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Genetically divergent algae shape an epiphytic lichen community on Jack Pine in Manitoba

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Abstract: Algal genotypes should freely associate with different lichen fungi that grow in the same confined habitat giving the appearance of low levels of selectivity and specificity. If genetic compatibility between algal and fungal partners limits the combination of partners, then some degree of taxonomic specificity should be evident. This study examined the photobiont composition in a community of epiphytic lichens on Jack Pine to investigate selectivity and specificity. The objectives of the study were to infer algal identity, to infer photobiont dispersal, and to investigate the distribution of algal genotypes relative to the fungal partner. Photobiont variability was determined by Restriction Fragment Length Polymorphism (RFLP) and nucleotide sequences of the Internal Transcribed Spacer (ITS) of ribosomal DNA (rDNA). Seven species of lichen-forming fungi are reported to associate with five divergent algal genotypes, with only one species, *Evernia mesomorpha*, showing some degree of selectivity and specificity. The algae represent at least two species (*Trebouxia jamesii* and *T. impressa*) for the area confined to 200cm^2 on the north side of 20 Jack Pine trees. Gene flow was inferred in this tightly defined community of lichenized algae. The algal sharing and inferred gene flow may suggest that soredia provide a means of algal transport and distribution among lichen-forming fungi in the habitat.

Key words: photobiont, selectivity, specificity, *Trebouxia impressa*, *T. jamesii*, *T. simplex*

Introduction

Even though lichens can be found in most ecosystems of the world (Nash 1996), many lichen-forming fungal symbionts cannot survive without the photobiont, reflecting the ecologically obligate nature of the fungal partner in the symbiosis (Honegger 1996). Unlike the mycobionts, many photobionts are thought to be free-living except members of *Trebouxia* De Puymaly (see Ahmadjian 1988; Mukhtar *et al.* 1994; Tschermak-Woess 1978). Flexibility in the taxonomic specificity and ecological selection of the photobiont must allow for alternative partnerships in different habitats. A highly selective lichen association may be recognized when the uncommon algal species in the pool of all available algae form lichen associations. Selectivity refers to the availability and preferential interactions of the symbionts in nature (Galun & Bubrick 1984). A low level of selectivity, reported for the genus *Umbilicaria*, was considered to be advantageous to the mycobiont where a high diversity of photobionts was available to associate with the fungi (Romeike *et al.* 2002). A highly specific lichen association may be recognized if one fungal taxon is always associated with one algal taxon and less specific when a fungal taxon associates with more than one algal taxon (Smith & Douglas 1987). Specificity refers to the range and taxonomic relatedness of symbiotic partners. For further explanation of selectivity and specificity in lichen associations see Honegger (2008).

Since the list of mycobionts and photobionts was published by Tschermak-Woess (1988), photobiont species are becoming known for a number of taxa of lichen-forming fungi. It has been shown that lichen fungi in the *Lecanorales* (except *Cladoniaceae* and *Stereocaulaceae*) associate with members

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of the algal genus *Trebouxia* (Beck 1999; Beck *et al.* 1998; Dahkild *et al.* 2001; Helms *et al.* 2001; Kroken & Taylor 2000; Ohmura *et al.* 2006). Fungi in the *Cladoniaceae* and *Stereocaulaceae* associate with green algae in the genus *Asterochloris* (*sensu* Tschermak-Woess 1980) (Nelsen & Gargas 2008; Piercey-Normore & DePriest 2001). Morphological and genetic characteristics of *Trebouxia* and *Asterochloris* have been described by Friedl (1989); Rambold *et al.* (1998) and Tschermak-Woess (1980). One species in *Trebouxia, T. jamesii*, is considered to be highly diverse (Kroken & Taylor 2000). Therefore, tentative names have been assigned to several clades within *T. jamesii* based on sequence variation and the fungal taxon associated with the alga (Hauck *et al.* 2007; Kroken & Taylor 2000).

The identity and variability in photobionts is commonly evaluated through nucleotide sequence polymorphism in the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA). The same region has also been used to examine variation among photobionts across large geographic regions (Ohmura *et al.* 2006; Kroken & Taylor 2000; Yahr *et al.* 2004) showing some differentiation. Population studies at smaller geographic scales using RFLPs (Robertson & Piercey-Normore 2007; Piercey-Normore 2006) showed less differentiation and high levels of gene flow suggesting that a diversity of algae is available to associate with lichen fungi at these smaller geographic scales. Therefore, on continuous substrata with distinct spatial boundaries, algae should become available to freely associate with any lichen fungal propagule that is present on the substratum, suggesting that algae are shared equally among lichen fungi within the same spatially defined habitat. However, if selectivity or specificity by the fungus constrains the photobiont, algae will not be shared equally within a lichen community.

The first objective of this study was to infer the taxonomic identity of the photobiont by comparing sequence similarity of photobionts in a lichen community with those from published taxa in NCBI GenBank from authentic morphological species. It is expected that the algae will be members of the genus *Trebouxia*. The second objective was to infer gene flow in the photobiont with the hypothesis that the photobiont will show low levels of population subdivision i.e. high levels of gene flow. The third objective of this study relied on the results of the first two objectives. The third objective was to investigate selectivity and specificity by examining the photobiont distribution in a community of lichens growing on continuous substrata with distinct spatial boundaries. Because algal dispersal has been shown to occur at small geographic scales, the hypothesis is that such a community of lichen-forming fungal species will share algal taxa suggesting low levels of selectivity and specificity.

Methods

Field collection

Epiphytic lichens were collected from the trunks of 20 Jack Pine trees (*Pinus banksiana* Lamb.) in Manitoba. Two transects were established, and each of ten trees within one metre of the transect was sampled. The location of the north end of transect 1 was N 51°7′35.3″ W 96°15′21.8″ and the location of the north end of transect 2 was N 51°7′54.5″ W 96°16′18.3″. Transect 1 was 60·1 metres long and transect 2 was 39·6 metres long. Both Jack Pine stands had similar ground vegetation, were both over 60 years old (80–90% Jack Pine and 10–20% trembling aspen), contained similar species of lichen-forming fungi, and were approximately 1 km apart. The lichen community on the tree trunk was confined to 20 cm to the left and 20 cm to the right of due north. and between 100 and 150 cm above ground level. Therefore, lichens were collected from an area of 200 cm^2 on each tree.

Two healthy thalli of each species were collected and were at least two centimetres long, in the case of fruticose species, or two centimetres in diameter, for foliose species. Vouchers of lichen thalli were air dried and deposited in the cryptogamic division of the University of Manitoba Herbarium (WIN). The collection numbers for the thalli collected in the present study were those of the first author and ranged from 06001 to 06203.

DNA extraction and amplification

A piece of young thallus, 0·5 to 1 cm, was used in the DNA extractions. The total cellular DNA was extracted with liquid nitrogen in a cetrytrimethylammonium bromide (CTAB) buffer following a protocol modified from Grube *et al.* (1995). The DNA pellet was suspended in 50 µl warm, sterile, distilled water and stored at −20°C.

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The algal internal transcribed spacer (ITS) of the nuclear ribosomal DNA (rDNA) and part of the small subunit (SSU) were amplified by PCR from 149 lichen specimens. All amplifications were in 20 µl reaction volumes. Ten to 150 ng of DNA were added to each PCR reaction, which contained amplification buffer (50 mM potassium chloride, 10 mM Tris-HCl (pH 8·3)), 200 µM of each dNTP, 2 mM magnesium chloride, 0·5 µM of each primer, and 2 units of Taq DNA polymerase (Invitrogen, Burlington, ON, Canada), The primers used were the algal specific primer AL1700F (5′-CCCACCTAGAGGAAGGAG-3′, Helms *et al.* 2001) located in the SSU rDNA and ITS4 (5′-TCC TCCGCTTATTGATATGC-3′, White *et al.* 1990) located in the large subunit rDNA. Amplifications were performed in a thermal cycler (Biometra T-Gradient; Tampa, FL, USA) with an initial denaturing of the template DNA for 5 minutes at 94°C followed by 34 cycles of denaturing for 1 minute at 94°C, annealing for 1 minute at 54°C, and extension for 2 minutes at 72°C. Samples were then stored at 4°C.

Restriction Fragment length polymorphisms (RFLP) and electrophoresis

Each 20 µl reaction included 2 units of the enzymes HaeIII and HpaII (Invitrogen, Burlington, ON, Canada), manufacturer's buffer, and approximately 200 ng of DNA, The reactions were carried out at 37°C following the manufacturer's directions. The presence or absence of an intron in the PCR product was recorded before the PCR product was digested allowing for similar banding patterns of the ITS-RFLP to be distinguished. Bands less than 100 base pairs were not scored for genotype determination. The RFLP genotype was based only on the ITS region.

PCR product was electrophoresed on a 1% agarose gel while the digested PCR product was electrophoresed on a 2% agarose gel. All gels were stained with ethidium bromide (0·5 µg ml^{-1}) and run at 130 volts until the loading dye (6 × bromophenol blue) was 1 cm from the bottom of the gel. The gels were electrophoresed in 1 × TBE buffer (89 mM Tris-HCl pH 8·0, 89mM boric acid, 20mM EDTA) and visualized with ultraviolet light in an Alphalmager 2200 transilluminator (Fisher Scientific, Nepean, ON, Canada). The fragment lengths of the digested PCR product were estimated by comparison with a 1kb DNA size standard (Invitrogen, Burlington, ON, Canada). A single representative sample of each RFLP banding pattern was selected for sequencing to show DNA variation in the algae from different lichens and to allow sequence comparison with other species of *Trebouxia* to infer algal identity. Identical RFLP patterns may not have identical DNA sequences but will only have the same four-base restriction enzyme recognition sequence in the same locations resulting in an underestimation of the number of algal taxa. Therefore other algal taxa may be present within each RFLP genotype. RFLP genotypes have previously been used for both symbionts in *Evernia mesomorpha* (Piercey-Normore 2006) and for the algal symbiont in Robertson & Piercey-Normore (2006). Samples that were selected for sequencing were 06019 (*T. impressa* B from *Parmelia sulcata*; EU683442), 06021 (*T. jamesii* B from *Tuckermanopsis americana*; EU683441), 06102 (*T. impressa* A from *Usnea lapponica*; EU683442), 06106 (*T. impressa* C from *Flavoparmelia caperata;* EU683444), and 06117 (*T. jamesii* A from *Evernia mesomorpha*: EU6S3440).

DNA sequencing

Eight replicate 50 µl volumes of PCR product of the RFLP representatives were pooled for sequencing. The pooled 400 µl of PCR product was precipitated by adding 0·2 volumes of 5M NaCl and 2·5 volumes of anhydrous ethanol, and centrifugation at 13 000 rpm for 10 min. The DNA pellet was resuspended in 20 µl of sterile, distilled water. The 20 µl of resuspended DNA was gel purified by excising the band from a 1% agarose gel, overnight freezing, crushing the frozen gel block in a folded parafilm strip, and retrieving the liquid containing the DNA by pipette transfer to a tube. DNA precipitation was repeated and the pellet was again resuspended in 20 µl sterile distilled water.

Cycle sequencing reaction volumes were 20 µl and contained 120 ng of purified PCR product, BigDye v. 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's directions, and the same PCR primers were used for sequencing. Post reaction cleanup followed the manufacturer's directions for the EDTA and ethanol precipitation, and the samples were dried in a DNA 120 SpeedVac (ThermoSavant, Holbrook, NY, USA). The dried product was resuspended in 20 µl of formamide and, after denaturation at 95°C for 5 minutes, the samples were immediately placed on ice and loaded into a 96-well plate for sequencing (3130 Genetic Analyser, Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were edited in Sequencher v. 4.6 (Gene Codes Corporation, Ann Arbor, ML USA) and all sequences were manually aligned using Se-Al v. 1.0 (Rambaut 2001). The ITS regions were delimited by comparison with GenBank sequences (accession numbers AJ249571, Z68698) and the ambiguous bases (approximately 100 bp) at each end of the IT S region were removed for the analyses. These ambiguous nucleotides resulted from sequencing ambiguity rather than taxon ambiguity.

Data analyses

Analysis of molecular variance (AMOVA) in GenAlEx version 6 (Peakall & Smouse 2005) was used to determine the population structure of the photobionts. The data were analysed by defining a population in terms of the number of trees and in terms of the algal taxonomic groupings inferred from similarity with GenBank sequences. Populations referring to number of trees were defined in four ways: 1) each tree being a population with 10 populations per transect, 2) every two trees being a population to give 5 populations per transect, 3) the first three, middle four, and last three trees being populations to give three populations per transect, and 4) the first five and last five trees being

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populations to give two populations per transect. In every instance, each transect was defined as a region. The taxonomic groupings were 1) data from all algal samples analysed together and 2) each algal species analysed separately. In the event that a population had a sample size of one associated with it, it was removed from the analysis. For all AMOVA analyses, 9999 permutations were used. The distribution of the algal genotypes was examined at three levels; within populations (ϕ_{PR}), among populations (ϕ_{PT}), and among transects (regions; ϕ_{RT}). ϕ_{PT} is a measure of population differentiation analogous to F_{ST} (Weir & Cockerham 1984). Euclidean pairwise differences between populations were obtained from AMOVA. A *P* value of 0·05 was used to determine significance for the values of the ϕ statistics and the pairwise differences. Since seven different population definitions were used, the Bonferroni correction for testing multiple hypotheses on the same data was applied by multiplying each of the *P*-values by seven.

The most likely identity of the photobionts was determined by a NCBI GenBank BLAST search (Altschul *et al.* 1997) of the ITS region using the nucleotide collection, optimizing for highly similar sequences. Phylogenetic analysis using Maximum Parsimony (MP) was performed in PAUP 4.0 (Swofford 2003) using a heuristic search with 1 000 replicates of random addition of taxa, tree-bisection-reconstruction (TBR) branch swapping, MulTrees off, and saving no more than 1 000 trees in each replicate. Bootstrap values were determined by using 500 bootstrap replicates (Felsensten 1985) with simple addition of taxa and TBR branch swapping. Nucleotide sequence similarities were determined using the 'uncorrected p' distance in PAUP 4.0. Maximum likelihood/Bayesian analysis, in MrBayes v3.1.2 (Huelsenbeck *et al.* 2001; Ronquist & Huelsenbeck 2003), was performed using 1 450 000 generations with random starting trees. A six-parameter GTR (General Time Reversible) model was applied with a gamma shaped parameter and proportion of invariable sites uniformly distributed. This was the best model estimated with Modeltest 3.7 (Posada & Crandall 1998) that was available in MrBayes. Both runs converged on similar likelihood values and were terminated when the standard deviation of split frequencies fell below 0·01. Posterior probability values greater than 99 are reported on the phylogeny. A neighbour joining tree was also created using the Kimura 2 parameter distance model (Kimura 1980). The Bayesian and NJ Trees are not presented.

Results

Genetic variation in the photobiont

Seven samples of 149 thalli produced multiple bands in PCR where a single band was desired and 19 samples did not produce any PCR product. As a result, algal rDNA was amplified from 123 thalli. Samples with multiple bands will be investigated in further studies. Two length classes of rDNA fragments were amplified from the algal DNA, an intron-plus fragment (1100 bp long) and an intron-minus fragment (700 bp long). Therefore the intron was approximately 400 bp long. The intron was determined to be located after position 1512 and was homologous to the SSU 1512 intron in algae (Friedl *et al* 2000).

Five algal ITS rDNA genotypes are reported (Fig. 1). Although the forward primer site was located at the 3′ end of the SSU, it amplified the 1512 SSU intron with the ITS region producing two length variants, one with and one without the intron. Therefore, sequenced representatives of each different RFLP pattern were used to determine ITS genotypes. The intron was present in *T. jamesii* A, *T. jamesii* B, and *T. impressa* A. It was absent from *T. impressa* B and *T. impressa* C. Each of the five ITS genotypes was defined by diagnostic ITS banding patterns: two fragments of lengths 490 and 216 bp (*Trebouxia jamesii* A); three fragments of lengths 329, 291 and 225 bp (*T. jamesii* B); four fragments of 405, 154, 149 and 147 bp (*T. impressa* A); three fragments of 256, 216 and 108 bp (*T. impressa* B); and two fragments of 517 and 131 bp (*T. impressa* C). Since fragments less than 100 bp were not used to determine the ITS genotype, the sums of the diagnostic fragment lengths are not consistent. The actual length of the ITS region in all five samples ranged from 489 to 504 bp. Identification of the algal taxa was based on significantly similar matches after BLAST searches were performed in GenBank. Although the names, *T. jamesii* and *T. impressa,* were used to refer to the five algal genotypes because of significant BLAST search results, morphology was not examined and the species epithet could not be confirmed.

The length of the ITS1 ranged from 267 to 276 bp and the length of the ITS2 ranged from 213 to 233 bp for the five samples sequenced in this study. The length of the 5.8S was constant at 157 base pairs. The length of the entire ITS region sequenced

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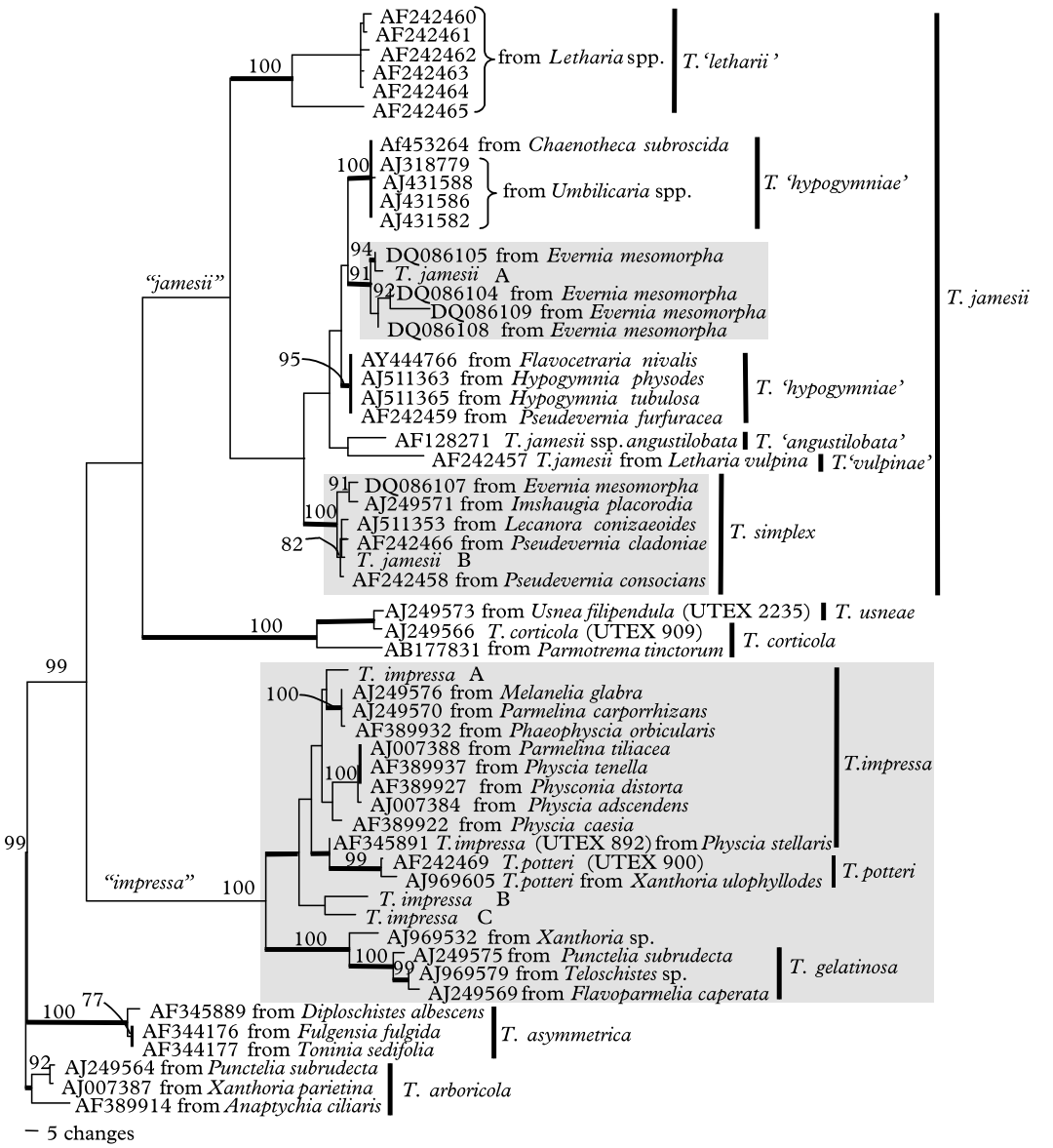


FIG. 1. One of 430 most parsimonious phylograms showing the phylogenetic placement of five algal symbionts in this study relative to species of *Trebouxia* from the NCBI GenBank database. The tree is based on the nucleotide sequence of the ITS rDNA amplified from the algal partner in epiphytic lichens. Only those bootstrap values greater than 70% are shown above the branches and posterior probabilities greater than 99% are indicated by thickened lines. *Trebouxia arboricola* is assigned as the outgroup. The tree is 674 steps long, CI=0.6053, RI=O.8782, and 212 characters are informative. Shaded boxes show the clades containing the algae sequenced in this study.

ranged from 637 to 664 base pairs from five representative algal genotypes. After removal of approximately 100 ambiguous bp at each end of the ITS region (see "DNA sequencing" in Methods), the alignment was 537 bp in length. Nucleotide sequence similarity

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within *T. jamesii* was 94%, within *T. impressa* was 86%–90%, and between *T. jamesii* and *T. impressa* was 80%–86%.

Photobiont evolution

The MP (Fig. 1), neighbour joining (NJ) and ML/Bayesian trees (data not shown) produced similar topologies with some exceptions. The main difference in topology between the NJ and MP trees was that in the NJ tree, *T. impressa* (UTEX 891) from *Physcia stellaris* was nested among members of *T. impressa* and two samples of *T. potteri* were sister to *T. gelatinosa*. In the MP tree two samples of *T. potteri* were sister to *T. impressa* (UTEX 891). The difference in topology between the ML/Bayesian and the MP trees was that two clades in the ML/Bayesian tree fell into different positions in the MP tree. The clade containing samples of *T. usneae* and *T. corticola* formed a sister clade with the "jamesii" clade in the MP tree, but was nested within the "jamesii" clade in the ML/Bayesian tree. The clade labelled as "jamesii" is paraphyletic with members of *T. simplex*, and the clade has low bootstrap and posterior probability support. A second clade in the MP tree, labelled as "impressa", consisted of members of *T. impressa, T. gelatinosa* and *T. potteri,* and was supported with 100% bootstrap but not with posterior probabilities (Fig. 1). The outgroup in the MP tree, *T. arboricola,* formed a sister clade with *T. asymmetrica* and both clades were sister to the "impressa" clade in the ML/Bayesian tree.

The five sequences from this study fell into either the "jamesii" or the "impressa" clade. *Trebouxia jamesii* A fell into a highly supported clade containing algae from *Evernia mesomorpha*. This clade was sister to a clade labelled as T. 'hypogymniae' and nested within a second clade labelled as T. 'hypogymniae'. *T. jamesii* B fell within a more diverse, but highly supported, clade labelled as *T. simplex* containing algae from *Evernia, Pseudevernia, Imshaugia*, and *Lecanora. Trebouxia impressa* A was nested among other members of *T. impressa. Trebouxia impressa* B and C fell basal to all the sequences of *T. impressa* and *T. potteri,* in the MP trees.

Distribution of photobiont ITS genotypes and mycobiont species

Five algal RFLP genotypes are reported to associate with seven mycobionts. *Trebouxia impressa* B was the most abundant alga, and *T. jamesii* A was the second most abundant alga (Table 1). *Trebouxia impressa* A and *T. impressa* C were rare, with each observed only three times (Table 1; Fig. 2). *Trebouxia jamesii* B was more common than *T. impressa* A and *T. impressa* C, but less common than *T. impressa* B and *T. jamesii* A. The most abundant lichen fungi in both transects were *Evernia mesomorpha, Parmelia sulcata, Tuckermanopsis americana* and *Usnea lapponica* (Table 1; Fig 2). Two thalli of *Flavoparmelia caperata* and one thallus of *Melanelia exasperatula* were present in transect 2 but were absent from transect 1. One thallus of *Ramalina dilacerata* was observed in transect 1, but it was not large enough to have allowed for a voucher specimen after DNA extraction and so DNA was not extracted from this lichen.

Evernia mesomorpha associated exclusively with *T. jamesii* A in both transects, even though *T. jamesii* A associated with four additional fungal species (Fig. 2). *Parmelia sulcata, Tuckermanopsis americana* and *Usnea filipendula* associated with a larger number of algal genotypes in the second transect than in the first transect. The same number of algal genotypes were associated with *U. lapponica* in both transects. *Trebouxia impressa* B, the most common alga, was associated with five of the seven fungi, and *T. jamesii* B was associated with three fungi.

Population structure

Gene flow was found among the algal partners under all population definitions (Table 2) The majority of the variation present, 99–100%, was within populations in all instances except one. *Trebouxia impressa* contained less variation within populations (94%) suggesting 6% variation among populations and transects. A pairwise comparison of ϕ_{PT} between each of the 20 trees studied did not produce any significant values suggesting gene flow among all populations

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TABLE 1. *Distribution of numbers of thalli of the 149 lichen forming-fungal species and 123 algal genotypes among two transects of Jack Pine trees*

	Transect 1	Transect 2	Total
Fungal taxa			
Evernia mesomorpha Nyl.	20	20	40
Parmelia sulcata Taylor	20	20	40
Tuckermanopsis americana (Sprengel) Hale	13	17	30
Usnea lapponica Vain.	12	11	23
Usnea filipendula Stirt.	7	6	13
Flavoparmelia caperata (L.) Hale	0	2	2
Melanelia exasperatula (Nyl.) Essl.	0	1	1
Algal taxa			
Trebouxia jamesii A	18	21	39
Trebouxia jamesii B	6	4	10
Trebouxia impressa A	1	2	3
Trebouxia impressa B	34	34	68
Trebouxia impressa C	1	2	3

based on these algal genotypes (data not shown). Even with the Bonferroni correction applied by multiplying the *P*-values by seven, there were no significant values.

Discussion

Photobiont identity

Photobionts associated with seven species of lichen-forming fungi probably constitute at least five species within the genus *Trebouxia* (Fig. 1). However, this may be an underestimation of the number of algal taxa present in this lichen community because of the low resolving power of RFLPs. Although the unknown species from this study are closely related to authentic species of *T. jamesii* and *T. impressa* in the tree (from UTEX Culture Collection; Hauck *et al.* 2007) based on ITS rDNA sequence, it does not confirm morphological species.

The clade of *T. jamesii* has been separated into a number of tentative species including T. 'letharii', T. 'vulpinae' (Kroken & Taylor 2000), T. 'hypogymniae', and T. 'angustilobata' (Hauck *et al.* 2007). Based on sequences compared with those reported in Hauck *et al.* (2007) and Kroken & Taylor (2000) the photobiont names have been applied to clades in Fig. 1. Since *Trebouxia jamesii* has been known as a highly variable species (Kroken & Taylor 2000), and has been named as *T. jamesii* or *T. simplex* in different studies (Hauck *et al.* 2007; Kroken & Taylor 2000; Opanowicz & Grube 2004; Piercey-Normore 2006), *T. simplex* is included in the *T. jamesii* clade in this study. *Trebouxia jamesii* A falls within a highly supported clade with sequences from algae of *Evernia mesomorpha* that does not contain morphological or accessioned species from other studies suggesting it may be a unique clade. Even though two clades sister to this clade contain sequences labelled as T. 'hypogymniae', together the three clades have low support and may represent three different algal lineages. Further sampling of algae representing T. 'hypogymniae' would be required to address the question of different lineages. *Trebouxia jamesii* B falls into another highly supported clade with sequences from *T. simplex* suggesting that it is *T. simplex.*

Trebouxia impressa A, B and C form a large group with high bootstrap support but low posterior probability support. This clade labelled as 'impressa' represents species of *T. impressa, T. potteri* and *T. gelatinosa* as indicated by the morphological species. The authentic species of *T. impressa*, UTEX892, is not clearly positioned with other morphological

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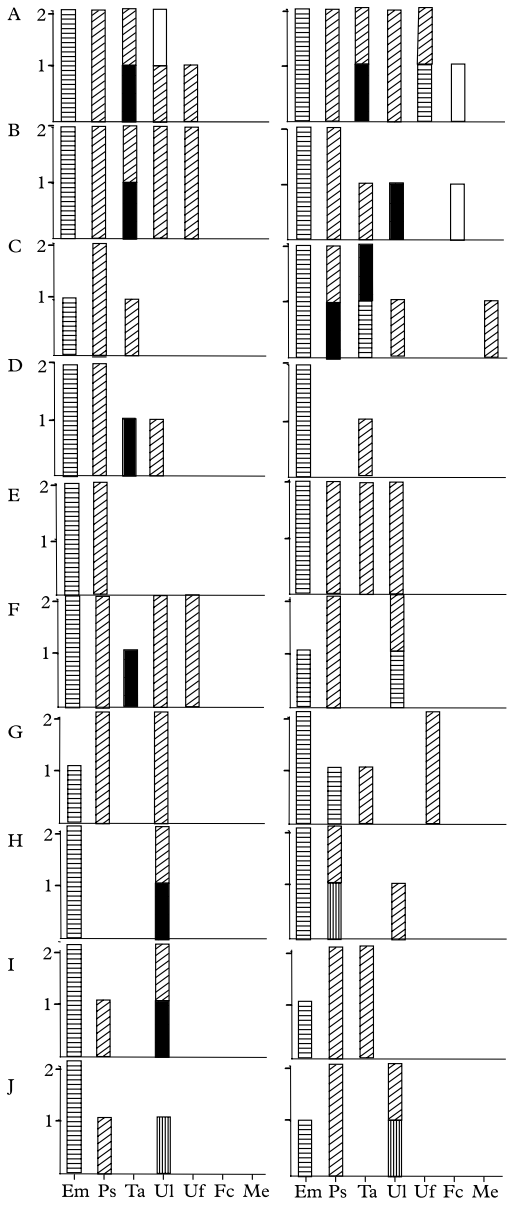


FIG. 2 Distribution of the lichen-forming fungal species (x-axis) and the algal partners among ten trees in each of two transects. The graphs in the left column represent the ten trees in transect 1 and those in the right column represent the trees in transect 2. Two graphs in each row, labelled A to J, represent each Jack Pine tree. The y-axis shows the number of samples, x-axis fungal species abbreviated by the first letters for the genus and species names (see Table 1). Horizontal hatching = *T. jamesi* A; solid black boxes = *T. jamesii* B; vertical hatching = *T. impressa* A; diagonal hatching = *T. impressa* B; solid white boxes = *T. impressa* C.

species of *T. impressa* (AJ007388, AJ007384; Beck *et al.* 1998) in the MP tree but they are together in the NJ tree. It is not known whether *T. impressa* B and C are *T. impressa* or another species of *Trebouxia* such as *T. flava* which is positioned as sister to *T. impressa* and *T. potteri* in Hauck *et al.* (2007).

Photobiont dispersal

Algal dispersal is supported in this study since no significant population subdivision existed for all definitions of populations (Table 2). This was not surprising because of the small geographic distances between and within transects. Comparison of dispersal distances with other studies show variable dispersal distances. Gene flow was inferred in *T. jamesii* associated with *E. mesomorpha* at distances of greater than 50 m (Piercey-Normore 2006). Wind dispersal of vegetative propagules was suggested to carry algae distances of 30m in *Ramalina farinacea* and *Evernia prunastri* (Tapper 1976). Printzen & Ekman (2003) inferred gene flow in the mycobiont of lichen propagules of *Cladonia subcervicornis* for populations that were 10–15 km apart, and Walser (2004) suggested the maximum dispersal distance for *Lobaria pulmonaria* propagules was 230 m. Dispersal of vegetative propagules would provide algae with a means of hitchhiking within a vegetative structure that might confer some protection to the alga and carry it longer distances than it might otherwise travel. In addition to vegetative propagules, algal dispersal may also occur as free-living algae. The occurrence of free-living colonies or 'escaped' algae of *Trebouxia* has been disputed (Bubrick *et al.* 1984; Mukhtar *et al.* 1994; Tschermak-Woess 1978), but zoospores have been observed (Slocum *et al.* 1980). If zoospores are produced inside the thallus, they can escape from the thallus through cracks in the cortex. These flagellated cells may form colonies close to the thallus in which they were produced (Slocum *et al.* 1980) and become available to associate with germinating fungal spores giving rise to entire populations of the species (Murtagh *et al.* 2000).

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TABLE 2. *Population structure of lichenized algae using four population definitions for each of three taxonomic analyses based on algal species inferred from GenBank BLAST search. Populations of n=1 were removed from the analysis. No values were significant (P=0·05)*

Algal species	Populations/transect	ϕ_{RT}	ϕ_{PR}	ϕ_{PT}	Variation within populations (%)
T. jamesii and *T. impressa*	10	0·000	0·000	0·000	100
	5	0·000	0·000	0·000	100
	3	0·000	0·011	0·000	99
	2	0·000	0·000	0·000	100
T. jamesii	8	0·012	0·000	0·000	99
	5	0·010	0·000	0·000	99
	3	0·000	0·008	0·000	99
	2	0·000	0·007	0·000	99
T. impressa	9	0·000	0·000	0·000	100
	5	0·000	0·000	0·000	100
	3	0·000	0·057	0·023	94
	2	0·000	0·000	0·000	100

Photobiont selectivity and specificity

Algal taxa were shared among seven lichen-forming fungal species supporting the hypothesis, but the algae were not equally distributed among the fungi. *Evernia mesomorpha* was associated with a single alga, *T. jamesii* A, but *T. jamesii* A also associated with four other species of fungi (Fig. 2). This distribution may be explained if *T. jamesii* A is the only alga that is genetically compatible with *Evernia mesomorpha* in this site. However, other algae are known to be compatible with *E. mesomorpha* (Piercey-Normore 2006), and one of these, *T. jamesii* B (*T. simplex* in Fig. 1), was present in this study. *Trebouxia jamesii* B was also reported to be associated with other species of *Pseudevernia, Imshaugia,* and *Lecanora* (Fig. 1). In this study *E. mesomorpha* appears to be highly selective of one photobiont and specific to *T. jamesii* A, but the alga is not specific to *E. mesomorpha.* Specificity has been shown among members of the *Physciaceae* (Dahkild *et al.* 2001) where four species in the *Physciaceae* associate only with *T. impressa.* In this study *E. mesomorpha* showed high selectivity, but in Piercey-Normore (2006) the same mycobiont showed low selectivity using the same molecular marker. Inconsistency in algal selectivity has been reported for three different species of *Cladonia* (Piercey-Normore 2004) where *C. multiformis* and *C. rangiferina* were highly selective in different geographic regions, but *C. gracilis* showed low levels of algal selectivity in one geographic area but high levels of selectivity in another geographic area. Habitat features, which were not measured in any of these studies, may be influencing fungal selectivity even within a single species.

Parmelia sulcata associated exclusively with *T. impressa* B, the most common alga, in transect 1, but it associated with three other algae in addition to *T. impressa* B in transect 2 (Fig. 2) suggesting low levels of selectivity and specificity. *Parmelia sulcata* is a pollution tolerant species (Hawksworth and Rose 1970), and is tolerant of many different environmental conditions and habitats. If pollution tolerant lichen-fungi are genetically compatible with many different algae or associate with widespread algae, they may have the capacity to survive in a wide range of habitats. Similarly, three photobiont species were reported to associate with another pollution tolerant lichen-forming fungus, *Protoparmeliopsis muralis* (Guzow-Krzeminska 2006) and 21 photobiont genotypes are reported to associate

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with *Parmotrema tinctorum* (Ohmura *et al.* 2006).

The algal distribution observed in this study may be explained by the replacement of locally adapted algae with a common genotype such as *T. impressa* B if no additional benefit is provided by the locally adapted algal genotype. Alternatively, the widely distributed *T. impressa* B may be replaced through the effective dispersal of algae that are adapted to particular micro-environmental conditions and confer advantageous properties to the lichen. In both scenarios neither the widely distributed genotype nor the locally adapted genotype may be better adapted in a moderated environment, such as this boreal Jack Pine stand, with low pollution levels. Moderated environments may provide suitable habitat for a higher diversity of algal genotypes by having less selection pressure on the photobiont than more extreme habitats such as iron rich rocks (Beck 1999) or polluted urban areas (Ohmura *et al.* 2006). These more extreme habitats may limit the available pool of algae only to those that can tolerate the more extreme conditions.

Conclusions

The community of algae housed in different lichen thalli within the same strictly defined habitat consisted of divergent algal lineages that were inferred to be at least two species and five genetically differentiated algae. Although these five algal lineages are probably an underestimate of algal diversity in this habitat because of the low resolving power of RFLPs, they show that algal diversity is present within the same confined habitat and that the algal taxa are shared among different mycobionts. This study showed low specificity and variable levels of selectivity with species of lichen-forming fungi. Perhaps in moderated habitats, such as this boreal Jack Pine forest, a large number of algae can survive with many fungi, while in extreme habitats, such as an iron-rich rock, only algae specialized to those conditions can survive. This must be studied further by sampling free-living algae in the same habitat. Even though the algal partners were highly divergent, gene flow was shown to occur under different population definitions in this tightly defined community of lichenized algae. Dispersal by soredia may provide an effective medium for algal dispersal and availability of algae to other fungi in the area. Individual algal and fungal partnerships may be a result of a combination of genetic compatibility between symbionts, algal availability and environmental conditions.

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