Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis

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Summary

- Multiple interacting factors may explain variation present in symbiotic associations, including fungal specificity, algal availability, mode of transmission and fungal selectivity. To separate these factors, we sampled the lichenized *Cladonia subtenuis* and associated *Asterochloris* algae across a broad geographic range.
- We sampled 87 thalli across 11 sites using sequence data to test for fungal specificity (phylogenetic range of association) and selectivity (frequency of association), fungal reproductive mode, and geographic structure among populations. Permutation tests were used to examine symbiont transmission.
- Four associated algal clades were found. Analysis of molecular variation (AMOVA) and partial Mantel tests suggested that the frequency of associated algal genotypes was significantly different among sites and habitats, but at random with respect to fungal genotype and clade. The apparent specificity for Clade II algae in the fungal species as a whole did not scale down to further within-species lineage-dependent specificity for particular algae. Fungal genotypes were not structured according to site and appeared to be recombining.
- We suggest that ecological specialization exists for a specific lichen partnership and a site, and that this selectivity is dynamic and environment-dependent. We present a working model combining algal availability, fungal specificity and selectivity, which maintains variation in symbiotic composition across landscapes.

Key words: symbiotic association, environmental specialization, *Cladonia subtenuis* (Cladoniaceae), lichenized fungus, specificity, selectivity.

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Introduction

The ecology and selective environment of all organisms include biotic interactions. Across these interactions, the processes shaping patterns of association among organisms are major forces shaping biodiversity. For example, strong specificity observed in insect herbivores in tropical forests has been suggested to be responsible for the huge diversity of herbivorous beetles (Erwin, 1982). Similarly, high estimates of fungal diversity (1.5 million species) originate from inferences about narrow specialization with plant hosts (Hawksworth, 2001). Roughly half the true fungi are known to be symbiotic with plants, as mycorrhizae, lichens or pathogens.

Specialization in symbioses can arise independently through direct selection on one or both partners, or via indirect selection on the interacting partners as a selective unit (the holobiont). However, the mechanisms shaping the associations in these symbioses are only beginning to be unravelled, and the relative contributions of phylogenetic history vs environmental variation are poorly understood in most groups.

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Lichens are a classic example of symbiosis, and an important and conspicuous part of biodiversity, representing approximately half the known ascomycetes and totaling more than 16 000 fungal species (Eriksson, 2006). The symbiont composition and diversity of lichen associations is poorly known, however. Although major macroevolutionary patterns (such as cospeciation and phylogenetic congruence) have been rejected for green algal (Kroken & Taylor, 2000; Piercey-Normore & DePriest, 2001) and cyanobacterial lichens (Rikkinen *et al.*, 2002; Lohtander *et al.*, 2003; Wirtz *et al.*, 2003), many fungal genera are found in nature only with specific photobiont genera (DePriest, 2004). In addition, many lichen species-level associations appear consistent (Paulsrud *et al.*, 1998; Kroken & Taylor, 2000; Paulsrud *et al.*, 2001), even across large geographic scales (Paulsrud *et al.*, 2000). Taken together, these results clearly demonstrate a role for at least some phylogenetically constrained associations in lichen fungi.

In addition to phylogenetic constraints on symbiotic associations, ecological mechanisms may also contribute to evolutionarily correlated patterns of association. For example, environmental variation (for example in geographic position, temperature or light conditions) correlates with temporally and spatially variable associations among horizontally transmitted symbionts in hard corals (Rowan & Knowlton, 1995; Rowan *et al.*, 1997; LaJeunesse & Trench, 2000; van Oppen *et al.*, 2001) and fungi (Taylor & Bruns, 1999). In these examples, associations with different symbionts may act to maintain variation among generalist host populations, allowing selective specialization for particular environments or conditions by associating with and exploiting symbionts differentially. In lichen associations, population-level studies indicate that lichen fungi specialize on particular algae despite the availability of other species (Beck *et al.*, 1998; Beck *et al.*, 2002; Romeike *et al.*, 2002; Yahr *et al.*, 2004), or form 'ecological guilds' of lichen fungi linked through shared photosynthetic partners (Rikkinen, 2003). Few studies have examined simultaneously the relative contributions of several possible mechanisms responsible for structuring symbioses. The elimination of one or more possible mechanisms can be achieved by taking an explicitly geographic approach, and by varying environmental factors (Brodie *et al.*, 2002).

Studies of other symbiotic systems provide a set of testable expectations for green algal–lichen associations, focusing on the roles of both phylogenetic and ecological hypotheses in determining the composition of the holobiont in nature. For example, at a single site a fungal species may be limited to a narrow group of algae by several possible mechanisms. First, dispersal limitation or strict vegetative reproduction may lead to a lack of, or infrequent, horizontal transmission, as seen in bacterial endosymbionts (Saffo, 1992; Moran & Baumann, 1994; Huigens *et al.*, 2000), grass endophytes (Schardl *et al.*, 1997, 2004) or vertebrate parasites (Clayton & Johnson, 2003). Second, phylogenetic constraint, or a narrow genetically defined compatibility, may limit potential interactions, as observed in hard corals (Santos *et al.*, 2004), fish parasites (Desdevises *et al.*, 2002) and mycorrhizal fungi (Bruns & Read, 2000; Bidartondo & Bruns, 2001, 2002). Third, ecological dominance, or the increased probability of encountering a single compatible type of symbiotic partner because of its high local frequency or fitness in a site, may result in a limited set of associations, as suggested for some coral communities (Rowan *et al.*, 1997; LaJeunesse & Trench, 2000; Toller *et al.*, 2001). Fourth, selectivity, or the symbiont's preference for a particular host in a particular site or habitat, may allow the symbiont to take advantage of variable fitness benefits of different hosts (Thompson, 1994; Desdevises *et al.*, 2002). Finally, random processes, including drift, lineage sorting and historical artifacts, may result in stochastic associations not related to any of the above factors. Few of these hypotheses have been examined explicitly in green algal lichens.

In lichens, the mechanism for symbiotic contact and thallus formation in nature is only partially understood. Honegger (1992) proposed a model for formation of symbiotic lichen structures in early developmental stages. In this model, an undifferentiated thallus stage exists in which fungal–algal contacts are formed in all sexual, and some asexual, lichens. Even in the case of asexual reproduction, where both partners are codispersed in specialized structures, de-differentiation (separation of algal and fungal partners) allows codispersed algae to be replaced by others available in the environment, or even 'robbed' from other nearby lichen associations (Friedl, 1987). The frequency of such algal substitution or 'switching' (Piercey-Normore & DePriest, 2001) in nature is unknown, but this strategy may provide a certain level of flexibility in early stages of symbiosis and, importantly, a mechanism for optimizing symbiotic composition in a local environment.

Cladonia species are symbiotic with green algal photobionts in the Asterochloris group of the genus *Trebouxia* (*sensu* Friedl, in Rambold *et al.*, 1998, hereafter referred to as Asterochloris). The lichen fungus *Cladonia subtenuis*, used as a model in the present study, provides the opportunity to test evolutionary and ecological mechanisms producing variable patterns of specificity and selectivity between symbionts. In Florida habitats, *C. subtenuis* was observed to associate with a single lineage of algae in Asterochloris Clade IIa (Yahr *et al.*, 2004), appearing to have relatively high specificity for this algal partner. This lineage, Clade IIa Asterochloris, has at the present time been found only in Florida, although the diversity and distribution of *Asterochloris* lineages is in general poorly known. Intriguingly, however, *C. subtenuis* is distributed over much of eastern North America, suggesting that this fungus may form associations with other algae in different regions.

In the present study, we investigate the geographic structure of genotypic variation among algal partners that are associated with *C. subtenuis*, and we compile and analyze data to create a conceptual model of factors responsible for structuring lichen associations. First, we compare fungal specificity across different geographic and taxonomic scales of study, comparing

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Table 1 Pairwise F_{ST} for nrITS of *Asterochloris* algal pools among sampling sites

	GA $n = 6$	FL-3 $n = 11$	FL-1 $n = 3$	FL-2 $n = 21$	NC-1 $n = 6$	SC $n = 7$	NJ $n = 2$	NC-2 $n = 7$	OZ $n = 6$	PA $n = 6$	VA $n = 9$
GA	0.000										
FL-3	0.051	0.000									
FL-1	−0.088	0.011	0.000								
FL-2	−0.002	0.143	−0.019	0.000							
NC-1	0.275	0.082	0.175	**0.476**	0.000						
SC	0.377	0.192	0.239	0.617	−0.206	0.000					
NJ	0.984	0.799	0.987	**0.968**	0.369	0.042	0.000				
NC-2	**0.950**	**0.814**	**0.943**	**0.953**	**0.491**	0.281	0.239	0.000			
OZ	**0.892**	**0.801**	**0.864**	**0.934**	**0.523**	0.386	0.216	0.136	0.000		
PA	**0.756**	**0.622**	**0.697**	**0.855**	**0.225**	0.003	−0.163	0.039	0.152	0.000	
VA	**0.880**	**0.790**	**0.859**	**0.921**	**0.529**	0.383	−0.003	−0.020	−0.075	0.074	0.000

Significantly positive values are shown in bold ($P < 0.05$). Comparisons across habitats are shaded. Overall $F_{ST} = 0.689$, $P = 0.00000$.
GA, Georgia; FL, Florida; NC, North Carolina; SC, South Carolina; NJ, New Jersey; OZ, the Ozark region of Missouri and Arkansas.

the results of Yahr *et al.* (2004) with this study; second, we test hypotheses concerning the roles of phylogenetic constraint, clonal reproduction, ecological dominance, selectivity, or random processes in explaining observed patterns of association. In particular, we (1) examine phylogenetic associations among lineages of *C. subtenuis* and its algal partners; (2) test for clonal reproduction and evidence of recombination in *C. subtenuis* and for clonal transmission of algal partners; and (3) investigate geographic variability across eastern North America in algal associations with *C. subtenuis* and other *Cladonia* species to seek evidence for ecological dominance of algal lineages. Random associations serve as our null hypotheses for each of these proposed mechanisms. Our goal was to produce a conceptual model to integrate the various factors that may explain observed patterns of association in *C. subtenuis*. This model may also be applied to other lichens.

Materials and Methods

Study species

Cladonia subtenuis (Abbayes) Mattick is an endemic of the West Indies and eastern North America, ranging from Florida westward to the Ozarks and north to the upper mid-west and Nova Scotia (Ahti, 1984). Populations of *C. subtenuis* can be found from open, acid, sandy soils in the coastal plain to granite outcrops and open pine forests over poor soil inland. Populations increase in density following fire (Hawkes & Menges, 1996) or disturbance. In open situations, it can occur with several other terrestrial lichen species, including several *Cladonia* species (Evans, 1952; Yahr, 2000).

Sampling

We sampled between three and 10 *C. subtenuis* thalli from each of 11 sites, in two habitats: coastal plain scrub and high pine vs inland pine forests (Table S1 in Supplementary Material). Collections were made between 2001 and 2004, except for recently collected herbarium material from the Ozarks, SC, and NJ (see Table 1). Sites FL-2 and FL-3 (both in Florida) are aggregates of five and two nearby locations, respectively, less than 50 km apart. All thalli were collected along meandering transects through each site, taking care to sample from noncontiguous thalli (at least 1 m apart if possible, typically > 5 m). We sampled a total of 87 thalli. Clean, apparently healthy branches of each specimen, weighing approx. 10–25 mg, were selected under a dissecting microscope and placed in 1.5-ml tubes for extraction. Vouchers for each specimen are housed at Duke University, except those from SC and NJ (housed at PH Academy of Natural Sciences, Philadelphia, PA, USA).

Molecular methods

DNA extractions were performed using cetyltrimethylammonium bromide (CTAB) and *N-tris*(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) extraction buffers, and a protocol modified from Grube *et al.* (1995) and Ivanova *et al.* (1999). DNA was resuspended in 50 µl dH_2O and frozen at −20°C until needed. Dilutions of total genomic extracts were used as a PCR template for all amplifications, totaling between 2 and 5 ng DNA per reaction. For internal transcribed spacers (ITS), we used 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 ITS4 (White *et al.*, 1990), at 10 mM each. We also sampled two single-copy nuclear genes, translation elongation factor 1 (*EF1α*) and RNA polymerase subunit II (*RBP2*) regions 5–7 (Liu *et al.*, 1999). For these, we used newly designed primers (forward *EF1α*, CLEF-3F: 5′-GGC AAA GGC TCC TTC AAG T-3′; reverse *EF1α*, CLEF-3R: 5′-GCC AAT ACC ACC GAT CTT GT-3′; forward *RBP2*, CLRPB5F: 5′-CTG

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TTT CGA ACG CTG TTT CA-3′; reverse *RBP2*, CLRPB7R: 5′-CGC ATC CAC GTA TTC AAC AA-3′), each encompassing an entire (*EF1α*) or partial (*RPB2*) intron. Each PCR reaction also included 0.1 mM dNTP and 2.5 units Klentaq with the manufacturer's buffer (AB Peptides, St. Louis, MO, USA). Fungal ITS was amplified using a 94°C 3-min denaturation step followed by 27 cycles of 94° for 1 min, 50°C for 1 min and 72°C for 2 min, with a 3-s autoextension. *EF1α* and *RPB2* were amplified using a 94°C 3-min denaturation step followed by 30 cycles of 95° for 1 min, 55°C for 30 s and 72°C for 1.5 min. Algal ITS was amplified using a 94°C 3-min denaturation step followed by 35 cycles of 94° for 1 min, 50°C for 30 s and 72°C for 1 min. PCR products were cleaned using Qiagen's QiaQuick kit (Qiagen, Valencia, CA, USA). Sequencing reactions were performed with PCR primers at 2 mM using BigDye dye-terminator chemistry (Perkin Elmer, Boston, MA, USA) with 10 ng PCR template. Sequences were determined on an ABI Prism 3700 (Applied Biosystems, Foster City, CA, USA), edited and assembled using SEQUENCHER ver. 3.1, and exported into PAUP*4.0b10 (Swofford, 2000). Sequence alignments were produced separately for each fungal and algal data set. For the fungal ITS data set, all sequences were compared with a database of *Cladonia* ITS sequences to verify field identifications. We used SITES (Hey & Wakeley, 1997) to identify identical sequences in both data sets, which were removed for phylogenetic analysis. For the aligned sequences, likelihood ratio tests were used to identify the best-fit model of sequence evolution using MODELTEST ver. 3.06 (Posada & Crandall, 1998). Bayesian phylogenetic analysis was conducted using MRBAYES ver. 3.0b4 (Huelsenbeck & Ronquist, 2001). Model parameters were estimated in each analysis and begun using random starting trees for 1 000 000 generations sampled every 100 for four complete runs for the fungi, and using 2 000 000 generations sampled every 100 for the algae. We examined plots of likelihood for each run to determine the number of generations required to reach stationarity (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, for each of the algal and fungal data sets a tree with the lowest log-likelihood was selected for illustration purposes. In addition, we assessed branch support by parsimony bootstrapping using 100 replicate heuristic searches using PAUP*4.0b10 (Swofford, 2000) in addition to Bayesian posterior probability.

Phylogenetic associations

Several terms have been used to describe patterns of association among interacting species. In this paper we use the term 'specificity' to refer to the phylogenetic range of compatible partners for a given symbiont (Smith & Douglas, 1987). Analogous to the concept of host range in parasitology, we use 'specificity' from the perspective of only one of the symbionts – the fungal partner. We adopt this perspective for two reasons. (1) In lichen algae the phylogenetic diversity is poorly known, and many divergent lineages of the commonest lichen algae, *Trebouxia* and *Asterochloris* (*sensu* Friedl, in Rambold *et al.*, 1998) lack names (Kroken & Taylor, 2000; Helms *et al.*, 2001; Piercey-Normore & DePriest, 2001). (2) Specificity of algae seems less restricted than fungal specificity (for example *Cladonia* is restricted to *Asterochloris*, while *Asterochloris* associates with several fungal genera; Piercey-Normore & DePriest, 2001).

We used several methods to test for phylogenetic correspondence between fungal and algal ITS variation as a description of specificity. First, to group algal genotypes we used analysis of molecular variation (AMOVA; Excoffier, 2000) with partitions structured according to statistically supported clades in the fungal ITS phylogeny. AMOVA partitions variation by calculating the proportion of covariance of components from user-defined categories (in this case, fungal clade). We tested two fungal clade groupings using either four or five clades: three well supported fungal clades plus the remaining *C. subtenuis* genotypes (four clades); or including the three supported clades plus the division of the unresolved genotypes into the two lineages found in lowest log-likelihood tree (five clades). A Mantel test was used to calculate the correlation between matrices of algal and fungal pairwise genetic distance. Permutation tests using 10 000 replicates were used to test significance (Smouse *et al.*, 1986) using ARLEQUIN (Schneider *et al.*, 2000).

Fungal reproductive mode and algal transmission

To test for recombining fungal population structure and reproductive mode, we used several methods. Clonal codispersive reproduction can be dismissed if either partner appears to have recombining population structure, or if partners appear to recombine with respect to one another. First, using SITES and RECOMBINATION DETECTION PROGRAM 2 (RDP2; Martin *et al.*, 2005), we examined a sample of 33 *EF1α* sequences plus one outgroup, and eight *RPB2* sequences plus one outgroup. Alignments were produced manually in PAUP. Using each data set alone and by concatenating the sample of eight *RPB2* sequences with the corresponding *EF1α* and fungal ITS, we used SITES to generate a table of site-by-site congruency and a table of the minimum set of recombination intervals (Hudson & Kaplan, 1985). As the number of sequences (eight for *RPB2*) or sites (642 bp for *EF1α*; 763 for *RPB2*) were in the range of values for which highly biased estimates of recombination parameters were expected, we did not estimate gamma (Hey & Wakeley, 1997) or Hudson's 4Nc. RDP (Martin & Rybicki, 2000) and MaxChi (Maynard-Smith, 1992; Posada & Crandall, 2001), which both performed well in empirical tests, including situations using sequences with low sequence divergence (Posada, 2002), were implemented in RDP2 to estimate the number of recombination events.

In addition, we used F_{ST} measures to test for population differentiation in the fungal populations. F_{ST} values range

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from 0 to 1, with 0 indicating no population differentiation caused by gene flow among populations, and 1 indicating complete isolation. F_{ST} values that are significantly different from zero can therefore demonstrate genetic subdivision and reject the null hypothesis of panmixia. In addition to seeking evidence for recombination within the fungal genome, we also sought evidence of nonclonal transmission of algal partners in *C. subtenuis*. Using known frequencies of fungal and algal ITS genotypes, we performed a permutation test for random association between these fungal and algal ITS genotypes. There were 77 pairs for which both fungal and algal genotypes were known in this study. We therefore sampled with replacement from the 77 algae for which the genotypes were known, and from the 77 fungi; randomly associated these sampled algae and fungi; and recorded the number of unique genotype pairs. We repeated this process 10 000 times. We then performed a one-tailed test to ask if the observed number of unique combinations was significantly lower than the fifth percentile (corresponding to a significance level of 0.05) of the distribution of random combinations, as would be expected if clonal propagation and codispersal of fungi and algae occur.

Geographic associations

In contrast with phylogenetically correlated specificity, selectivity describes the frequency of association with any of the compatible partners (Rambold *et al.*, 1998), and represents the combined effects of phylogenetically determined specificity and ecologically determined holobiont fitness. Selectivity, then, is a measure of ecological specialization. We used F_{ST} tests, AMOVA and partial Mantel tests to test geographic patterns of variation in symbiotic associations. Sites < 50 km apart were tested for differentiation using F_{ST} tests, and were combined if no significant structuring among sites was observed. Using the entire sample of either fungal or algal sequences separately, we calculated the overall F_{ST} to test for variation corresponding to geographic population structure. Significant values of F_{ST} are nonzero, indicating genetic subdivision. Exact tests of pairwise F_{ST} are calculated by generating replicated permuted data sets representing the null hypothesis of random association in ARLEQUIN. *P* values were adjusted for multiple tests using a Bonferroni correction to adjust the threshold for significance proportional to the number of tests performed.

Where significant population structure was detected in an analysis, Mantel tests were used to describe the correlation between genetic and geographic distance/habitat, or between the genetic distance of algal and fungal partners (Smouse *et al.*, 1986), using ARLEQUIN. For the algal and fungal genetic distance matrices, we used the pairwise genetic distance calculated using the K2P model with $\gamma = 0.0167$ and $\gamma = 0.2215$, respectively. Geographic distances were calculated based on latitude and longitude using a javascript program (http://www.wcrl.ars.usda.gov/cec/java/lat-long.htm). For all distance measures, we tested untransformed and log-transformed distances to ensure the measures used for correlation tests fitted the assumptions of linearity, and used untransformed geographic and genetic distance. The habitat matrix was constructed by assigning 0 to all coastal plain sites and 1 to all sites outside the coastal plain, using the classification of Fenneman & Johnson (1946). We use these broad physiographic regions, coastal plain vs inland, as proxies for direct habitat measures. Partial correlations were calculated using Mantel tests in ARLEQUIN with either algal or fungal pairwise distance matrices as the *Y* matrix, geographic distance as matrix X_1 and habitat as X_2. The null hypothesis tested was whether a random value for association between matrix measures was higher than the observed association. For significance of all tests, we used 10 000 permutations.

Results

We examined a total of 87 thalli of *C. subtenuis* from 11 sites (Table S1). A total of 55 new algal ITS sequences were produced. Aligned sequences were 568 bp with 38 variable and 30 parsimony-informative sites. Of the 81 algal ITS samples, a total of 24 unique algal genotypes were present, all from Asterochloris Clade II (Piercey-Normore & DePriest, 2001; Yahr *et al.*, 2004). The most abundant algal genotypes were found 17 (J) and 12 (E) times, respectively, with another three common genotypes found eight, eight and seven times (II, AA and Q), respectively. The other 19 algal genotypes were observed four or fewer times. The 24 algal genotypes were members of three well supported clades: IIa, IIb (those identified in Yahr *et al.*, 2004) and IIc, and one genotype of uncertain placement (genotype II, Fig. 1, inset), Clade IId.

We sequenced the fungal ITS of 79 samples: 77 of these have corresponding algal sequences. Aligned fungal ITS was 566 bp, with 112 variable characters of which 58 were parsimony-informative. We resolved 32 unique fungal ITS genotypes, distributed in three well supported clades plus several unresolved genotypes (Fig. 1). Sequences representing each fungal and algal ITS genotype in Fig. 1 have been deposited in GenBank (algal ITS accession nos DQ482671–82; fungal ITS accession nos DQ482683–3711).

In addition, for *C. subtenuis* we generated 33 *EF1α* and eight *RPB2* sequences. *EF1α* sequences were 643 bp with nine variable positions and five parsimony-informative sites (GenBank accession nos DQ490091–DQ4900105). These were sampled from five populations (FL-2, FL-3, GA, NC-1 and PA) and from four of the five fungal ITS clades found (excluding the clade with only two representative genotypes; Table S1). A total of 15 genotypes were detected, all with > 99.7% similarity. The *RPB2* data set included seven genotypes from eight sequences of 763 bp, nine variable positions and two parsimony-informative sites, all with > 99.3% similarity (GenBank accession nos DQ522282–DQ522289).

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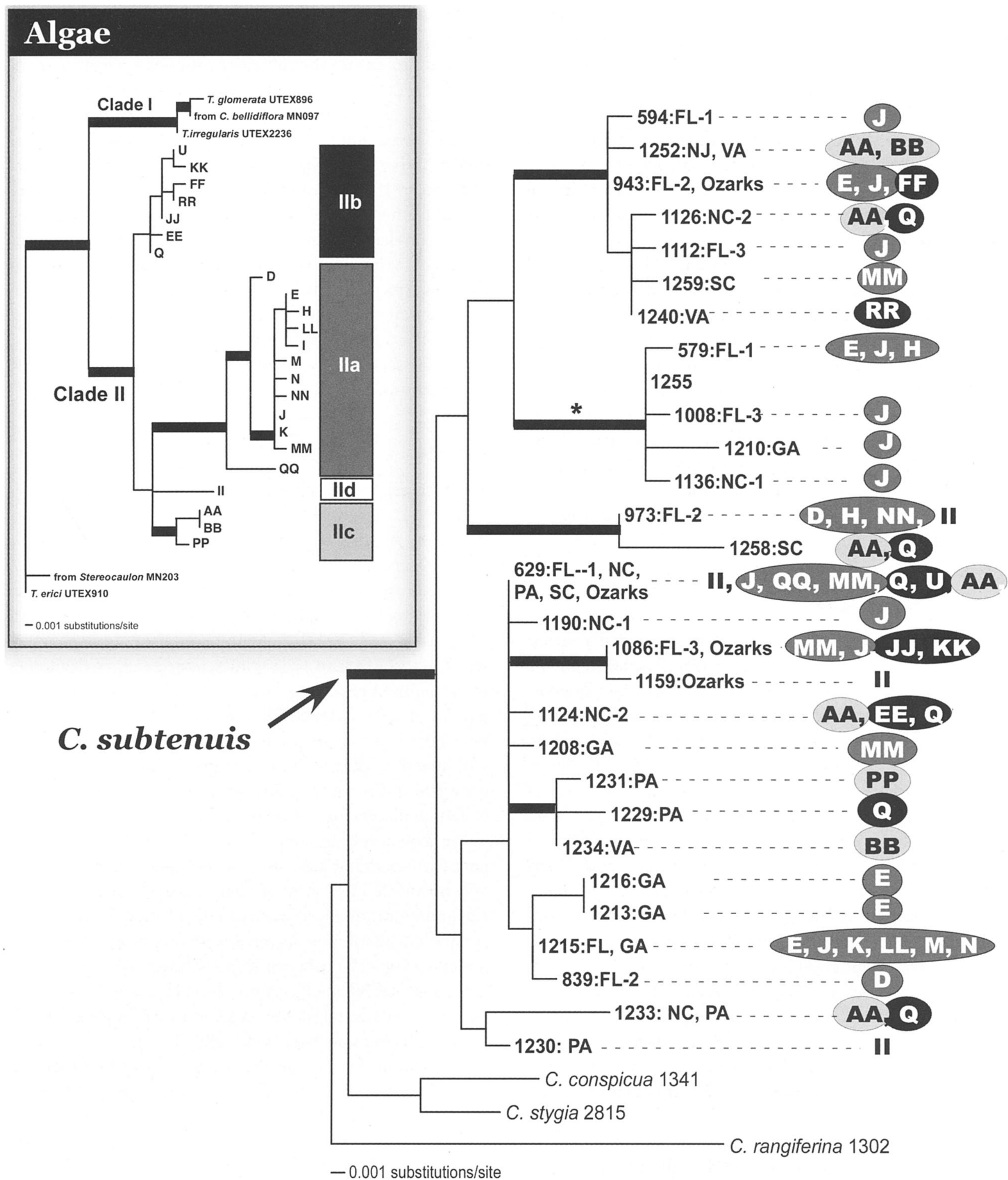

Fig. 1 Phylogenetic relationships of fungal and algal partners and their associations. Each tree shown has the lowest log likelihood from each (algal or fungal) set of trees in the posterior distribution. Branch support of over 95% posterior probability is shown as thickened branches. Trees were rooted with *Cladonia conspicua*, *Cladonia stygia* and *Cladonia rangiferina* (fungal), or *T. erici* (algal). (a, inset). Estimated internal transcribed spacer (ITS) phylogeny of unique *Asterochloris* algal genotypes. Genotypes of *Asterochloris* from *Cladonia subtenuis* are in capital letters and grouped by clade. (b) Estimated phylogeny of unique *C. subtenuis* ITS genotypes (numeric code according to voucher number) with their algal genotypes, corresponding to lettered codes in (a). States in which fungal genotypes were found are listed by standard two-letter abbreviations (see Table 1 footnote) following voucher numbers. Several sampling sites shown with single fungal voucher numbers indicate that the same genotype was found in samples from multiple sites. Asterisk indicates a fungal clade specific to algal Clade IIa.

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In no case did we find evidence for more than a single copy of any sequenced region.

Phylogenetic associations

There is no clear association of fungal and algal genotypes overall, on the basis of mapping algal genotypes and clades onto the estimated fungal ITS phylogeny (Fig. 1). In three of four main fungal lineages, fungal relationships do not predict algal partners. For example, for eight distinct and unrelated fungal genotypes (Fig. 1, fungal genotypes 943, 1126, 973, 1258, 629, 1124, 1086, 1233), associations were detected with at least two, and up to four, separate algal clades. No correlation between genetic distance of algae and fungi was found with a Mantel test ($r = -0.079$, $P = 0.739$; 0.06% of variation explained). One fungal clade, however, shows a relatively narrow host range, including only Clade IIa algae (asterisk in Fig. 1), although the algal partner of one member of this clade was not sequenced. This clade is found throughout Florida, northward along the coastal plain into New Jersey.

An additional test of association was performed using an AMOVA to examine the genetic structure of algae according to fungal clade membership. We tested algal partitioning according to either four or five fungal clades. In both tests, only 2–5% of algal genetic variation was distributed among fungal clades, whereas 95–98% of variation was found within fungal clades, resulting in an overall algal F_{ST} (according to fungal clade) not significantly different from zero (5 clade: $F_{ST} = 0.0500$, $P = 0.110$; 4 clade: $F_{ST} = 0.0188$, $P = 0.252$). We therefore found little evidence of multiple cryptic fungal lineages specific to narrow but different algal host ranges.

Fungal reproductive mode and algal transmission

Two hypotheses can be tested in relation to fungal reproductive mode: H1, clonal reproduction; and H2, random mating. Evidence of recombination can reject the null hypothesis of clonality (H1), while evidence against panmixia rejects the null hypothesis of random mating (H2). We use F_{ST}, a measure of gene flow (Cockerham & Weir, 1993), as a proxy for random mating. F_{ST} values approaching 0 indicate unlimited gene flow, whereas F_{ST} values approaching 1 indicate complete isolation of populations caused by the absence of gene flow among sites. Using the *EF1α* data, SITES detected a minimum set of one recombination interval, shown between positions 114 and 254. No recombination events were detected in the *RPB2* data. Using the combined data set (*EF1α*, *RPB2* and ITS) with 1973 positions, two recombination events were detected, one at each of the boundaries between loci, with significant linkage disequilibrium detected only within loci. However, using RDP2 and MaxChi with single and concatenated data sets, no recombination events were detected. Fungal populations were poorly but significantly differentiated according to ITS variation ($F_{ST} = 0.091$, $P = 0.008$) rejecting the hypothesis of panmixia. Finally, in a permutation test for association between fungal and algal genotypes, we found that the observed number of unique combinations ($n = 56$) was not significantly different from random ($P = 0.360$). If clonal propagation and codispersal of fungi and algae occurred, we would expect significantly fewer combinations than random.

Algal geographic associations

Algal ITS genetic variation was significantly structured by site, with overall $F_{ST} = 0.687$, $P < 0.00001$. Sites FL-2 and FL-3 (both in Florida) are aggregates of five and two nearby locations, respectively, < 50 km apart. These locations were tested for differentiation using F_{ST} tests, and combined as no significant differentiation among them was observed. Among all sites studied, pairwise comparisons showed especially strong differences between southern coastal plain (FL-1, FL-2, GA, FL-3) vs inland (NC-2, VA, PA, OZ) sites, with 15 of 16 comparisons being statistically significant (Table 1, shaded). Nine of 12 comparisons between mid-Atlantic coastal plain (SC, NC-1, NJ) vs inland sites, and 12 of 21 within coastal plain comparisons, also had high F_{ST} values (> 0.15), although most were not statistically significant. Both geographic position and habitat predict variation in algae, with > 40% of the genetic variation we detected in algal symbionts explained by geographic distance (23.5%) and habitat (19.8%) in partial Mantel tests (Table 2). Geographic distance and habitat were themselves weakly correlated ($r_{12} = 0.296$), and both the partial correlations for geographic distance and habitat with algal genetic variation were significant (Table 2).

Figure 2 shows the frequency of algal ITS clades associated with *C. subtenuis* for each sampling site, and highlights the unequal distribution of clades across sites. For example, all Clade IIa genotypes (dark gray) occur in the southern coastal

Table 2 Partitioning of internal transcribed spacer (ITS) genetic variation in *Asterochloris* algae of *Cladonia subtenuis* according to partial Mantel tests, using pairwise genetic distance of algae (K2P + $\gamma = 0.0167$) vs geographic distance (X_1) and habitat (coastal plain vs inland; X_2)

Source of variation	Correlation coefficient	Percentage variation explained	*P*
Geographic distance	0.5449	23.5	0.000000
Habitat	0.5137	19.8	0.01
Geographic distance by habitat	0.2957	na	na
Geographic distance (habitat fixed)	0.4796	na	0.000000
Habitat (geographic distance fixed)	0.4402	na	0.019

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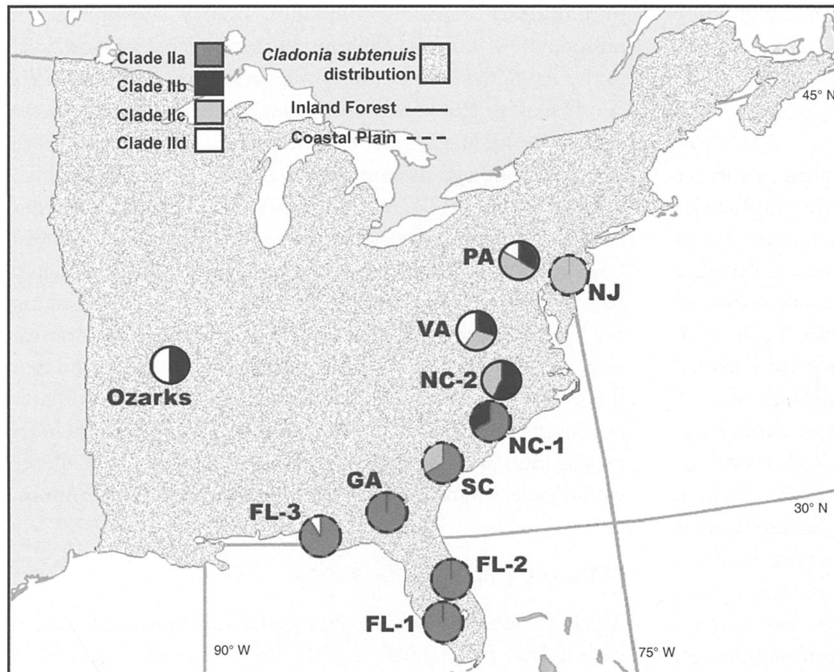

Fig. 2 Distribution (stippled) and collection sites for *Cladonia subtenuis* with proportion of major algal clades sampled in each site. Pie charts for coastal plain sampling sites are shown with dashed lines; solid lines surround inland sites. Colors of major algal clades correspond to Fig. 1: dark gray, Clade IIa; black, IIb; light gray, IIc; white, IId.

Table 3 Partitioning of internal transcribed spacer (ITS) genetic variation in *Cladonia subtenuis* according to partial Mantel tests, using pairwise genetic distance of fungal ITS (K2P + γ = 0.3639) vs log (geographic distance) and habitat (coastal plain vs inland)

Source of variation	Correlation coefficient	Percentage variation explained	*P*
Log (geographic distance)	−0.232	7.0	0.903
Habitat	0.226	6.7	0.059
Geographic distance (habitat fixed)	−0.300	na	< 0.957
Habitat (geographic distance fixed)	0.296	na	0.020

plain south of 35° N, and most of Clades IIb (black) and IIc (light gray) algal genotypes occur northwards. Sites that harbor different proportions of the same or partially overlapping algal clades (e.g. OZ vs NC-2; FL-2 vs FL-3, NC-1 or SC) also contain significantly different algal associates according to F_{ST} tests (Table 1).

Fungal geographic associations

We detected high diversity of fungal ITS genotypes within sites, with gene diversity (equivalent to heterozygosity estimates for haploid organisms) values between 0.44 and 0.78, calculated according to Weir (1996). This is also consistent with gene diversity of 0.48 reported in an earlier study of *C. subtenuis* at NC-2 (Beard & DePriest, 1996). In comparison with the algal partners, *C. subtenuis* has little population genetic structure as measured by pairwise F_{ST} or corresponding to geographic position. Only one of 55 pairwise comparisons shows significant structuring as measured by F_{ST}: between PA vs GA (data not shown). The overall measure of population differentiation using F_{ST} was 0.092 (P = 0.008). This value represents approx. 9% of the total genetic variation across all *C. subtenuis* samples, compared with the average > 90% of variation found within populations: on average, any single population of *C. subtenuis* fungi contains > 90% of that found across the entire species. This genetic structure among fungal ITS genotypes is not significantly correlated with geographic distance (r = –0.232, P = 0.903). Fungal genetic structure is marginally significantly associated with habitat differences (r = 0.226, P = 0.059; Table 3), explaining approx. 6.7% of the variation in fungal ITS distribution. The partial correlation of habitat with the fixed effect of geographic distance (r = 0.296, P = 0.020) was significant, but the converse was not (r = –0.300, P = 0.957).

Discussion

The phylogenetic breadth of a species' potential symbiotic partners – its specificity – may be a product of intrinsic or extrinsic factors. The combination of intrinsic, genetically determined constraints with extrinsic factors such as availability of partners or fitness in a particular site leads to observed patterns of association among symbionts. Selectivity is defined as the frequency of observed association among

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compatible partners (Rambold *et al.*, 1998), and may be a function of these interacting forces. In the lichenized fungus *C. subtenuis*, significant differences among sites in the associations with Asterochloris algae exist in eastern North America (Fig. 2). This geographic structure in algal associations occurred in the context of limited population structure in the fungal partners, and without obvious genetic correlations between fungi and algae – without evidence of clonal transmission of partners or within species clade-level specificity.

Among sites, a comparison of the distribution of known compatible algal genotypes for *C. subtenuis* with the patterns of association with these genotypes shows that not all compatible algae present in a particular site are associated. Instead, a degree of ecological specialization exists between the partnership of fungus plus alga for a particular site, and this specialization appears to depend on the local environment.

Across the range of *C. subtenuis*, we found four associated algal clades. This algal specificity is much lower than that for a smaller sampling area (Yahr *et al.*, 2004) for the same fungal species, reinforcing the observation that sampling scale has important implications for studies of ecological interactions (Thompson, 1997). In addition, despite high algal clade diversity within *C. subtenuis* (Fig. 1), we cannot detect specificity of these fungal lineages for particular algal clades. Instead, in our sample of *C. subtenuis*, both geographic position and habitat are good predictors of algal genotype distribution. This result is consistent with the selective associations suggested for algae in *Cladonia rangiferina* (Piercey-Normore, 2004) and for '*Trebouxia* sp. 3' in subalpine *Letharia* populations of the Pacific north-west (Kroken & Taylor, 2000). We found that algal associations are not predicted by fungal lineage, as distribution of fungal genetic variation was not, or was only weakly, structured by geographic factors, and fungal populations appear panmictic across their range.

Fungal specificity decreases over increasing geographic area

Fungal specificity is the pattern of taxonomically limited associations and may indicate genetically determined compatibility, a mechanism that may result in tight associations between symbionts. In *C. subtenuis*, algal associations vary across its range, but appear specific for members of Clade II. The apparent specificity for Clade II algae in the fungal species as a whole does not scale down to further within-species, lineage-dependent specificity for particular algae. An example of within-species, clade-level specificity is illustrated by the *C. subtenuis* clade indicated by an asterisk in Fig. 1, which associated with a single clade of algae (IIa). In contrast, we found that most (four of five) lineages within *C. subtenuis* were compatible with several distinct Clade II algae.

Although almost all samples of *C. subtenuis* in Florida are apparently restricted to Clade IIa algae, this study demonstrates that association of *C. subtenuis* with Clade IIa algae in Florida cannot be explained solely by phylogenetic correlation or specificity below the species level. First, most of the lineages of *C. subtenuis* detected in our sample across eastern North America were found in Florida, ruling out the possibility that fungal lineages are geographically restricted within the area sampled. Second, identical lineages (and genotypes) of fungi restricted to Clade IIa algae in Florida are found with different Clade II lineages in other parts of the geographic range of *C. subtenuis* (Fig. 1), demonstrating a lack of specificity for individual fungal lineages. In the light of these new observations, *C. subtenuis* appears specific for the large group of Clade II algae in general, not with a single lineage within that group. In addition, these observations exclude the possibility that cryptic species that may be currently included in the concept of *C. subtenuis* are specific for different algal lineages. These Clade II Asterochloris algae may represent a very disparate group, containing several unnamed clades (IIa, IIb, IIc) as well as sequences belonging to *Trebouxia magna* and *Trebouxia excentrica* (Piercey-Normore & DePriest, 2001; Yahr *et al.*, 2004).

Because determination of specificity here is based on molecular phylogenetic criteria, failure to resolve fine-scale associations between fungal and algal lineages is highly dependent on obtaining appropriate phylogenetic resolution, in this instance using ITS sequences to estimate phylogenies. For some fungal studies, ITS has proved a less variable marker than other single-copy nuclear genes such as *EF1α*, *RPB2* or even anonymous DNA markers (Kroken & Taylor, 2001). However, screens of *EF1α* and *RPB2* have so far revealed less variation than in ITS for *C. subtenuis* (see Results). Although we cannot rule out further resolution altering these conclusions, we believe the associations suggested by fungal ITS are conservative. The amount of apparent algal sharing indicated by Fig. 1 suggests that, even if additional lineages could be resolved, they would also be likely to associate with multiple algal genotypes (e.g. fungal genotype *629*; Fig. 1). The resolution in our algal phylogeny is similarly restricted to information from the ITS. Although we did not sample any other markers, other researchers have used actin-coding regions and introns for studies of *Trebouxia* photobionts; these markers have been largely congruent with, and less variable than, ITS (Kroken & Taylor, 2000; Piercey-Normore, 2006).

Clonal propagation

A simple model of tight associations between symbionts predicts that the partners cannot be separated, or are only infrequently separated. In some symbiotic systems, such as maternally inherited endosymbionts (Saffo, 1992; Moran & Baumann, 1994; Huigens *et al.*, 2000) or grass endophytes (Clay, 1990; Saikkonen *et al.*, 2002), strong population structure and shared phylogenetic history of symbionts are expected because of the vertical transmission of symbionts (Brem &

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Leuchtmann, 2003). In lichens, vegetative fragmentation of thalli is likely within populations, producing physically separate but genetically identical thalli, or 'symbiotic individuals', including genetically identical fungal and algal components (Paulsrud *et al.*, 1998). Although *C. subtenuis* has no specialized vegetative propagules, we detected 11 such putative clones, unique combinations of fungal and algal genotypes, found more than once in a single site or among sites (a total of 32 samples), consistent with that expectation. The average geographic distance between putative clones was approx. 160 km, with only nine of 45 pairs sampled from the same site. In some cases, indistinguishable symbiotic individuals were found widely distributed among distant sites, for example between North Carolina's Coastal Plain and Florida's North Gulf Coast, > 550 km apart. This distribution may reflect either widespread dispersal of clones; lack of resolution in these markers; or, perhaps most likely, chance reassociation with identical genotypes.

Despite the repeated occurrences of symbiotic individuals in *C. subtenuis*, the absence of association between fungal and algal genetic variation using both Mantel tests and AMOVA illustrates the reassorting of algal and fungal lineages, and suggests horizontal transmission of symbionts (any transmission not between parents and offspring). Furthermore, the number of unique symbiotic individuals is well within the number expected under random association of fungal and algal genotypes (Fig. 2). Finally, the occurrence of seven different algal genotypes with a single fungal genotype (629 in Fig. 1) further supports the hypothesis of horizontal transmission of symbionts. This indication of horizontal algal transmission is also consistent with observations suggesting *de novo* lichenization in this species, for example frequent observations of juvenile recruitment in the field, presumably from spores.

Although clonal propagation cannot be rejected as contributing to the patterns of association between fungal and algal symbionts, it does not appear to be a major factor in this species. In addition to the evidence above, the sexual strategy inferred from the evidence of recombination between sampled gene regions (and within EF1a) using SITES also suggests that re-lichenization must occur at each new generation. The lack of significant evidence for recombination using the other two algorithms implemented in RDP probably indicates insufficient statistical power, as the ability to detect recombination in empirical studies relies especially on the amount of sequence divergence (Posada, 2002). Sexual reproduction has been demonstrated in several other species of *Cladonia* using fingerprinting techniques (Seymour *et al.*, 2005).

Algal availability does not limit lichen associations

The geographic distribution of algal strains associated with lichen fungi is so far incompletely known. Trebouxioid symbionts may not require lichenization for all or even part of their life cycle, and may frequently occur as free-living (Mukhtar *et al.*, 1994). Inferences from recent molecular work also stress the likelihood of a pool of free-living green algae in explaining observed lichen associations (Beck *et al.*, 1998; Beck, 1999; Kroken & Taylor, 2000; Piercey-Normore & DePriest, 2001; Romeike *et al.*, 2002). However, even if some algal strains are capable of surviving as free-living, they may not be frequent enough to be encountered by a *Cladonia* spore or fragment (Courtney & Chew, 1987). Therefore the frequency and distribution of available algae in each site may determine algal abundance in lichen associations.

In studies of algal associations, some geographic differences in algal genotypic distribution have been detected. Clade II algae are widely distributed and genetically diverse, with a clade of neotropical genotypes (Piercey-Normore & DePriest, 2001) and a clade known only from the south-eastern USA, Clade IIa (Yahr *et al.*, 2004). Although in Florida *C. subtenuis* associates almost exclusively with Clade IIa algae, Clade IIb algae are also known to be compatible (Fig. 1) and abundant in Florida, being the most common symbionts of other co-occurring *Cladonia* species: *C. leporina*, *C. pachycladodes*, *C. perforata* and *C. prostrata* (Yahr *et al.*, 2004). Therefore the almost exclusive association of *C. subtenuis* with Clade IIa algae in Florida cannot be explained by either its absence or low frequency, at least across the coastal plain in Florida. Likewise, in the Ozarks, Clade IIa algae have been found in *Cladonia caroliniana*, although they are absent from *C. subtenuis* in that area (R.Y., unpublished results). Finally, single algal genotypes can sometimes be found across several continents (Piercey-Normore & DePriest, 2001), indicating that although narrow algal distributions are possible, they are clearly not solely responsible for observed associations between lichen symbionts.

Random processes

Historical artifacts such as population bottlenecks are expected to produce patterns of low diversity and strong, correlated geographic structure. In *C. subtenuis*, there is no evidence of low diversity in subsets of sites or genetically isolated populations, as expected with bottlenecks or founder events. Furthermore, random processes cannot explain the observed geographic patterns in algae, as the same patterns of association across distant sites (e.g. associations with only Clade IIa across multiple sites in the south-eastern Coastal Plain) would not be expected. The finding of strong patterns with respect to geographic associations suggests active processes beyond those with a stochastic explanation (Fig. 2).

Fungal selectivity

The variable fitness of symbiont associations across a range of ecological settings may determine their success, and therefore their frequency of association, in a site or habitat (Fox & Morrow, 1981). Although the distribution of fungal variation

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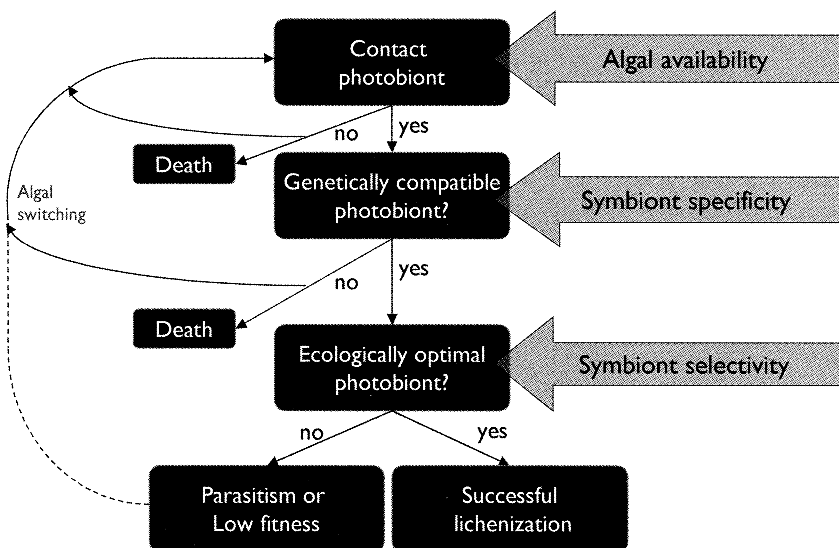

Fig. 3 A hypothetical model of fungal–algal association for lichens, showing the interactions between partners and the mechanisms that structure them. (a) Algal availability determines which algae can be contacted. (b) Fungal specificity determines the ability to form prethallus stages. (c) Fungal selectivity, dependent on ecological context, determines the final composition of observed lichens.

is weakly correlated with habitat differences, this factor explains only about 7% of the variation observed (Table 3). Therefore the strong relationship between geographic position and *C. subtenuis* algal associations shown here could be caused by either algal factors alone (i.e. fitness or abundance of free-living algae), or mechanisms that act on the composite symbiotic thallus. These dynamic species interactions may produce evolutionarily specialized lineages and coevolution between partners (e.g. Clade IIa in Florida sites; Thompson, 1994; Thompson, 1999), and empirical studies have shown geographic structure in the organization (Taylor & Bruns, 1999) and outcomes of other symbiotic interactions (Lively, 1999; Brodie *et al.*, 2002). Although the role of genetically structured populations is well understood in establishing and maintaining mosaics of differentially specialized species interactions, studies of lichens have so far not investigated this factor in detail.

Two possibilities exist to explain local patterns of specialization in *C. subtenuis* for algae. (1) Given both the presence of, and the specificity for, Clade II algae, *C. subtenuis* may associate randomly with compatible Clade II algae it encounters at a site. Following initial contacts and establishment, varying site-specific selective forces may remove unfit symbiotic combinations, leaving only the subset of successful thalli with an apparently specialized symbiotic composition. (2) Alternatively, the fungus may recognize and 'select' one partner over another (for example via more efficient interactions; Schaper & Ott, 2003). Such local and variable selection is akin to the local specialization of insects for host plants, which is known to occur on small spatial scales (Jaenike, 1990). In our study, the habitat division tested is broad, but is known to be important for the distribution of variation between *Cladonia polycarpia* and *Cladonia polycarpoides*, with soil type explaining distributional differences in sibling species (Park, 1985). Habitats in the coastal plain are well differentiated from inland sites in terms of both geology and vegetation, with the former typically characterized by sandy, acidic and nutrient-poor soils, supporting open vegetation types such as coastal dune scrubs and sandhills (Fenneman & Johnson, 1946). The differences in symbiotic associations detected here between coastal plain and inland sites may reflect the local specialization of the holobiont to selective factors in these different habitats. For example, varying forest light levels have been hypothesized to be important for determining mixtures of algal genotypes within thalli optimal for photosynthesis in a recent study of an epiphytic lichen association (Piercey-Normore, 2006).

A model of lichen associations in nature

Based on the observations above, we propose the following generalized model for factors involved in lichen associations (Fig. 3). This model is similar to Combes's 'filter model', which emphasizes those mechanisms – 'filters' – determining encounter and compatibility between parasites (Combes, 2001). However, we add an important new ecological filter determining fitness of the symbiotic association. Pre-thallus stages have not been studied and are not included.

Algal availability First, algae disperse widely, perhaps globally, providing a continuous and diverse source of potential algal partners. Studies of wind patterns (Munoz *et al.*, 2004) and airborne algae and soredia (Tormo *et al.*, 2001) support this idea. These are the algae available for contact by fungal partners, although algal frequency and habitat suitability may also play a role in what algae are available. Certain habitats may prove unsuitable for a portion of this algal 'rain', and these genotypes are not available in these sites, or at such low abundance as to be undetectable. Clade IIc algae may fit this scenario: in a study of eight species of *Cladonia* in Florida's

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coastal plain with over 200 samples investigated, no genotypes of this clade were detected (Yahr *et al.*, 2004). As shown in this study, its absence from such sampling may be caused by those algae not being 'selected' by the sampled fungi in these sites. An empirical study of algal frequency in environmental (e.g. soil, air) samples is needed to address this aspect of the model, for example by PCR detection (Walser *et al.*, 2001). Nonetheless, it is possible that certain algae are habitat specialists to some extent, with such low fitness in others as to be functionally absent from the lichen association.

Fungal specificity Following thinning of available genotypes by environmental selection, the inherent and phylogenetically determined compatibility of fungal propagules may limit the algae with which they can associate. *In vitro* studies have shown that compatibility varies between fungal and algal species, with variable efficiency of interactions in terms of timing of contact and establishment of haustoria (Schaper & Ott, 2003), and with variable outcomes for the algae and fungi. For example, a fungus equally compatible with two algal strains, but more efficient in terms of establishing contacts and conduits for symbiotic interactions with one, will probably be more frequent in nature with that strain. Likewise, some fungi are able to parasitize nonsymbiotic algae temporarily, while in some instances lichen symbiosis seems unlikely to establish at all (Ahmadjian *et al.*, 1980; Schaper & Ott, 2003). In this example, *C. subtenuis* may be phylogenetically compatible with any of the available Clade II algae, but apparently not with Clade I algae, to the extent that this association has not yet been detected. Lack of association at this stage may be a product of failure to recognize, communicate with, or establish stable associations with potential partners.

Fungal selectivity Lastly, even compatible pairings in one habitat may not be optimal in another, leaving only a portion of any possible associations detectable at any site. Ecological context is known to be an important variable determining the evolutionary outcomes (Bronstein, 1994) and distribution of interacting species (Fox & Morrow, 1981; Thompson, 1999; Desdevises *et al.*, 2002). For example, the importance of spatial scale has been shown in several coral symbioses (Rowan & Knowlton, 1995; Rowan *et al.*, 1997; Rowan, 1998), where strong patterns of variation in algal associations correlate with ecological variables (e.g. light, depth, latitude). In the lichen model, a compatible – but nonoptimal – photobiont may be parasitized by the fungus, or an association of low fitness may form, resulting in poor performance and low abundance. Such an association may persist, or algal switching may occur (dashed line, Fig. 3).

Therefore the frequency of associations in a site is not necessarily determined by the frequency of those partners that arrive and survive by themselves, but rather by the environmentally determined success of the partnerships that form.

In conclusion, we have provided indirect evidence that differential selection maintains variation among populations for symbiotic interactions in the field, and that the variation in associations among populations can be explained by ecological determinants. We suggest that the variation in ecological parameters is a frequently ignored, yet important factor in determining associations in lichens and should be the subject of experimental tests in future studies.

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Supplementary Material

The following supplementary material is available for this article online

Table S1 Collection information for specimens sampled, with algal and fungal internal transcribed spacer (ITS) genotypes (vouchers are housed at DUKE unless otherwise indicated)

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