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Research

The lichen-forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*

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Summary

• The epiphyte *Evernia mesomorpha* forms a lichen association with green algae in the genus *Trebouxia*. Little is known about the population structure of *E. mesomorpha*. Here, population structure of the algal and fungal symbionts was examined for 290 lichen thalli on 29 jack pine (*Pinus banksiana*) trees in Manitoba.

• Through phylogenetic analysis of internal transcribed spacer (ITS) nuclear ribosomal DNA (rDNA) sequences, five algal genotypes were detected that were nested within *T. jamesii*. Two fungal genotypes were detected that formed a clade with two other *Evernia* species. The genus *Evernia* was paraphyletic with *E. prunastri*, sister to *Parmelia saxatilis*. Restriction fragment length polymorphism (RFLP) of ITS rDNA showed multiple algal genotypes in 45% of the 290 lichen thalli collected, whereas all thalli only contained one fungal genotype.

• Low population subdivision of algal and fungal genotypes among trees suggested that the algal symbiont was being dispersed in the lichen soredium.

• Low fungal specificity for multiple algal genotypes and a hypothesized algal switch may be important life history strategies for *E. mesomorpha* to adapt to changing environmental conditions.

Key words: algal dispersal, algal switch, *Evernia mesomorpha*, fungal specificity, lichen algae, population structure, *Trebouxia jamesii*.

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Introduction

Lichens are symbiotic associations between a fungal (mycobiont) and a photosynthetic (photobiont) partner, which can be a cyanobacterium or a green alga. The photobiont is retained within the thallus of the lichen fungus, providing carbon to the mycobiont. The mycobiont is thought to control the growth and reproduction of the alga. Because of the intimate association between the alga and fungus it is thought that they coevolve. Coevolution is often defined as reciprocal evolutionary change between two organisms and can include directional selection by one symbiont, co-ordinated speciation or adaptation, or patterns in the evolution of host specificity (Thompson, 1994). Ahmadjian (1988) maintains that *Trebouxia*-containing lichens are obligate associations and highly coevolved. The genus *Trebouxia* de Puymaly is one of the most common green algal genera that form lichen associations (Friedl & Büdel, 1996) and is not considered to be free-living (Ahmadjian, 1988, but see Tschermak-Woess, 1978; Mukhtar *et al.*, 1994).

Fungal selection for the algal partner is not a random process (Beck *et al.*, 1998; Rambold *et al.*, 1998; Dahlkild *et al.*, 2001; Piercey-Normore, 2004). Selectivity is defined as a preferential interaction between two organisms (Galun & Bubrick, 1984). High selectivity is when free-living *Trebouxia* spp. always associate with a lichen-forming fungus, even when other algae are more common in the same habitat. Low selectivity is when a lichen-forming fungus associates with the more common algal species in the habitat. Low selectivity in *Umbilicaria* has been suggested to reflect the ability of the lichen to adapt to harsh or changing environmental conditions (Romeike *et al.*, 2002). With a change in environmental conditions, selection of the algal symbiont may be redirected towards a different species or genotype appearing as a preference by the fungal partner. This concept also underlies the Adaptive Bleaching

Hypothesis for algal partners in coral symbioses (Kinzie *et al.*, 2001; Rowan, 2004). When environmental conditions change, some algal partners are lost. This is followed by a new consortium of different algal partners that are more suited to the environmental conditions in the habitat of the host.

The degree of fungal specificity to an algal taxon reflects the coevolutionary pattern between symbionts. High specificity is when one fungal taxon or genotype always associates with one algal taxon or genotype. If species of lichen-forming fungi exhibited high specificity, a comparison of algal and fungal phylogenies would reflect co-ordinated speciation events between alga and fungus, depicting reciprocal evolutionary change. For example, the lichen-forming ascomycete, *Fulgensia fulgida* (Nyl.) Szatala, is highly specific to *T. asymmetrica* Friedl et Gärtner (Beck *et al.*, 2002). Low specificity is when more than one fungal taxon or genotype associates with more than one algal taxon or genotype. On the other hand, low specificity may be complicated by taxonomic diversity in the fungal partner. For example, the genus *Chaenotheca* Th. Fr. associates with four different photobiont genera but has a history of taxonomic changes (Tibell, 1984). See Beck *et al.* (2002) for an explanation of selectivity and specificity in lichen associations.

The lichen-forming fungus, *Evernia mesomorpha* Nyl., associates with green algae in the genus *Trebouxia*. *E. mesomorpha* is one of five North American species in the genus (Esslinger & Egan, 1995). It has a circumboreal distribution and is a highly branched fruticose lichen forming a shrubby tuft on conifers and hardwoods. The angular wrinkled branches produce abundant coarse soredia, which are vegetative propagules containing both fungal hyphae and algal cells. Apothecia are rarely seen and therefore *E. mesomorpha* disperses predominately by vegetative symbiotic propagules. The lichen is considered to be pollution tolerant, being one of the first species to recover after pollution damage (Gunn *et al.*, 1995). Studies that have included *E. mesomorpha* are a description of the North American species of the genus (Howe, 1911; Bird, 1974), a list of the chemical substances in the genus (Culberson, 1963), and isozyme variation among populations of *E. mesomorpha* and other lichens (Fahselt, 1988). The majority of studies in the genus have focused on the economically important sister species, *E. prunastri* (L.) Ach.

In the past, lichen algae were identified based on morphological and culture characteristics. The ability to study algae in field-collected lichens has become more common with the development of taxon-specific primers (Beck *et al.*, 1998; Kroken & Taylor, 2000; Dahlkild *et al.*, 2001; Piercey-Normore & DePriest, 2001; Piercey-Normore, 2004). Algal-specific primers can be used to investigate the population structure in lichen algae using field-collected lichen thalli. Genetic variation is generally assumed to be important in determining the ability of a species to adapt to new habitats by increasing the chance of population survival (Mitton & Grant, 1984). The ability of the alga to adapt to new or changing habitats would also influence the ability of the lichen to adapt to changing habitats. The corticolous habitat of *E. mesomorpha* provides an ideal environment for wind dispersal of sexual or vegetative propagules. As many lichen fungi release sexual spores without the photobiont, the spores must land and germinate in the vicinity of a compatible photobiont before lichenization can proceed. This presents a challenge for the lichen, as two compatible partners must come together before the lichen can develop. The challenge is lessened by the production of vegetative propagules of lichens, such as soredia, where the two partners are already together and compatible and they will develop into a new lichen thallus. This ensures maintenance of the symbiotic system, but reduces the level of genetic variation in both symbionts.

Knowledge is severely lacking regarding the population structure of lichen photobionts. There has been some knowledge gained about population structure (Fahselt, 1988; DePriest, 1993; Fahselt *et al.*, 1995; Zoller *et al.*, 1999; Walser *et al.*, 2003), breeding systems (Murtagh *et al.*, 2000; Kroken & Taylor, 2001; Honegger *et al.*, 2004; Seymour *et al.*, 2005) and dispersal strategies (Walser *et al.*, 2001) of lichen-forming fungi. Few articles have reported studies of population variation or fungal selection of green algal photobionts (Kroken & Taylor, 2000; Tibell, 2001; Romeike *et al.*, 2002; Piercey-Normore, 2004) or cyanobacterial photobionts (Paulsrud & Lindblad, 1998; Oksanen *et al.*, 2002). Genetic variation within the symbiont genomes of a lichen population may be generated by sexual reproduction of each of the alga and fungus. Symbiont variation in a lichen population may be generated by relichenization after sexual reproduction of the fungal partner and release of fungal spores. Symbiont variation may also be maintained within a population by algal switching (Piercey-Normore & DePriest, 2001) from established or decaying lichen thalli or through soredia (Honegger, 1993; Beck, 1999). The dispersal distance of soredia may influence the level and pattern of symbiont variation within and among populations.

The goal of this study was to examine fungal selection of the alga of *E. mesomorpha*, within and among trees, in three locations in Manitoba. The first objective was to identify the species of alga associated with *E. mesomorpha* by comparison of nucleotide sequences of internal transcribed spacer (ITS) nuclear ribosomal DNA (rDNA) and type 1 actin-coding regions with those of other algae. The second objective was to determine the number of algal and fungal ITS genotypes in the lichen association. The third objective was to determine the population structure of the algal and fungal genotypes on jack pine trees in Manitoba.

Materials and Methods

The study sites were in Sandilands Provincial Forest, Manitoba, Canada (Table 1). Sandilands Provincial Forest is a mixed deciduous-coniferous forest consisting of dry sandy ridges of trembling aspen (*Populus tremuloides* Michx.), jack pine (*Pinus banksiana* Lambert), and white birch (*Betula papyrifera* Marshall)

Table 1 Site description and location for collections of *Evernia mesomorpha* in Sandilands Provincial Forest, Manitoba, Canada

Transect number	Site description	Latitude, longitude and elevation
1	Sandilands Road near Trail 20, Jack Pine forest; 10% shrubs; 40% *Pleurozium schreberi* and *Dicranum* spp.; 50% *Cladonia rangiferina*, *Cladonia uncialis*, *Cladonia mitis*, *Cladonia stellaris*; Canopy 50% open	49°20'3.8" N 96°14'45.3" W 412 m
2	Whitemouth lake Road near Trail 31. Jack pine forest; 30% low shrub; 30% *Pleurozium schreberi* and *Dicranum* spp.; 30% *Cladonia mitis*, *Cladonia rangiferina*, *Cladonia multiformis*; Canopy 30% open	49°19'44.5" N 96°7'38.2" W 418 m
3	Whitemouth lake Road. Jack Pine forest; 30% vascular plant, 90% *Pleurozium schreberi* and *Dicranum* spp.; 5% *Cladonia rangiferina*, and *Cladonia mitis*; Canopy 60% open	49°23'53.5" N 96°15'35.7" W 420 m

See methods for a list of corticolous species.

interspersed with wetter lowlands of black spruce [*Picea mariana* (Miller) Britton, Sterns and Poggenberg], larch [*Larix laricina* (Du Roi) K. Koch], white cedar [*Chamaecyparis thuyoides* (Linnaeus) Britton, Sterns and Poggenberg, and black ash (*Fraxinus nigra* Marshall)]. Corticolous lichens that were common on jack pine trees with *E. mesomorpha* included *Flavopunctelia flaventior* (Stirton) Hale, *F. soredica* (Nyl.) Hale, *Hypogymnia physodes* (L.) Nyl., *Lecanora circumborealis* Brodo & Vitik., *Melanelia exasperatula* (Nyl.) Essl., *Parmelia sulcata* Taylor, *Punctelia subrudecta* (Nyl.) Krog, *Ramalina dilacerata* (Hoffm.) Hoffm., *Tuckermannopsis americana* (Sprengel) Hale, *Usnea glabrata* (Ach.) Vainio, *U. hirta* (L.) F. H. Wigg., and *Vulpicida pinastri* (Scop.) J.-E. Mattsson & M. J. Lai. *Physcia adscendens* (Fr.) H. Olivier, *P. stellaris* (L.) Nyl., and *Xanthomendoza hasseana* (Räsänen) Søchting, Kärnefelt & S. Kondr. (Syn. *Xanthoria hasseana* Räsänen) were common on shrubs. Ten samples of *E. mesomorpha* were removed from each of 10 jack pine trees (nine trees in transect three), in each of three transects, providing 290 samples of lichen thalli. Transects were at least 50 m long and were composed of 10 trees that were all > 5 m apart. The diameter at breast height (DBH) was measured by placing a tape around the tree at a height of 1 m from the ground. Intact thalli were removed from the branches and trunk of jack pine trees and placed in labelled paper bags. All specimens (*Normore* 3200: Em1 to Em290) were air dried and deposited in the University of Manitoba Herbarium (WIN). *E. mesomorpha* is widespread throughout the Sandilands Provincial Forest and is predominantly sterile, implying low levels of gene flow and algal switching by relichenization. Detection of secondary compounds was performed by thin-layer chromatography (TLC) based on the methods described by Culberson (1972).

Total DNA was extracted from a 2-cm thallus branch using a cetyltrimethylammonium bromide (CTAB) extraction buffer and a protocol modified from Grube *et al.* (1995). Although each thallus branch was examined to avoid contaminating particles or areas of discoloration, it could not be guaranteed that all algal PCR products obtained originated from the algal layer proper. DNA was resuspended in sterile distilled water and amplified by the polymerase chain reaction (PCR). Amplification of the ITS of rDNA of the algal partner was from an algal specific primer, nr-SSU-178005'-Algal (Piercey-Normore & DePriest, 2001), and a universal primer, ITS4-3' (White *et al.*, 1990). Amplifications were performed in 20-μl reaction volumes for restriction endonuclease digestions, and in 300-μl volumes for DNA sequencing. Amplifications consisted of amplification buffer [200 mM Trizma base (pH 8.4) and 500 mM potassium chloride], 2.5 units of *Taq* DNA polymerase (GibcoBRL, Burlington, ON, Canada), 200 μmol l^{-1} of each dNTP, 2.0 mmol l^{-1} $MgCl_2$, 0.5 μmol l^{-1} of primer, and 10–50 ng of DNA. Amplification conditions in the Techne Genius thermal cycler (Fisher Scientific, Nepean, ON, Canada) were: initial template denaturing at 94°C for 5 min, then 33 cycles of denaturing at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 1.5 min. PCR products were agarose-gel purified by crushing frozen blocks of agarose, pipetting the liquid, and subsequent precipitation with 0.2 volumes of 5 M NaCl and 2.5 volumes of 100% (v/v) ethanol. DNA was quantified on a 1% agarose gel and stained with ethidium bromide. Amplification of the type I actin gene, including the intron at position 526 of the algal partner, was performed using the algal-specific primers, Act1T and Act2T (Kroken & Taylor, 2001). Amplification conditions for the actin gene were the same as those for the algal ITS rDNA except that the annealing temperature was 56°C. Amplification of the ITS rDNA of the fungal partner was performed using a fungal-specific primer located at position 1566 in the small subunit of rDNA, nr-SSU-1566-5' (Gargas & DePriest, 1996) and ITS4-3' (White *et al.*, 1990). Amplification conditions for the fungal ITS rDNA were the same as those for the algal ITS rDNA.

Restriction endonuclease digestion

PCR product of 290 samples of the algal and fungal partners of *E. mesomorpha* was quantified on a 1% agarose gel, and 300 ng was digested in 20-μl volumes. The endonuclease used to digest the algal ITS rDNA was 2 U of *Hpa*II (Gibco BRL) per reaction. The algal restriction enzyme was chosen based on

the variation, in GenBank sequences, of *T. jamesii*. Therefore, algal RFLP patterns were determined before the nucleotide sequencing was performed. The endonuclease used to digest the fungal ITS rDNA was 2 U of *Xmi*I (*Acc*I) per reaction (MBI Fermentas Inc., Burlington, ON, Canada). Fungal ITS sequences were required before selection of the enzyme (see the Results section). Restriction fragment length polymorphisms (RFLP) were electrophoresed in a 2% agarose gel run at 150 V until the loading dye was 1 cm from the bottom of the gel, stained with ethidium bromide, and visualized by ultraviolet light using an Alpha Imager 2200 transilluminator (Fisher Scientific). Fragment lengths were determined by comparison with a 100-bp DNA size standard (Gibco BRL).

DNA sequencing and sequence alignment

Double-stranded amplification products were sequenced using BigDye Terminators, Version 3.0, on a 377 and 377XL ABI DNA Sequencing Instrument (University Core DNA and Protein Services, University of Calgary, Calgary, AB, Canada). Sequencing primers were the same as those described for PCR. Sequences were assembled into full-length sequences using Sequencher 4.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Both strands were sequenced from two PCR primers. Sequences representing the primer sites were removed and only the remaining nucleotide sequences were used for the analysis. Algal and fungal ITS rDNA and type I actin sequences were aligned manually in Se-Al v. 1.0 (Rambaut, 2001) and imported into PAUP* 4.0b10 (Swofford, 2003). Representative samples from each of the five algal ITS genotypes were chosen, and the ITS rDNA and actin gene were sequenced for these. As detection of multiple algal ITS genotypes in the same sample was initially revealed when double peaks were present in the chromatograms, representative samples showing RFLP patterns with two bands were sequenced (Em1, Em2, Em86, Em 214, Em 217), reflecting the five algal genotypes containing only one sequence trace. These samples produced unambiguous sequences, with no double peaks in the chromatograms, indicating that a single genotype was present. Nested primers based on these polymorphic regions (Forward primers: EmITS2A 5′-CTACCTCAAAATGATGTT-3′ and EmITS3A 5′-CTACCTCAAAACTATTT-3′) were designed and used to amplify (with ITS4-3′ as the reverse primer) random samples of the algal population to confirm the presence or absence of multiple ITS genotypes. Location of the restriction enzyme recognition sites were determined for each sequenced ITS genotype, and calculated fragment lengths were compared with those detected by agarose gel electrophoresis to confirm the predicted banding pattern.

Data analyses

The algal ITS rDNA phylogeny was inferred from seven specimens of *E. mesomorpha* and 36 accessioned DNA sequences retrieved from NCBI GenBank. The actin phylogeny was inferred from the protein-coding portion of the gene without the intron for seven algal sequences from the same specimens of *E. mesomorpha* (except Em98 and Em207) and four sequences from GenBank. The fungal ITS rDNA phylogeny was inferred from 14 samples of *E. mesomorpha* and 24 sequences from GenBank. All nucleotide sequences generated in this study have been deposited in NCBI GenBank and accession numbers are indicated on the phylogenies. Each of the algal and fungal ITS rDNA trees were midpoint rooted with no assigned outgroup. The actin tree was rooted with *Nannochloris* Naumann (Bhattacharya *et al.*, 1996). Aligned sequences were subjected to phylogenetic analyses using PAUP* 4.0b10 (Swofford, 2003). Analyses were performed on three sets of data: (1) the entire algal ITS region – ITS1, 5.8S and ITS2; (2) the algal actin-coding region without the intron; and (3) the entire fungal ITS region – ITS1, 5.8S and ITS2. Phylogenetic determinations were based on maximum parsimony (MP) analyses using the option tree bisection and reconnection (TBR) branch swapping. Heuristic searches were conducted using 500 random addition replicates with a limit of 1000 trees per search and bootstrap searches of 500 resamplings (Felsenstein, 1985).

Distribution of algal and fungal ITS genotypes among populations was examined by analysis of molecular variance (AMOVA) at three population levels – within populations (ϕ_{PR}), among populations (ϕ_{PT}), and among transects (ϕ_{RT}) – for each of the algal and fungal symbionts using GenAlEx 6.0 (Peakall & Smouse, 2001). ϕ_{PT} is a measure of population differentiation that is analogous to F_{ST}, a measure of coancestry in populations (Weir & Cockerham, 1984). In this study, a population was defined as the 10 thalli sampled from each jack pine tree. For algal genotypes, a distinction was made between population size of the alga and population size of the lichen. As multiple algal genotypes were found within the same thallus, the algal population size (number of genotypes per tree where algal mixtures were coded as separate genotypes) was larger than the lichen population size (number of thalli per tree where algal mixtures were coded as mixtures). Algal population size (368 genotypes in 290 thalli) was used in the AMOVA analysis only. All other comparisons used algal genotypes in lichen population size (290 thalli). Pairwise comparisons with Spearman's correlation coefficient, using SPSS (SPSS Inc., Chicago, IL, USA), were determined between each of the genotype frequencies, DBH and tree. Minimum spanning trees were produced from genotype frequencies for each population of algal and fungal symbionts using Euclidean distances in SYN-TAX v. 5.02 (Podani, 1994). The mantel test in GenAlEx provided a statistical comparison between genetic distance matrices of the algal and fungal partners.

Results

The thallus of *E. mesomorpha* forms shrubby tufts with wrinkled and ridged branches irregularly divided, and with coarse

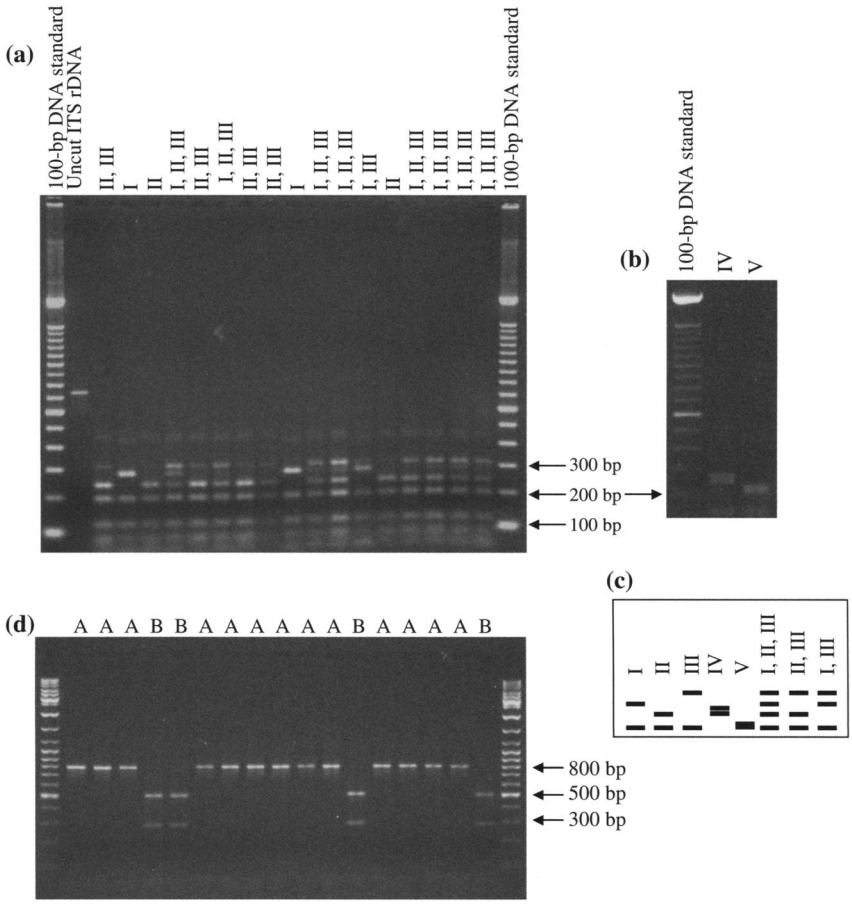

Fig. 1 Restriction fragment length polymorphism (RFLP) of internal transcribed spacer (ITS) nuclear ribosomal DNA (rDNA) amplified with taxon-specific primers from thalli of *Evernia mesomorpha* used to determine genotypes in algal and fungal partners. Genotypes are indicated above the lanes. (a) Fragments between 200 and 350 bp were the RFLP patterns used to determine algal genotypes. The first and the last lanes contain the 100-bp DNA size standard; lane 2 contains an uncut fragment of the algal ITS rDNA at 720 bp in length; the remaining lanes contain sample numbers 115–131, consecutively, showing genotypes I, II and III that are present in each sample. (b) Section of a different gel showing genotypes IV and V, which were not shown in (a). (c) Illustration of banding patterns for algal genotypes I to V when detected alone in the thallus, and the three combinations when detected as mixtures in the thallus. (d) Fragments between 300 and 800 bp were the RFLP patterns used to determine fungal genotypes. The first and the last lanes contain the 100-bp DNA size standard; the remaining lanes contain sample numbers 3–19, consecutively, showing the genotypes A and B that are present in each sample.

soredia developing along the ridges. In this study, abundant soredia were present on all samples. Six fungal apothecia were present on four samples [Em151 and Em154 (tree 6, transect 2), Em174 (tree 8, transect 2) and Em205 (tree 1, transect 3)]. All specimens were examined using TLC to determine secondary compounds. Divaricatic and usnic acids were consistently present in all specimens examined. Weak traces of an unidentified compound were also present.

Algal RFLP patterns

The amplified algal ITS rDNA was first subjected to RFLP analysis for all 290 thalli producing eight RFLP patterns (Fig. 1a,b,c), representing eight genotypes. Representative samples chosen from seven of the eight patterns were sequenced, two producing superimposed peaks in the sequence chromatograms (suggesting multiple sequence traces) and five producing single peaks (one sequence trace). By comparing RFLP patterns and nucleotide sequences of the seven algal samples it was determined that those RFLP patterns with two bands contained single algal genotypes and those with more than two bands contained multiple algal genotypes in the same thallus. Samples Em1, Em2, Em86, Em214 and Em217 were sequenced for algal ITS rDNA and algal actin because they represented each of the five algal ITS genotypes (genotypes I, II, III, IV and V) in ITS–RFLP patterns. Samples Em4 and Em6 were two of the first samples to be sequenced and revealed that multiple algal ITS and actin genotypes were present in the same thallus. Samples Em98 and Em207 were chosen for sequencing of the algal actin gene because they contained a single ITS genotype.

Confirmation of the presence of multiple sequences in a sample was performed by designing new nested primers using the six ambiguous sites in Em4 and Em6 with superimposed peaks in the sequence chromatograms. Similar superimposed peaks were found in the actin type 1 intron, supporting the ITS results of multiple genotypes in the samples. Although the preferential nature of PCR in different reactions may result in various amplification products, repeated amplifications were performed (three times on randomly selected samples) always producing the same genotype in the samples tested. If all 290 samples were sequenced there may have been a larger number of algal genotypes.

The lengths of the digested DNA fragments in genotype I were *c.* 285, 200, 105, 80, 30, and 20 bp. The lengths of the fragments in genotype II were *c.* 245, 200, 105, 80, 70, and 20 bp. The lengths of the fragments in genotype III were 314, 200, 105, 80 and 20 bp. The lengths of the fragments in

Table 2 Summary of percentages of the five algal genotypes detected alone, in mixtures, and the total percentage of each genotype in 290 lichen thalli in this study

Genotype	Alone	Mixture	Total
I	20	5	25
II	33	18	51
III	1	22	23
IV	0.3	0	0.3
V	0.3	0	0.3

Percentages for single genotypes are based on algal population size (368 genotypes). Percentages for mixture of genotypes are based on lichen population size (290 thalli).

genotype IV (Em214) were 270, 250, 110, 80, and 20 bp. The lengths of the fragments in genotype V (Em217) were 215, 200, 90, 80, 70, 30, and 20 bp. For each of genotypes IV and V, both diagnostic bands migrated close together in the gel, appearing as a broad single band (Fig. 1). The underscored fragment lengths were used to define genotypes.

The five ITS genotypes of lichen algae were distributed among the 290 lichen thalli in varying combinations. Three algal genotypes (genotypes I, II and III) were common in association with *E. mesomorpha* and were detected alone and together in the lichen thallus (Table 2). Genotype I was recovered as the only genotype present in the thallus in 20% of the samples, genotype II in 33% of the samples, genotype III in 1% of the samples, and a mixture of two or three genotypes was present in 45% of the samples. Two genotypes were rare (genotypes IV and V) occurring only one time each in 290 samples. Where mixtures of algal genotypes were present in the same thallus, the dominant type of mixture was genotypes II and III together (63% of mixtures), whereas mixtures of genotypes I and III (18%), and genotypes I, II and III (18%) were less common, and genotypes I and II were never found together.

Fungal RFLP patterns

In contrast to the algal RFLP analysis, sequencing for the fungal ITS rDNA was performed prior to RFLP analysis so that a restriction enzyme could be chosen based on variation in the fungal ITS sequences. Samples of lichen thalli chosen to sequence the fungal ITS rDNA were based on the greatest ITS diversity detected in the algal populations. This included representative samples from thalli containing each of the five algal ITS genotypes (Em1, Em2, Em86, Em214 and Em217) and thalli containing multiple algal genotypes (Em4 and Em6).

The low level of variation in the fungal ITS sequences provided few options for selection of an enzyme. After the enzyme was chosen and RFLP analyses performed, additional fungal sequencing included thalli that represented each of the two fungal ITS genotypes (F-A: Em80 and Em161; F-B: Em87, Em92 and Em162). A synapomorphy at position 61 of the ITS1 provided the recognition sequence for *Xmi*I when a 'T' was present in 58% of the 12 samples, and no recognition site when a 'C' was present in 42% of the samples. Only two other synapomorphies were present (positions 33 and 320) and represented 25% and 75% of the samples. Although the chosen enzyme was a six-base cutter, it distinguished the samples into two fungal genotypes and allowed the detection of more than one genotype in a single thallus if they were present. However, only one fungal genotype was present in each thallus. Two fungal ITS genotypes were detected with *Xmi*I in all 290 samples; these consisted of genotype A (300 bp and 500 bp bands), present in 79%, and genotype B (uncut 800 bp band; Fig. 1d) present in 20% of the samples. These bands included *c.* 300 bp of the SSU because they were amplified between primer sites nr-SSU-1566-5′ and ITS4-3′. Although these results detected two fungal genotypes, they were based on RFLP of the ITS region of the fungus. If all 290 samples were sequenced there may have been additional genotypes present.

Algal and fungal sequence similarity

Pairwise similarities and amplified fragment lengths among algal and fungal ITS rDNA and algal actin exon nucleotide sequences are shown in Table 3. There was more ITS variation within algal species (*T. jamesii*), and even among members of *T. jamesii* from *E. mesomorpha*, than there was within fungal species (*E. mesomorpha*). Although pairwise similarities among six algal type I actin exon sequences from *E. mesomorpha* (excluding Em86; genotype III) were > 99%, the inclusion of the actin sequence from ITS genotype III reduced the similarity to 90%. All seven algal actin sequences from *E. mesomorpha* contained an intron at position 569 in the actin gene, based on the sequence of *T. jamesii* (Hildreth and Ahmadjian) Gartner from *Letharia* (Kroken & Taylor, 2000). Pairwise similarity of the intron among all algal sequences from *E. mesomorpha* (excluding Em86; genotype III) was > 97% and the intron sequences were 192-bp long. The algal actin intron from Em86 (genotype III) was 228-bp long and was too variable to align with the intron from other *E. mesomorpha* photobionts.

Population structure

The population structure was examined for each of the algal (algal and lichen population sizes) and fungal partners separately, and then a comparison was made between the symbionts to determine the specificity of the relationship at the population level. Based on algal population size (368 genotypes) there was no population subdivision among transects ($\phi_{RT} = 0$; $P = 0.96$) or among individuals within populations ($\phi_{PR} = 0.03$; $P = 0.08$). Even though only 3% of the variation was attributed to variation among populations,

Table 3 Nucleotide sequence similarity and length for internal transcribed spacer (ITS) nuclear ribosomal DNA (rDNA) of the algal (seven samples) and fungal partners (12 samples) and the actin exon from the alga (seven samples)

	Sequence similarity (%)		Fragment length (bp)	
	ITS rDNA	Actin exon	ITS rDNA	Actin exon
Alga				
Within algae from *Evernia mesomorpha*	> 91.3	> 90	622–670	199–258
Within *Trebouxia jamesii*	> 86	> 85	622–671	170–258
Within *Trebouxia*	> 80	–	581–678	–
Fungus				
Within *Evernia mesomorpha*	> 99.8	–	491–502	–
Within *Evernia*	> 91	–	483–509	–

Ranges are shown for different taxonomic breadths, establishing more similarity within species than between species of *Trebouxia*. Fungal fragment length is based on the ITS1, 5.8S and ITS2 regions, with surplus SSU sequence removed.

AMOVA detected genetic structure among algal populations (ϕ_{PT} = 0.03; P = 0.04). Most of the variation (97%) was attributed to individuals within populations. Based on lichen population size (290 thalli with algal mixtures) there was no algal genetic structure among transects (ϕ_{RT} = 0.00; P = 0.53), but there was structure among populations (ϕ_{PT} = 0.10; P = 0.01) and among individuals within populations (ϕ_{PR} = 0.10; P = 0.01). Most (90%) of the variation occurred within populations and less variation (10%) occurred among populations (Fig. 2). The discrepancy between algal and lichen population sizes suggested that the presence of several algal genotypes in the same thallus ('mixture' of algal genotypes) may play a role in the lichen life history strategy. The discrepancy may be explained by examination of the pairwise lichen population values (ϕ_{PT}) where 13% of the pairwise comparisons produced significant population structure (Fig. 3). A single tree was homogeneous with algal genotype II (tree 15), and four trees had more than 70% of the thalli containing a single genotype (trees 3, 5, 7 and 28). Tree 4 contained 60% of the less common genotype I and showed significant differences with other trees. Genetic differentiation was highest in comparisons with these four trees, suggesting population subdivision and a low level of dispersal. The remaining 25 heterogeneous populations had lower ϕ_{PT} values, suggesting low population subdivision and higher levels of dispersal. Based on fungal population size, there was no population subdivision among regions (ϕ_{RT} = 0.00; P = 0.22), among populations (ϕ_{PT} = 0.00; P = 0.96), or among individuals within populations (ϕ_{PR} = 0.00; P = 0.96), suggesting a high level of dispersal among the fungal genotypes.

The Mantel test produced a positive correlation (R_{xy} = 0.05; P = 0.02) between distance matrices of algal and fungal genotypes. However, the Spearman's correlation coefficient showed no significant correlation between algal and fungal genotypes and therefore no coevolutionary pattern at the genotypic level. Minimum spanning trees (Fig. 4), produced from frequencies of each of the algal and fungal ITS genotypes, showed a random distribution (i.e. no clustering) of the trees in each transect, suggesting widespread dispersal. If dispersal was restricted to neighbouring jack pine trees, the same genotype would be expected to remain common in trees located next to one another. The algal minimum spanning tree shows a dominance of genotype II with homogeneous populations on the far left, heterogeneous populations with dominant genotype II clustering in the centre of the tree, and mixtures of genotypes on the far right in the tree. The fungal tree shows populations containing less than 70% genotype A on the far right, 80% genotype A (the most common arrangement) in the middle, and 90% genotype A on the far left of the tree. There were no samples that contained both fungal genotypes in the same thallus. The branching patterns in the algal and fungal trees are different from one another.

Spearmans rank correlation coefficient analysis was conducted on algal genotype frequencies and tree DBH to test for relationships. The significantly negative correlation between the frequencies of genotypes I and II (P = 0.000, r = −0.780) and between genotypes I and III (P = 0.039, r = −0.386) may suggest some level of competition or strong fungal selection between the algal genotypes. The significantly positive correlation between frequency of genotype III and the mixture of genotypes (P = 0.000, r = 0.916) is a result of genotype III being found predominantly within a mixture of genotypes rather than alone in the thallus. Correlations between all other combinations of frequencies and tree DBH were not significant. Distribution of the algal genotypes is shown in Fig. 2.

Photobiont identity and phylogenetic histories

To determine the closest relative of the algal partner in *E. mesomorpha*, ITS rDNA sequences representing the five genotypes were subjected to a BLAST search (Altschul *et al.*, 1997), resulting in significant e-scores with GenBank sequences from *T. jamesii*. The midpoint rooted algal ITS phylogeny was one of 54 MP trees with 524 steps (Fig. 5). In addition, a BLAST search with the coding region of algal type I actin sequences resulted in fewer matches that included two species

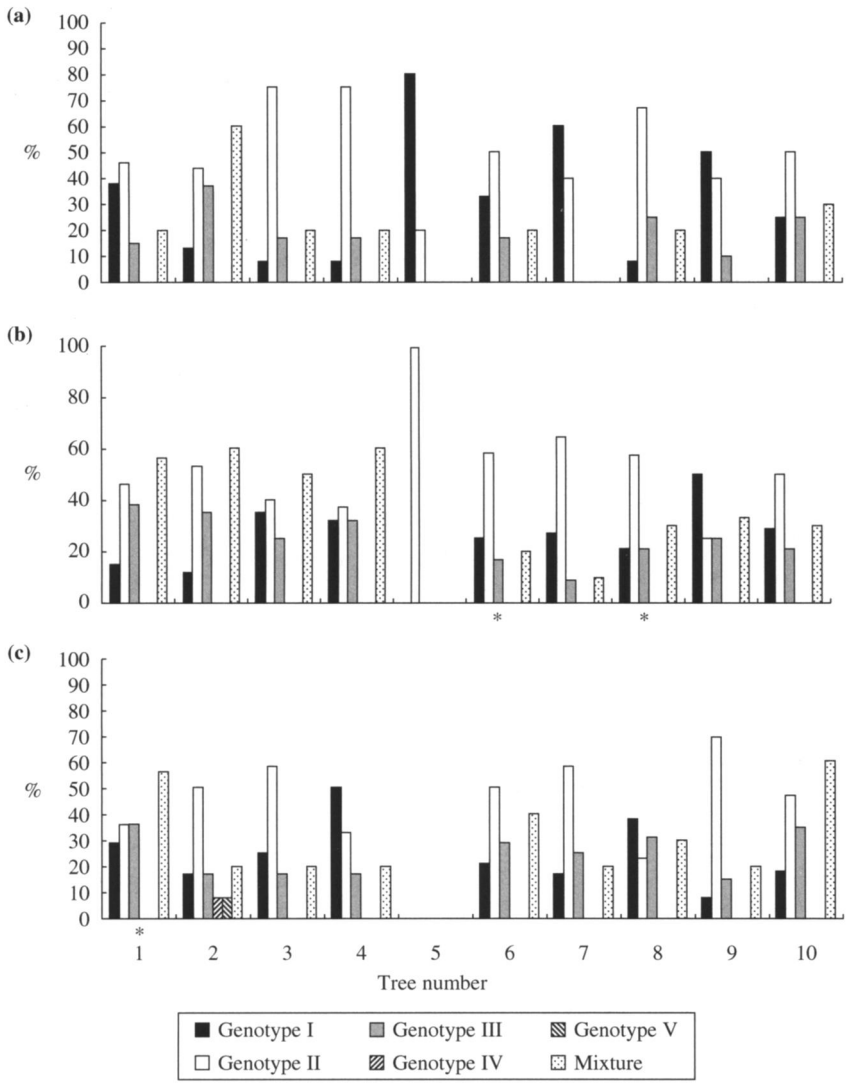

Fig. 2 Bar graphs showing percentage (%), on the *y*-axes, of each algal genotype (algal population size) and mixtures of algal genotypes (see legend) among 10 trees in each of transects (a) 1, (b) 2 and (c) 3. Each bar represents the percentage value of number of each genotype out of the total number for that tree. The occurrence of fungal apothecia is indicated by an asterisk below the tree.

of *Trebouxia* (*T. jamesii* and *T. erici* Ahmadjian). The intron sequence was too variable to detect significant homology in a BLAST search. A MP analysis of 11 taxa and 258 aligned nucleotides from the actin gene produced 76 most parsimonious trees of 130 steps (Fig. 6). All algal sequences clustered within *T. jamesii*, forming monophyletic groups with 97% bootstrap support in the ITS rDNA and 80% bootstrap support in the actin gene tree. Phylogenetic trees from both loci were in agreement with respect to the clustering of algal genotypes I, II, IV and V, and also with the separation of genotype III basal to the clade containing the alga from *L. vulpina* (L.) Hue (Figs 5 and 6).

Results from a BLAST search of the fungal ITS rDNA of *E. mesomorpha* produced significant e-scores with similarities to two other species of *Evernia* – *E. esorediosa* and *E. divaricata*. The ITS rDNA of *E. prunastri* was not detected in the BLAST search. The fungal genus *Evernia* is paraphyletic with *E. prunastri*, forming a sister species to *P. saxatilis* (Fig. 7) and differed from the other species of *Evernia* by 8–9% sequence divergence. Within the genus *Evernia*, *E. divaricata* was separated from both *E. mesomorpha* and *E. esorediosa* but all three species formed a clade with 100% bootstrap support. The sample, Em86, was not separated from the genus *Evernia*, in contrast to the situation with the algal partner, which was separated in the algal tree. *Pseudevernia cladionia* and *P. consocians* were separated from *P. furfuracea*, both forming clades with 100% bootstrap support.

Discussion

Fungal selection of algal genotypes

E. mesomorpha was not limited to one ITS genotype of *T. jamesii* in the lichen association. In fact, the fungus formed an association with more than one algal genotype in the same branch of a thallus (Figs 1 and 2). This is one of the first studies to report multiple genotypes of algae within the same

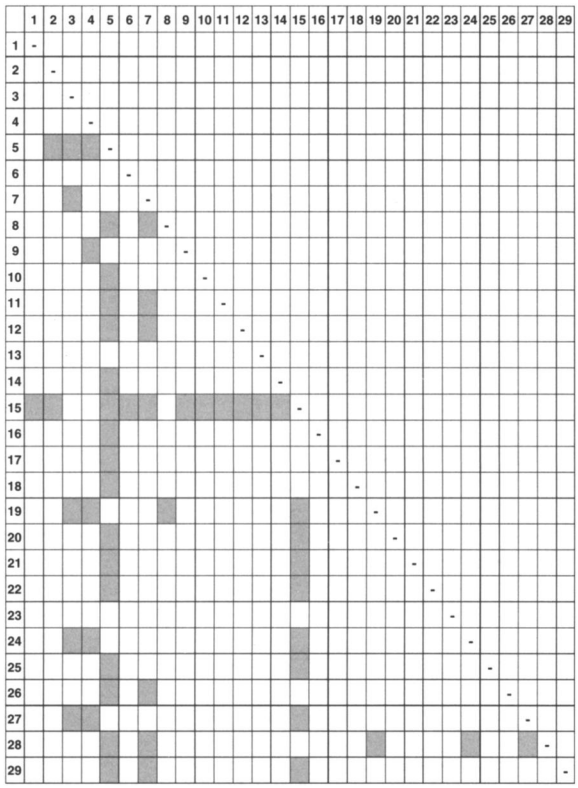

Fig. 3 Pairwise comparison of populations of algal and fungal genotypes showing significant ϕ_{PT} values (shaded boxes) and non-significant ϕ_{PT} values (open boxes). (ϕ_{PT} is the population structure among populations. See data analyses in the Materials and Methods section.) Numbers from 1 to 29 in the left column and the top row represent populations (i.e. jack pine tree with 10 thalli). Comparisons below the diagonal are for algal populations, and above the diagonal are for fungal populations.

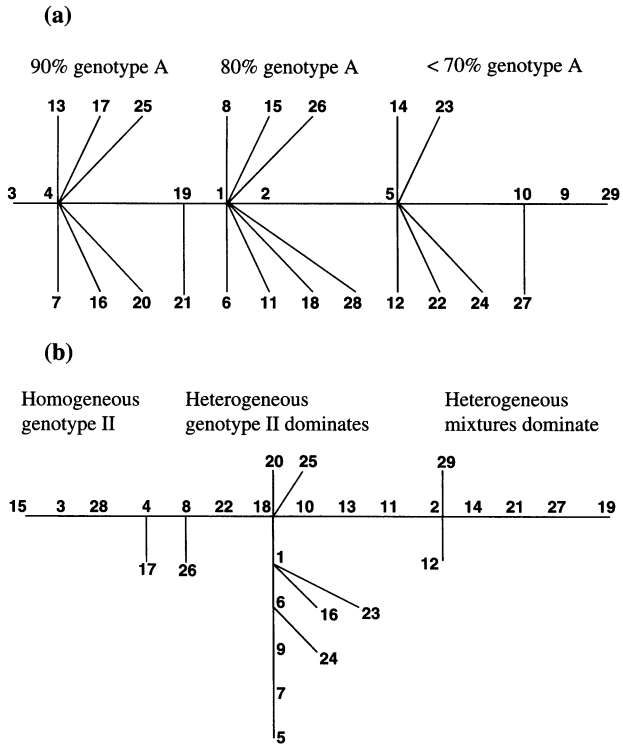

Fig. 4 Minimum spanning trees for (a) fungal and (b) algal genotypes, showing the distribution of populations (trees 1–29) based on frequency of genotypes. If a dominant genotype was shared among neighbouring trees they would cluster together.

lichen tissue in a high proportion of samples. However, it has been suggested that a single thallus may consist of more than one individual (Fahselt, 1996), and there have been reports of the occurrence of more than one algal genotype in species of *Rinodina* (Helms *et al.*, 2001) but the number of samples containing genotype mixtures was low. Romeike *et al.* (2002) also reported several algal variants within the same species of *U. decussata* (Vill.) Zahlbr., just 30 m from one another, with two algal variants in different parts of the same thallus. A mixture of algal genotypes within a single thallus containing one fungal genotype reflected low specificity for *E. mesomorpha*, despite the fact that all the algal genotypes came from the same species, *T. jamesii*. On the other hand, high specificity was evident in the family Physciaceae (Helms *et al.*, 2001), and high selectivity in *Cladonia rangiferina* (L.) Nyl., and *C. multifomis* G. Merr. (Piercey-Normore, 2004). Single algal genotypes were reported from each thallus of *Letharia* (Kroken & Taylor, 2001), for the most part in *Umbilicaria* (Romeike *et al.*, 2002) and in *Cladonia* (Piercey-Normore & DePriest, 2001; Piercey-Normore, 2004). Interpretation of fungal specificity may vary depending on the taxonomic range studied. Although there were five algal genotypes associated with *E. mesomorpha*, they were nested within the 21 monophyletic *T. jamesii* genotypes. Therefore, *E. mesomorpha* associates with five ITS genotypes of *T. jamesii* as the algal partner.

Mixtures of algal genotypes in the thallus may be an important life history strategy because most of the variation was attributed to individuals within populations. The mixture of algal genotypes in the same thallus of *E. mesomorpha* may be an adaptive advantage (Romeike *et al.*, 2002) to harsh or changing environmental conditions. *E. mesomorpha* is also thought to be pollution tolerant (Gunn *et al.*, 1995) and therefore may associate with several algal genotypes as an adaptation to changes in environmental conditions. An additional hypothesis is that the fungus may utilize several algal genotypes to achieve maximum photosynthesis with varying light levels in the forest canopy. Change in the morphology of *Pseudevernia furfuracea* (L.) Zopf., a species related to *Evernia*, occurred with an unequal distribution of solar radiation in the forest canopy (Rikkinen, 1997). Changes in solar radiation would directly affect the algal partner, perhaps influencing the dominant algal genotype in the thallus. Corresponding changes in the algal symbiont with changes in the environment forms the adaptive bleaching hypothesis with *Symbiodinium* symbionts and coral (Kinzie *et al.*, 2001; Rowan, 2004). The occurrence of multiple algal

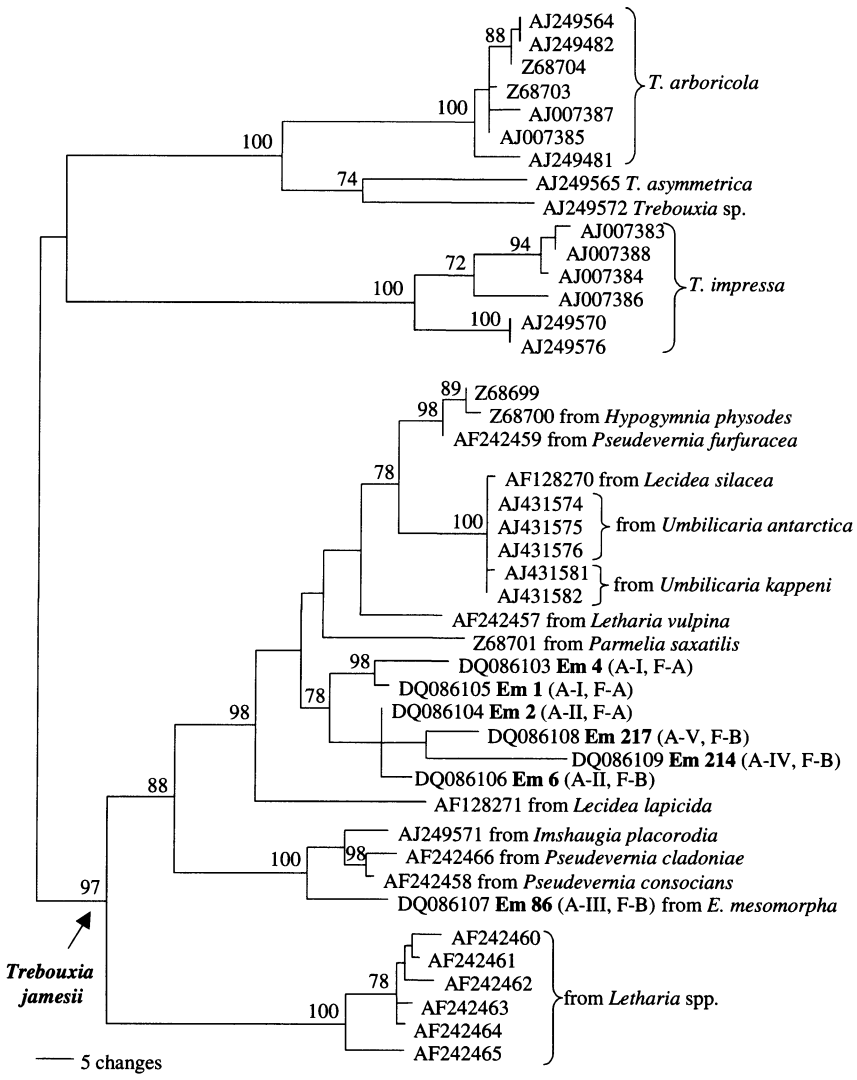

Fig. 5 One of 54 most-parsimonious trees of algal internal transcribed spacer (ITS) nuclear ribosomal DNA (rDNA) sequences from seven samples of *Evernia mesomorpha* (bold) and 36 additional sequences from NCBI GenBank. Algal (A) and fungal (F) genotypes are shown in parentheses after each sample (Em). The tree length was 524 steps with 195 informative characters; CI (consistency index), 0.6889; RI (retention index), 0.9062; and the tree was midpoint rooted. Numbers above lines are bootstrap values from 500 resamplings. Only bootstrap values of > 70% are shown.

genotypes in the same thallus may also be related to the age of the thallus (Fahselt *et al.*, 1995). An older thallus would have had a longer time to accumulate multiple genotypes from dispersed soredia. If the soredium already contained several algal genotypes before it landed on a thallus, *E. mesomorpha* would rapidly accumulate multiple genotypes. The rare genotypes IV and V may be founder events, or they may be intolerant of the microhabitat and are decreasing in number. If maintained within the population, they may provide flexibility of the population to rebound after environmental changes.

Fungal phylogeny and coevolutionary patterns

Low ITS sequence divergence (1%) among sequences of *E. mesomorpha* and the GenBank sequences of *E. esorediosa* and the unknown species *Evernia* sp. (DQ001282) suggest that they are the same species. The ITS1, ITS2 and 5.8S regions were identical among all specimens of *E. mesomorpha* and *E. esorediosa* (Müll. Arg.) Du Rietz, except for four nucleotide substitutions. Although both are northern species with similar habitats, *E. esorediosa* is found in eastern Siberia and Japan and has abundant apothecia (Bird, 1974), while *E. mesomorpha* is circumboreal, sorediate and rarely has apothecia. The low level of variation in the ITS of *E. mesomorpha* is similar to that reported for the threatened *Lobaria pulmonaria* (L.) Hoffm. in Switzerland (Zoller *et al.*, 1999). Fahselt (1988) reported that isozyme variation in *E. mesomorpha* was the lowest of five genera examined. The level of variation among *E. mesomorpha*, *E. esorediosa*, and *E. divaricata* (L.) Ach. was low (1.4%), but, including *E. prunastri* (L.) Ach. (8–9%), was similar to that found in other genera of lichen fungi in this study, such as 10% in *Pseudevernia* and 5% in *Umbilicaria*, and 19% in the widespread genus *Cladonia* (Piercey-Normore & DePriest, 2001). Fungal variation in the genus *Letharia* revealed five to six phylogenetic species (Kroken & Taylor, 2001). Based on the information in this study, there may be three phylogenetic

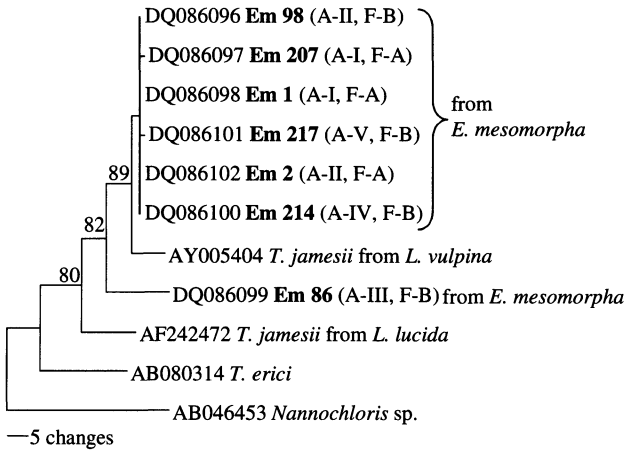

Fig. 6 One of 76 most-parsimonious trees of the algal type I actin-coding region from seven samples of *Evernia mesomorpha* (bold) and four samples from NCBI GenBank. Algal (A) and fungal (F) genotypes are shown in parentheses after each sample (Em). The tree length was 130 steps with 34 informative characters; CI, 0.8692; RI, 0.6531; and *Nannochloris* sp. was designated as the outgroup taxon. Numbers above the lines are bootstrap values from 500 resamplings. Only bootstrap values of > 70% are shown.

species in the genus *Evernia*: (1) *E. mesomorpha*, *E. esorediosa*, and the unknown species; (2) *E. divaricata*; and (3) *E. prunastri*. A larger number of independent loci must be examined before phylogenetic species can be proposed for *Evernia*.

The fungal ITS rDNA phylogeny did not agree with the algal ITS rDNA phylogeny (Figs 5 and 7). The sample Em86 was nested within the *E. mesomorpha* fungal clade, but fell outside the *E. mesomorpha* algal clade with *Pseudevernia* and *Imshaugia*, suggesting that the lichen-forming fungus of Em86 belongs to the species *E. mesomorpha* but that the alga from Em86 may be a different phylogenetic species within *T. jamesii*. Even though the Mantel test produced a significant correlation between symbiont distance matrices at the population level, there was no correlation between the algal and fungal genotypes and there was disagreement between the minimum spanning trees (Fig. 4), suggesting a lack of genotypic specificity within *E. mesomorpha*. The low level of genotypic coevolution between symbionts was supported by the above tests and by the number of genotypes in the thallus, where 45% of the thalli contained multiple algal genotypes and none contained multiple fungal genotypes. Few studies have shown multiple fungal genotypes in the same thallus (Murtagh *et al.*, 2000). It is thought that the thalli of some *Xanthoria* species may consist of two fungal mating types (Honegger *et al.*, 2004). The pattern of a slowly evolving fungal partner (1.4% sequence divergence) and a faster evolving algal partner (8.7% sequence divergence) within *E. mesomorpha* is contrary to findings in *Omphalina* (Zoller & Lutzoni, 2003) and *Cladonia* (Piercey-Normore & DePriest, 2001).

Phylogenetic species and algal switching in *T. jamesii*

Algae associated with *E. mesomorpha* may represent two phylogenetic species because of the higher level of variation in the *T. jamesii* clade (8.7%) than that reported for species delimited by gene genealogies (Kroken & Taylor, 2001). The first phylogenetic species, represented by the algae of the *E. mesomorpha* clade, is monophyletic with 78% bootstrap support and consists of genotypes I (Em1 and Em4), II (Em2 and Em6), IV (Em214), and V (Em217). The second phylogenetic species is represented by the clade containing genotype III (Em86), *P. consocians* (Vainio) Hale & Culb., *P. cladonia* (Tuck.) Hale & Culb., and *I. placorodia* (Ach.) S. F. Meyer, with 100% bootstrap support. At least six to seven phylogenetic species of *T. jamesii* have been reported to associate with *Letharia* (Kroken & Taylor, 2000). Algal genotype III from *E. mesomorpha* may have switched from *P. consocians*, *P. cladonia* or *I. placorodia* to colonize *E. mesomorpha*. It is possible that the algal switch resulted from soredia of *E. mesomorpha* landing on the thallus of a species of *Pseudevernia* or *Imshaugia* after which the fungal hyphae (from the soredium) incorporated the alga from *Pseudevernia* or *Imshaugia* into its developing thallus. An alternative explanation is that ascospores from the apothecia of *E. mesomorpha* lichenized with a free-living alga or incorporated an alga from the thallus of another species. Although this may be an unlikely explanation, the low level of sexual reproduction detected in *E. mesomorpha* (six apothecia in a population of 290 thalli) suggests that the fungus has the potential to switch algal partners. This algal switch may be a recent algal switch in which the alga is not yet fully acclimatized to the thallus of *E. mesomorpha*. Evidence for this recent algal switch includes: (1) a positive correlation between genotype III and the mixtures of genotypes among 29 trees, suggesting that genotype III requires the presence of another genotype; (2) the topological agreement between the actin and algal ITS rDNA phylogenies (Figs 4 and 5) placing genotype III distant to the other algae from *E. mesomorpha*; and (3) the highly variable actin intron in Em86, suggesting a different phylogenetic origin for the actin intron in genotype III.

Resource tracking may also explain the divergent algal genotype III. The sample, Em86 (genotype III), clustered with algal strains that associate with *P. consocians*, *P. cladonia*, and *I. placorodia*. These species colonize woody substrata similar to *E. mesomorpha*. Perhaps the ancestral *T. jamesii* genotype was tracking this type of habitat, and the genotype diverged to associate with *Pseudevernia*, *Imshaugia*, and *Evernia*. The superficial morphological resemblance of *Pseudevernia*, *Imshaugia* (Ach.) S. F. Meyer, and *Evernia*, and their occurrence in similar habitats on conifers, support the clustering of algae (100%) from the three genera (Fig. 5), even though the three fungal genera were separated in a combined phylogeny based on three unlinked nuclear genes (Thell *et al.*, 2004). As the lichen chemistry and habitat may also be a reflection of the fungal taxonomy, the alga may appear to be tracking

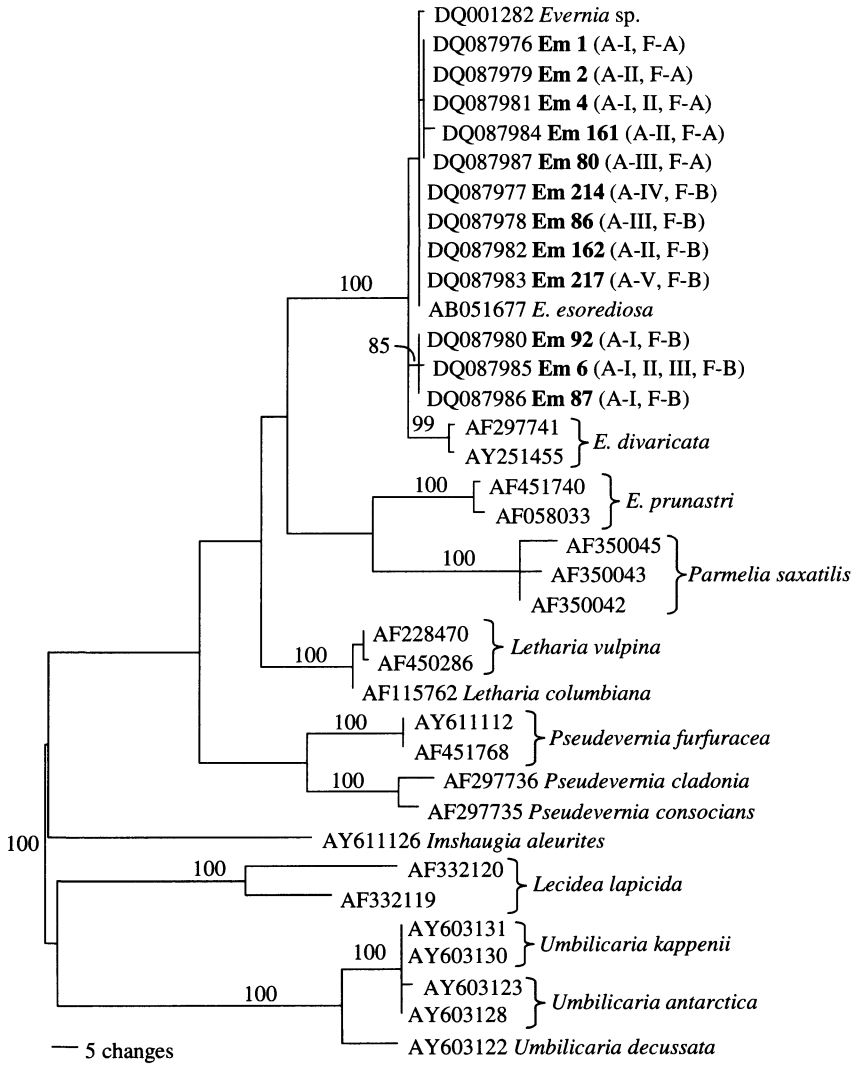

Fig. 7 One of 30 most-parsimonious trees of fungal internal transcribed spacer (ITS) nuclear ribosomal DNA (rDNA) sequences from 12 samples of *Evernia mesomorpha* and 24 additional sequences from NCBI GenBank. Algal (A) and fungal (F) genotypes are shown in parentheses after each sample (Em). The tree length was 419 steps with 178 informative characters; CI (consistency index), 0.7208; RI (retention index), 0.8848; and the tree was midpoint rooted. Numbers above the lines are bootstrap values from 500 resamplings. Only bootstrap values of > 70% are shown.

the taxonomic unit, but is actually tracking characters that are used to define the taxonomic unit (Kethley & Johnston, 1975). A study on the algae of the Parmeliaceae that colonize a variety of substrates may provide more insight into the resource that the alga is tracking, its specificity to the fungal taxonomic unit and coevolutionary patterns.

Dispersal and competition in the algal partner

Dispersal by lichen soredia was interpreted to be common because most of the variation was attributed to individuals within populations (90–97%) when both algal population data and lichen population data were examined. In addition, algal genotypes appeared to be evenly distributed among populations (Fig. 2). Dispersal distances of 30 m were reported for the soredia of *R. farinacea* (L.) Ach. and *E. prunastri* (Tapper, 1976). As transects in this study were approx. 50 m long, dispersal in *E. mesomorpha* was > 30 m. In addition, dispersal may have occurred for a long time period, permitting mixing of genotypes over a large distance. Although soredia are produced abundantly on *E. mesomorpha* and may be wind dispersed through the open jack pine forests in summer and across the frictionless snow surface in winter, the algal genotypes found in *E. mesomorpha* have not been reported in other species, except genotype III in *Pseudevernia*, despite the apparent ease of dispersal. Dispersal and relichenization of fungal spores from the low number of sexual reproductive structures may contribute to the level of algal variation observed. However, it is more likely that an alga will be taken up from another thallus, or soredium (Ahmadjian, 1993). If sexual reproduction was occurring at a high level in the algal population, incongruent algal phylogenies would be expected (Kroken & Taylor, 2001) but were not detected in this study (Figs 5 and 6). Therefore, most of the mixing of algal genotypes probably occurs by fungi in one soredium taking up algae from another source. The algae may rely on the abundantly produced lichen propagules in *E. mesomorpha* for gene flow between trees. As the decline in gene flow with distance (Wright, 1943) is thought to occur at a very slow rate

in highly 'mobile' species, a larger geographical area must be studied before changes in the proportions of genotypes relative to geographical distance can be detected.

Although overall gene flow for the algal populations was low, indicating increased dispersal, there were significant levels of genetic structure among pairs of populations (Fig. 3). The high ϕ_{PT} between population 5 and other populations suggested that dispersal from other trees to tree 5 was low. On the other hand, none of the fungal populations produced significant population subdivision (Fig. 3), suggesting that dispersal was high. With only two fungal loci, the ITS rDNA may have provided insufficient variation to detect the same level of population subdivision as in the alga. Nevertheless, if population subdivision was significant, a larger number of trees with homogeneous genotypes would be present for both symbionts. Only one of the 29 trees contained 10 thalli with a single algal genotype and no tree contained a single fungal genotype, even with only two ITS genotypes detected from all the samples.

Competition may play a role in the population dynamics of the algal partner. The two significant negative correlations between algal genotypes (genotypes I and II, genotypes I and III) may present at least three different scenarios.

(1) Fungal preference, where one genotype may be preferred by the fungus or have the ability to tolerate microenvironmental stress or undergo seasonal influences (Fiechter & Honegger, 1988) better than another genotype.

(2) Fungal rejection, where one genotype may be more limited in growth by the fungus than another genotype.

(3) Algal competition, where genotypes I and II, and genotypes I and III may form antagonistic relationships with each other while in the same lichen thallus with little influence from the fungus.

Any one or a combination of the three scenarios may be possible. The first two scenarios would result from fungal–algal interactions, rather than direct competition between two algae. The third scenario may be a consequence of one genotype having a negative effect on neighbouring genotypes by capturing all resources, or by releasing inhibitory compounds, as in allelopathy (LeGrand *et al.*, 2003). Competition or stress tolerance among algal genotypes may play a role in the absence of more than one genotype reported for many other lichen associations. In the coral–*Symbiodinium* symbiosis, it is thought that fluctuating water temperatures on the reef influence survival of the algal symbionts, shuffling the algal community within the coral body (Kinzie *et al.*, 2001).

Conclusions

In conclusion, two ITS genotypes of *E. mesomorpha* associate with five ITS genotypes of *T. jamesii*. Combinations of the five algal genotypes were detected together in 45% of the samples, but the two fungal genotypes were never found together in a thallus. Although the genotypes are based on RFLP of the ITS rDNA sequences from seven algal and 14 fungal samples, sequences of all 290 samples may reveal other genotypes. Based on this study, the less variable fungal partner may take advantage of the more variable algal partner for adaptation to changing environmental conditions, similar to the *Symbiodinium*–coral symbiosis. Coevolutionary studies (such as this one) at the population scale may provide insight into adaptations of rare or invasive species, and why some have difficulty surviving or are widespread and can tolerate a wide range of environmental conditions species (such as *E. mesomorpha*). The lack of coevolution detected in lichens at higher taxonomic levels may, in part, result from low specificity at the population level. Low sample size may also contribute to the lack of evidence for coevolution, especially if the samples include recent algal switches or rare genotypes. If the degree of fungal selectivity among populations has an influence on the detection of coevolution at higher taxonomic levels, only species with high selectivity and few algal switching events should show evidence for coevolution. A larger number of species must be examined at the population level before this hypothesis can be tested.

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