify fungal species into specificity groups according to the pairwise sequence diversity of the

**Table 2** Table of photobiont ITS genotype frequencies by sites and fungal species

| | Sorted by sites | | | | | | | Sorted by fungal species | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | ABS-S | ABS-N | CS CS | LW-RSF | ACR | NGC | Total | *C.d.* | *C.ss.* | *C.pa.* | *C.pe.* | *C.pr.* | *C.l.* | *C.e.* | *C.st.* | Total |
| Clade I | | | | | | | | | | | | | | | | |
| A | 11 | 8 | 8 | | 4 | | 31 | 7 | 23 | 1 | | | | | | 31 |
| B | | | 1 | | | | 1 | | 1 | | | | | | | 1 |
| C | | | | 7 | | | 7 | 2 | 4 | 1 | | | | | | 7 |
| Clade IIa | | | | | | | | | | | | | | | | |
| D | 1 | | 3 | | | | 4 | | | | | | | | 4 | 4 |
| E | 13 | 3 | 4 | 7 | 2 | | 29 | | | | | | 5 | 15 | 9 | 29 |
| F | 1 | | | | | | 1 | | | | | | | 1 | | 1 |
| G | | 1 | | | | | 1 | | | | | | | 1 | | 1 |
| H | | 1 | | 1 | 1 | | 3 | | | | | | | 1 | 2 | 3 |
| I | 2 | | | | 2 | | 4 | | | | | | 1 | 2 | 1 | 4 |
| J | 5 | | 4 | 1 | 3 | 4 | 17 | | | | | | 1 | 7 | 9 | 17 |
| K | | | | | | 2 | 2 | | | | | | | 1 | 1 | 2 |
| M | | | | | | 1 | 1 | | | | | | | | 1 | 1 |
| MM | | | | | | 1 | 1 | | | | | | | | 1 | 1 |
| N | 1 | 1 | 1 | | | | 3 | | | | | | | 1 | 2 | 3 |
| Clade IIb | | | | | | | | | | | | | | | | |
| O | | | | 2 | | | 2 | | | | 2 | | | | | 2 |
| P | | | 1 | | | | 1 | | | | | 1 | | | | 1 |
| Q | 7 | 2 | 3 | 8 | 4 | 18 | 42 | | | 7 | 13 | 10 | 12 | | | 42 |
| R | 2 | 13 | | | | | 15 | | | 3 | 10 | 2 | | | | 15 |
| S | | | | | | 1 | 1 | | | | | 1 | | | | 1 |
| T | | | 1 | | | | 1 | | | 1 | | | | | | 1 |
| U | 14 | 7 | 3 | 5 | 2 | | 31 | | | 5 | 13 | 8 | 5 | | | 31 |
| V | 1 | 1 | | | | | 2 | | | | | | 2 | | | 2 |
| W | | | 3 | | | | 3 | | | | | 2 | 1 | | | 3 |
| X | 2 | | | | | | 2 | | | 1 | | 1 | | | | 2 |
| Y | | | | | 1 | | 1 | | | 1 | | | | | | 1 |
| Totals | 60 | 37 | 32 | 31 | 19 | 27 | 206 | 9 | 28 | 20 | 38 | 25 | 27 | 29 | 30 | 206 |

*C.d.*, *Cladonia dimorphoclada*; *C.ss.*, *C. subsetacea*; *C.pa.*, *C. pachycladodes*; *C.pe.*, *C. perforata*; *C.pr.*, *C. prostrata*; *C.l.*, *C. leporina*; *C.e.*, *C. evansii*; *C.st.*, *C. subtenuis*.

Genotypes are grouped into clades by inferred phylogenetic relationships. Clade IIb has no statistical support. The same 206 samples are sorted by site and fungal species.

photobiont genotypes with which they were associated in this sample. Three specificity groups were described, for each of which the members were significantly different from the members of the two other groups, with $P < 0.001$ for all pairwise *t*-tests (Fig. 5). Fungi with both the lowest and intermediate values of pairwise sequence diversity among photobiont partners were termed photobiont specialists. Each of these groups of fungi associated with only a single clade of photobiont partners, but differed by which clade and the corresponding sequence diversity among genotypes. We therefore refer to these fungi as Clade I or Clade II specialists, with $\pi < 1$ and $1 < \pi < 2$, respectively. *Cladonia dimorphoclada* and *C. subsetacea* were Clade I specialists, while the four Clade II specialist species, *C. evansii*, *C. perforata*, *C. prostrata* and *C. subtenuis*, associated with photobionts from either Clade IIa or IIb, but not both. The third group comprised the photobiont generalists, *C. leporina* and *C. pachycladodes*. These species associated with photobionts from two separate clades (Fig. 3) and had significantly higher pairwise sequence diversity among their photobiont partners, $\pi > 4$ (Fig. 5).

Differences in photobiont pools among fungi can also be examined using $F_{ST}$, which simultaneously incorporates genetic similarity and frequency of genotypes. More than 85% of tests comparing the genetic structure of photobiont genotypes among fungal species showed significance greater than $P < 0.00001$ (Table 3, overall $F_{ST} = 0.795$). Although some fungi often shared the same algal genotypes,

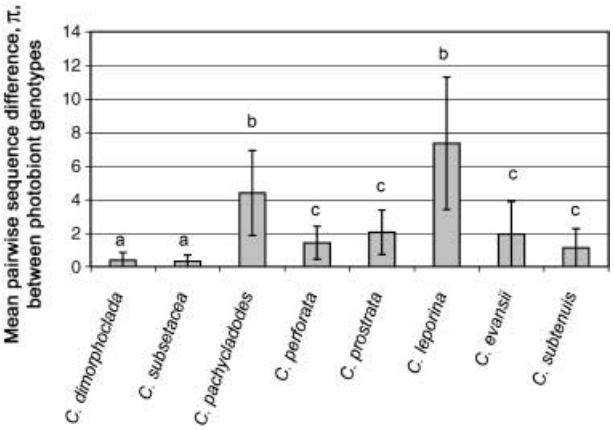


**Fig. 5** Diversity of photobionts by fungal partner, π, mean pairwise difference of photobiont genotypes. Error bars are standard deviations and different letters above bars indicate significantly different diversities according to *t*-tests, with $P = 0.0002$ after applying the Bonferroni correction. Two comparisons are only significant at the 0.001 and 0.01 level, respectively, *C. perforata* vs. *C. prostrata* and *C. subtenuis* vs. *C. leporina*.

e.g. A, E, J, Q, R and U, their frequencies of association with these genotypes differed, accounting for highly structured $F_{ST}$ partitions among species (Table 2). For example, *C. pachycladodes* associated with two major clades of photobionts found in Florida, but was more often found (90% of samples) with Clade IIb. Similarly, 75% of *C. leporina* associations were with Clade IIb and only about 25% were with Clade IIa. This pattern extends to the preference of fungal species for particular photobiont genotypes within clades for the six photobiont specialists (Table 2). In four cases, fungal species shared statistically equivalent photobiont pools, indicated by nonsignificant *P*-values in pairwise $F_{ST}$ tests (Table 3).

## Discussion

### *Specificity*

In our study of the ITS genotype of more than 200 photobionts from eight lichen fungi among six disjunct Florida scrub sites, we found strong patterns of fungal specificity (the phylogenetic range of potential partners) for their algal photobionts. Only two of eight species associated with more than a single phylogenetic clade of photobiont genotypes. In addition, fungi exhibit strong selectivity by associating in unequal frequencies with compatible algal photobiont genotypes and clades. In contrast, we found only weak patterns of differentiation among most of the Florida scrub sites. One site, NGC, was an obvious exception to this trend (Fig. 2), in that it lacked Clade I algal genotypes. However, this may be accounted for by the absence of two *Cladonia* species, *C. subsetacea* and *C. dimorphoclada*, which are Clade I specialists, in the site. Although these observations do not rule out ecological explanations of associations, taken together, they suggest a dominant role for specificity rather than geographical processes in structuring lichen symbioses in these Florida rosemary scrub sites. Despite using only a single genetic marker, ITS, specificity appears high and photobiont genotypes are very closely related. Resolution may be increased by adding other markers such as the introns in nuclear genes (e.g. actin introns in *Trebouxia*, Kroken & Taylor 2000), although such markers would be unlikely to change markedly the observed specificity at the clade level.

### *Evolutionary benefit vs. stochastic association*

Even with the overall pattern of homogeneous photobiont pools among sites, we found evidence for within-site

**Table 3** Comparisons of photobiont genetic differentiation by fungal partner

| | *C.d.* | *C.ss.* | *C.pa.* | *C.pe.* | *C.pr.* | *C.l.* | *C.st.* | *C.e.* |
|---|---|---|---|---|---|---|---|---|
| *C. dimorphoclada* | 0.000 | | | | | | | |
| *C. subsetacea* | −0.054 | 0.000 | | | | | | |
| *C. pachycladodes* | **0.791** | **0.862** | 0.000 | | | | | |
| *C. perforata* | **0.926** | **0.943** | 0.034 | 0.000 | | | | |
| *C. prostrata* | **0.911** | **0.938** | 0.030 | 0.016 | 0.000 | | | |
| *C. leporina* | **0.713** | **0.794** | **0.103** | **0.203** | **0.165** | 0.000 | | |
| *C. subtenuis* | **0.934** | **0.952** | **0.823** | **0.900** | **0.887** | **0.636** | 0.000 | |
| *C. evansii* | 0.967 | 0.975 | 0.859 | 0.927 | 0.920 | 0.677 | 0.077 | 0.000 |

*C.d.*, *Cladonia dimorphoclada*; *C.ss.*, *C. subsetacea*; *C.pa.*, *C. pachycladodes*; *C.pe.*, *C. perforata*; *C.pr.*, *C. prostrata*; *C.l.*, *C. leporina*; *C.e.*, *C. evansii*; *C.st.*, *C. subtenuis*.

ARLEQUIN was used to test the structure of genetic variation across algal samples, using a Tamura & Nei model to calculate the distance matrix. Values are $F_{ST}$, or among-partition photobiont genetic variation, with bold entries significantly different from zero.

patterns that may point to the possibility of ecological specialization or local adaptation. For example, several photobiont genotypes were restricted to a single site, either within or among fungal species (genotypes C, K, O, W and X). Thompson (1999) has suggested that such patterns are consistent with local specialization. For example, the frequency of a newly derived algal genotype may increase in a given site because it confers greater benefit to its fungal symbionts or because it is a better competitor in that site. Alternatively, such patterns may only arise from stochastic processes like drift and dispersal.

Our data offer two cases that may suggest either local specialization or stochastic processes. (i) Algal genotype C appears to fit a scenario of specialization. This genotype occurred only in site LWRSF, where all Clade I specialist fungi associated with this genotype exclusively. In most other sites Clade I specialists associated with algal genotype A. One interpretation is that other Clade I genotype photobionts, such as genotype A, are not available in the site because they never dispersed there. In fact, this explanation seems unsatisfactory because the Clade I genotype A is widely dispersed, having been found in at least 16 fungal species (of *Cladonia* and *Sterocaulon*) across North America and Japan (Piercey-Normore & DePriest 2001 and this study), occurs in nearby site CS, and is most probably locally available. (ii) Algal genotype R frequencies appear to fit a scenario of chance association. Algal genotype R is found in both sites ABS-N and ABS-S and accounts for the significant site-level differences between them. This genotype differs significantly between these sites in its frequency of association with particular fungal species. For example, *C. perforata* associates with only genotype R in ABS-N, and with only genotype U in ABS-S (Table 2). Reducing or removing *C. perforata*-associated genotypes from the analysis eliminates the significant site-level differences. Since *C. perforata* is predominantly a clonally reproducing species, these photobiont differences may reflect only different chance founder events in the sites. Still, these few site-restricted genotypes do not overwhelm the general pattern of homogeneous photobiont pools among most LWR and ACR sites (Table 2). With the exception of site NGC, both clade and genotype diversities of photobionts in each sampled site are indistinguishable from those of a random sample of genotypes available in these Florida sites.

Although photobiont pools do not vary significantly among most sites when all *Cladonia* species are examined together, they do vary among sites for individual fungal species. Even with our low sampling at this geographical scale (usually between three and five samples per site), *C. subsetacea*, *C. perforata*, *C. leporina* and *C. subtenuis* had significant site-level differences in their photobiont genotypes using analysis of molecular variance and pairwise $F_{ST}$ (data not shown). Preference for a particular genotype at a given site (e.g. Law & Lewis 1983; Bruns *et al*. 2002), or specialization for one of several possible compatible photobionts is consistent with short-term processes of adaptive evolution. Either a fungus is adapted to a particular photobiont in a site, or a photobiont is adapted to a particular site. It is possible that the coastal plain species studied here have specialized in a relatively short time, because Florida and its scrub habitats have existed *in situ* for only approximately 2 million years (White 1970).

### *Fungal specificity, selectivity and evolutionary specialization*

Of the eight fungal species sampled, six were photobiont specialists, showing specificity for a single clade of algae, and two were photobiont generalists, showing associations with divergent clades of photobiont genotypes. However, all of the species, whether photobiont specialists or generalists, showed a strong pattern of population structuring and selectivity for algal genotype, or clade in the case of the photobiont generalists. This selectivity may be a product of genetically determined physiological tolerance for certain partners, which could be tested experimentally (e.g. Schaper & Ott 2003). In contrast, these patterns may be the product of the reproductive mode of the fungus, or a flexible response to the environment. For example, specialized lichen propagules may codisperse the fungus with one of its photobiont partners (vertical transmission), resulting locally in unequal frequencies of some pairs of symbionts. Codispersal at first seemed a likely mechanism for the generation of these patterns because reproduction in many *Cladonia* species is presumed to be predominantly via vegetative fragmentation; however, selectivity is not limited to species that reproduce exclusively by asexual modes.

Furthermore, a comparison of fungal and algal genotypes among sites (Table 2) shows combinations of genotypes that can only arise from occasional horizontal switching. For example, algal genotype K is found only in NGC where it is shared by both *C. evansii* and *C. subtenuis*. The simplest explanation for the distribution of this genotype, is that it has being taken up — switched to — by one or both of these fungi in that site. Therefore, it seems likely that that the selectivity must be explained, at least in part, by factors other than strict vertical transmission through vegetative reproduction and clonal populations. An alternative mechanism, evolutionary specialization of one lineage for another (i.e. fungal specialization for suitable algal photobionts), can also produce the observed unequal frequency of association among suitable algal types.

Evolutionary specialization occurs when a symbiont adapts to and attains increased benefit from a host when that host is readily available (Douglas 1998; Bruns *et al*.

2002). This strategy may be employed by each of the six photobiont specialist fungi in this study, because their photobionts appear common, and therefore probably available, in the sites. For example, *C. dimorphoclada* and *C. subsetacea* are highly specific for photobionts found world-wide in several *Cladonia* species (Piercey-Normore & DePriest 2001), while *C. perforata*, *C. prostrata*, *C. subtenuis* and *C. evansii* are specific to photobiont lineages which appear to be restricted in distribution, but are very frequent in the sampled sites. Strong specificity in lichens has also been suggested by some studies of green-algal lichens (Kroken & Taylor 2000; Helms *et al*. 2001) and cyanolichens (Paulsrud *et al*. 1998, 2000; however, see Rikkinen *et al*. 2002; Wirtz *et al*. 2003). This pattern of specificity may arise as adaptive specialization and may eventually result in physiological or genetic constraints for symbiotic pairings (Wilkinson *et al*. 1996; Bidartondo & Bruns 2002; Desdevises *et al*. 2002) and sometimes codiversification (Currie *et al*. 2003; Pellmyr & Leebens-Mack 1999). In our study, despite this strong species-level specificity, algal genotypes mapped onto phylogenies of individual fungal species show no evidence of further specificity at the subspecific fungal level (data not shown).

Even generalist species, associating with multiple and divergent algal genotypes, may be evolutionarily specialized. This would allow symbionts to take advantage of the differential fitness of potential partners among differing sites or environments (Bruns *et al*. 2002). Photobiont generalists, such as *C. leporina* and *C. pachycladodes*, associate preferentially with only one of the two clades with which they are found and therefore show strong selectivity for only a subset of suitable algal photobionts. It is important to note that the observed specificity may differ with sampling effort and location because different photobionts may be available, or may offer variable fitness benefits, among different habitats or microsites. Sampling outside the range of Florida scrub may reveal new associations with respect to the photobiont partners of a given fungal species. In contrast, the distribution of specialist fungi may be limited by the distribution of their photobionts. For example, a range extension of the endangered lichen *C. perforata* might not be possible because it appears to be a specialist for photobionts that are currently known only from Florida.

### *Conclusions*

The genus *Cladonia* illustrates the multiple scales at which symbiotic interactions occur. Short-term ecological interactions may result in local specificity and specialization as a product of local adaptation (Thompson 1999). In contrast, over deeper evolutionary scales, horizontal sharing may link ecologically similar groups of related species interacting within communities (Thompson 1989). For example, *Cladonia* is apparently highly specific for Asterochloris-group algae, whereas individual species and even genotypes from across the genus may range from high to low specificity for individual clades or genotypes of the Asterochloris group. In addition, photobiont pools appear to be shared among fungi, with the same algal genotypes found with several fungal species both within and among sites. Within sites, some *Cladonia* species may be selective for a particular genotype of the Asterochloris group. The relatively shallow evolutionary timescale in this study suggests that we may have detected a rapid, local evolutionary process.

Our investigation of the *Cladonia* species of rosemary scrub also provides a context for understanding other photobiont-containing symbioses, e.g. in different lichens (Beck *et al*. 1998; Paulsrud *et al*. 1998; Kroken & Taylor 2000) or invertebrates (LaJeunesse 2002). We demonstrate a range of specificity between *Cladonia* lichen fungi and their algal photobionts that varies with the studied scale of interaction. At long evolutionary timescales, the genus *Cladonia* is specific for the Asterochloris group of the genus *Trebouxia*, but this strict specificity does not necessarily scale down to the fungal-species level. Interestingly, this specificity shows a strong taxonomic effect without a pattern of cospeciation, supporting a strong role for specialization for particular algal photobionts after diversification of these fungal species. We propose that fungi may select their favourite algal photobiont from the locally available pool.

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## Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2350/MEC2350sm.htm

**Appendix S1.** List of fungal taxa and collections or cultures used for sequencing of algal nuclear rDNA ITS, with ITS genotype. Culture or collection data includes country, state and site (for Florida scrub sites only), collection date, collection number and location of voucher (in parentheses). GenBank accession numbers are listed for representative sequence types.

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