The Bryologist 103(4), pp. 645–660

Phylogenetic Species, Reproductive Mode, and Specificity of the Green Alga *Trebouxia* Forming Lichens with the Fungal Genus *Letharia*

SCOTT KROKEN[1] AND JOHN W. TAYLOR[2]
Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720 U.S.A. [1]e-mail: kroken@nature.berkeley.edu, [2]jtaylor@socrates.berkeley.edu

Abstract. *The green algal symbionts of the lichenized fungus* Letharia *are identified as members of the morphospecies* Trebouxia jamesii, *based on ITS sequences that place them in a molecularly diverse clade with other lichen algae also identified as* T. jamesii. *However, based on additional sequence from a second locus, actin,* T. jamesii *is seen to consist of many phylogenetic species. A clade of six to seven closely related phylogenetic species of* T. jamesii *are symbiotic with five of the six phylogenetic species of* Letharia. *Within this broad level of specificity, there are also narrower patterns of specificity. For example, three cryptic species of* T. jamesii *were found only in two species of* Letharia *in California, a pattern that may be due either to biogeography, or to* Letharia *phylogenetically tracking* Trebouxia. *Allopatric* Letharia vulpina *has switched to a genetically distant phylogenetic species within the* T. jamesii *morphospecies, suggesting that habitat plays a role in specificity. One phylogenetic species of* T. jamesii *was found to have a recombining population structure, as two alleles of two loci were found in all four pairwise combinations among the haploid genotypes of that species. This result suggests that* Trebouxia, *a genus in which sexual structures have never been observed, is nevertheless recombining.*

Trebouxia (*sensu lato,* including *Asterochloris*) is the photobiont found in 20% of all lichens (Rambold & Triebel 1992). *Trebouxia* is in the order Trebouxiophyceae (Friedl 1995), which includes the symbiotic algae for 50–70% of all lichen species (Ahmadjian 1982), including the Lecanorales (Friedl & Büdel 1996). In this study, we determine the identity and phylogeny of green algal symbionts of the lichenized fungus *Letharia,* compare the algal phylogeny to the fungal phylogeny to examine patterns of specificity (the range and taxonomic relatedness of acceptable partners, Rambold et al. 1998), and investigate the population structure of the algae to determine if recombination contributes to the reproduction of lichenized algae. The study uses variation in DNA-based loci to identify phylogenetic species using concordance of gene genealogies, and to infer reproductive mode from comparison among gene genealogies within phylogenetic species. This same approach has been used to study the speciation and reproductive mode of *Letharia* (Kroken & Taylor 2001).

Letharia was selected for this study among the many members of the largest order of lichens, the Lecanorales, because we have shown that it is composed of at least six phylogenetic species (Kroken & Taylor 2001). To permit comparison of mycobiont and phycobiont, the six fungal species are provisionally identified as follows. *Letharia 'lucida'* is strictly apotheciate. *Letharia 'barbata,' L. 'gracilis,'* and *L. 'rugosa'* are always apotheciate, and sometimes also isidiate. *Letharia vulpina* and *L. 'lupina'* are always sorediate and rarely apotheciate.

In spite of the fact that 20% of all described species of fungi are lichenized, their photobionts remain poorly understood in terms of the number and identity of species, the reproductive mode of those species, and the degree of specificity in the associations between lineages of photobionts and lichenized fungi. Approximately 13,500 lichen species have been named, corresponding to the diversity of the fungal component (Hawksworth 1988). Of these, 12,500 lichen species have a green alga as a photobiont and 1,000 lichen species have a cyanobacterium (Tschermak-Woess 1988). The photobionts of most lichen species have been assigned to a genus based on morphology. However, only 200 photobionts have been described to species, also based on morphology, with 100 species of cyanobacteria, and 100 species of green algae (Tschermak-Woess 1988). If these morphological species are indeed phylogenetic species, then for each cyanobacterial photobiont there would be 10 compatible fungi, and for each green algal photobiont there would be 1,250 compatible fungi! However, each photobiont species as defined by morphology may comprise many cryptic species that are compatible only with a much smaller range of fungal species. DNA sequence data are being used to determine the

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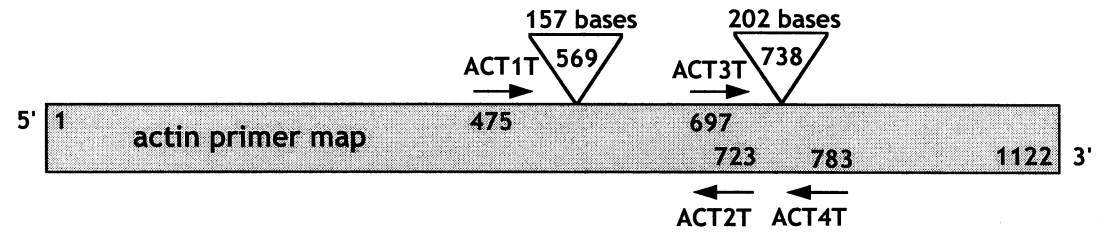

FIGURE 1. Primer map for type I actin.

identity of photobiont species and their specificity to lichenized fungi. One such study used DNA sequence data to suggest specificity among strains of the cyanobacterium *Nostoc* and species of the Peltigeralean lichenized fungi in the genera *Peltigera* and *Nephroma* (Paulsrud & Lindblad 1998).

Most green algae include both clonal and sexual reproduction in their life cycles. However, physical evidence of sex (e.g., fusion of gametes, meiotic tetrads) has not been observed in any green algal photobiont (Friedl & Rokitta 1997), and the green algal class Trebouxiophyceae has been described as "sexual reproduction unknown" (Friedl 1995). It has been suggested that the fungus suppresses sexual reproduction in the phycobiont to prevent it from producing novel genotypes that would be less suited to symbiosis (Ahmadjian 1993*a*). This effect has been demonstrated for the bacterium *Wolbachia* that infects female wasps and in which sexual recombination is suppressed, thereby causing the wasps to reproduce parthenogenetically (Rousset et al. 1992). Although clonality may be advantageous over short periods, clonal lineages are predicted to be doomed for extinction (Muller 1964), and no green alga has been demonstrated to have a clonal population structure. With molecular methods, we no longer need to observe sex directly to negate the hypothesis of clonality, because we can infer that recombination is or is not occurring based on comparison of genotypes.

MATERIALS AND METHODS

DNA extraction.—The whole lichen DNA extracts of *Letharia* that were used to study the mycobiont (Kroken & Taylor 2001) were also used here, which allowed for comparison of co-existing fungal and algal genotypes in single lichen thalli. Whole lichen DNA extracts were also obtained from *Usnea arizonica* (U.S.A. CALIFORNIA. Napa), *Pseudevernia furfuracea* (AUSTRIA. STYRIA. Handalpe), *P. cladonia* (U.S.A. NORTH CAROLINA. Mt. Mitchell), and *P. consocians* (U.S.A. NORTH CAROLINA. Overlook Pass). DNA vouchers are stored by the authors at −20°C, and phenotypic vouchers have been deposited in the University of California Herbarium (UC).

Six *Trebouxia* species were obtained from the UTEX Culture Collection and cultured on agarized medium, according to UTEX's instructions (http://www.bio.utexas.edu/research/utex/). The algal DNA was extracted with a boiling water procedure (C. F. Delwiche pers. com.). Into each 1.5-mL microcentrifuge tube containing one ml of purified water, 10 μL of algal cells and 50 μL of PVPP (polyvinylpolypyrrolidone) powder were added. The open tubes were secured in a floating rack and placed into a beaker of boiling water for two min., and then placed on ice to chill. One μL of the resulting solution was used directly as template for each PCR reaction.

Primer design, PCR and DNA sequencing.—Algal-specific primers were necessary to amplify *Trebouxia* DNA from the whole lichen DNA extracts. PCR primers were designed to amplify ITS from the nuclear ribosomal array, and a fragment of the actin gene that contained two introns. Fungal sequences were aligned to algal sequences available in Genbank to identify primer sites that would discriminate against fungal ITS. The algal-specific ITS primers ITS1T (gga agg atc att gaa tct atc gt), ITS2T (ttc gct gcg ttc ttc atc gtt), ITS3T (aac gat gaa gaa cgc agc gaa), and ITS4T (ggt tcg ctc gcc gct act a) are based on nuclear ribosomal sequences from the Trebouxiophyceae, including species of *Trebouxia* and *Asterochloris* (Bhattacharya et al. 1996; Friedl 1995; Friedl & Rokitta 1997; Friedl & Zeltner 1994). The initial set of heterologous algal-specific actin primers were based on sequences from *Chlorella vulgaris* (Chow & Chan 1997), a member of the Trebouxiophyceae (Friedl & Zeltner 1994),*Chlamydomonas reinhardtii* (Sugase et al. 1996) a member of the Chlorophyceae (Friedl & Zeltner 1994), and several representative fungal actin sequences. The *Trebouxia*-specific actin class I primers ACT1T (cac acr gtr ccc atc tay gag g), ACT2T (agg tag ctc ata gtt ctt ctc aat), and ACT3T (acy att gag aag aac tat gag cta), ACT4T (gtt gaa cag cac ctc agg gca) are based on *Trebouxia* sequence obtained from the initial primers (Fig. 1).

PCR was performed to bias the reaction toward algal amplicons with a TD62 (touchdown) program that consists of initial two min. treatment at 94°C, followed by of 10 cycles of one min. at 94°C, one min. at 62°C– 0.5°C per cycle), and one min. at 72°C. It is followed by 35 cycles of one min. at 94°C, one min. at 57°C, and one min. at 72°C, followed by seven min. at 72°C. DNA sequencing and alignment of those sequences was performed as described in Kroken and Taylor (2001). Representative sequences have been deposited in the Genbank (accession numbers for ITS are listed in Table 1, and accession numbers for actin are given in the results section). All alignments have been deposited in TreeBASE under accession number SN420.

Analysis of phylogeny and reproductive mode.—Phylogenetic analyses of DNA sequences were performed using PAUP 4.02ba (Swofford 1998). Maximum parsimony (MP) was used as the working criterion, and results were corroborated with maximum likelihood and neighbor joining to show that results are not specific to parsimony analysis. MP analysis was done with data sets that included and excluded gaps, to detect any character type-specific results and to allow comparison to results from ML anal-

TABLE 1. *Trebouxia* species investigated. [[1]UTEX is the University of Texas Culture Collection. [2]Accession number for ITS sequence. [3]UBT is the University of Bayreuth Culture Collection. [4]*Pseudotrebouxia* (Archibald 1975) has been merged back into *Trebouxia* (Gärtner 1985). [5]Bhattacharya et al. 1996. [6]Friedl et al. 2000. [7]This study. [8]Beck 1999.]

Trebouxia sp.	Genbank #[2]	From Lichen	Collection #	Taxonomic Reference
T. arboricola[4]	Z68703[5]	?	92.001C3[3]	Archibald 1975
	AJ249564[6]	*Punctelia subrudecta*	87.060E1[3]	
	AJ249481[6]	*Pleurosticta acetabulum*	92.011A1[3]	
T. asymmetrica	AJ249565[6]	*Diploschistes diacapsis*	2507[1]	Friedl & Gärtner 1988
T. corticola[4]	AJ249566[6]	?	909[1]	Archibald 1975
T. flava	AF242467[7]	*Physcia pulverulenta*	181[1]	Archibald 1975
T. galapagensis[4]	AJ249567[6]	*Ramalina* sp.	2230[1]	Hildreth & Ahmadjian 1981
T. gelatinosa	Z68697[5]	?	86.108B2[3]	Archibald 1975
	Z68698[5]	?	905[1]	
	AJ249568[6]	*Flavoparmelia caperata*	87.015A1[3]	
	AJ249575[6]	*Flavoparmelia subrudecta*	87.072B1[3]	
T. gigantea[4]	AJ249577[6]	*Caloplaca cerina*	2231[1]	Hildreth & Ahmadjian 1981
	AF242468[7]		2231[1]	
T. higgensiae[4]	AJ249574[6]	*Buellia straminea*	2232[1]	Hildreth & Ahmadjian 1981
T. jamesii[4]	Z68699[5]	?	86.011E1[3]	Hildreth & Ahmadjian 1981
	Z68700[5]	?	86.132E2[3]	
	Z68701[5]	?	86.156C3[3]	
	AJ249571[6]	*Imshaugia placorodia*	85.073A1[3]	
	AF242466[7]	*Pseudevernia cladoniae*	(none)	
	AF242458[7]	*Pseudevernia consocians*	(none)	
	AF242459[7]	*Pseudevernia furfuracea*	(none)	
subsp. *jamesii*	AF128270[8]	*Lecidea silacea*	97.017A2[3]	Beck 1999
subsp. *angustilobata*	AF128271[8]	*Lecidea lapicida*	97.027B3[3]	Beck 1999
'vulpinae'	AF242457[7]	*Letharia vulpina*	(none)	
'letharii' sp. #1	AF242460[7]	*Letharia 'lupina,' L. 'gracilis,' L. 'barbata,' L. 'lucida,' L. 'rugosa'*	(none)	
'letharii' sp. #2	AF242461[7]	*Letharia 'lupina,' L. 'barbata,' L. 'gracilis'*	(none)	
'letharii' sp. #3	AF242462[7]	*Letharia 'lupina,' L. 'barbata,' L. 'lucida,' L. 'gracilis'*	(none)	
'letharii' sp. #4	AF242463[7]	*Letharia 'lucida'*	(none)	
'letharii' sp. #5	AF242464[7]	*Letharia 'barbata'*	(none)	
'letharii' sp. #6	AF242465[7]	*Letharia 'lucida,' L. 'barbata'*		
T. impressa[4]	AJ249570[6]	*Parmelina carporrhizans*	87.026A6[3]	Archibald 1975
	AJ249576[6]	*Melanelia glabra*	87.017E1[3]	
T. potteri[4]	AF242469[7]	*Lecanora rubina*	900[1]	Archibald 1975
T. showmanii[4]	AF242470[7]	*Lecanora hageni*	2234[1]	Hildreth & Ahmadjian 1981
T. sp.	AJ249572[6]	*Parmelia pulla*	98.003B2[3]	(none)
T. sp.	AF242471[7]	*Usnea arizonica*	(none)	
T. usneae[4]	Z68702[5]	?	87.019A1[3]	Hildreth & Ahmadjian 1981
	AJ249573[6]	*Usnea filipendula*	2235[1]	

ysis, which does not allow gaps as character states. The branches in the resulting phylogenies were tested for robustness with 1,000 bootstrap replicates (Hillis & Bull 1993). The congruity of ITS and actin data sets was examined using the Partition Homogeneity Test (PHT) (Incongruence Length Difference test, Farris et al. 1994), and with the Kishino-Hasegawa test (Kishino & Hasegawa 1989), both which were implemented in PAUP 4.02ba. The algal phylogeny was compared to the previously determined fungal phylogeny with the PHT using two partitions: the 1,227 algal nucleotides from two loci found in this study, and the 1,970 fungal nucleotides from six loci (Kroken & Taylor 2001). Here, the null hypothesis is that the two phylogenies are congruent. Reproductive mode was also investigated with the PHT. In this case, the null hypothesis to be tested is clonality, indicated by congruity of loci (Taylor et al. 1999).

RESULTS

ITS Phylogeny.—The algal-specific primer pair ITS1T/ITS4T was used to amplify a ca 730 nucleotide PCR product from 36 of the 51 lichen thalli studied in Kroken and Taylor (2001). The primers ITS1 (5') and ITS2T (3') were used to sequence ITS1, and the primers ITS3T (5') and ITS4 (3') was used to sequence ITS2 (ITS1 and ITS4 from White et al. 1990). Only one algal ITS allele was recovered from each lichen thallus and there were no ambiguous sequences with strong double peaks to suggest the presence of more than one genotype. To determine the closest relative of the green algae

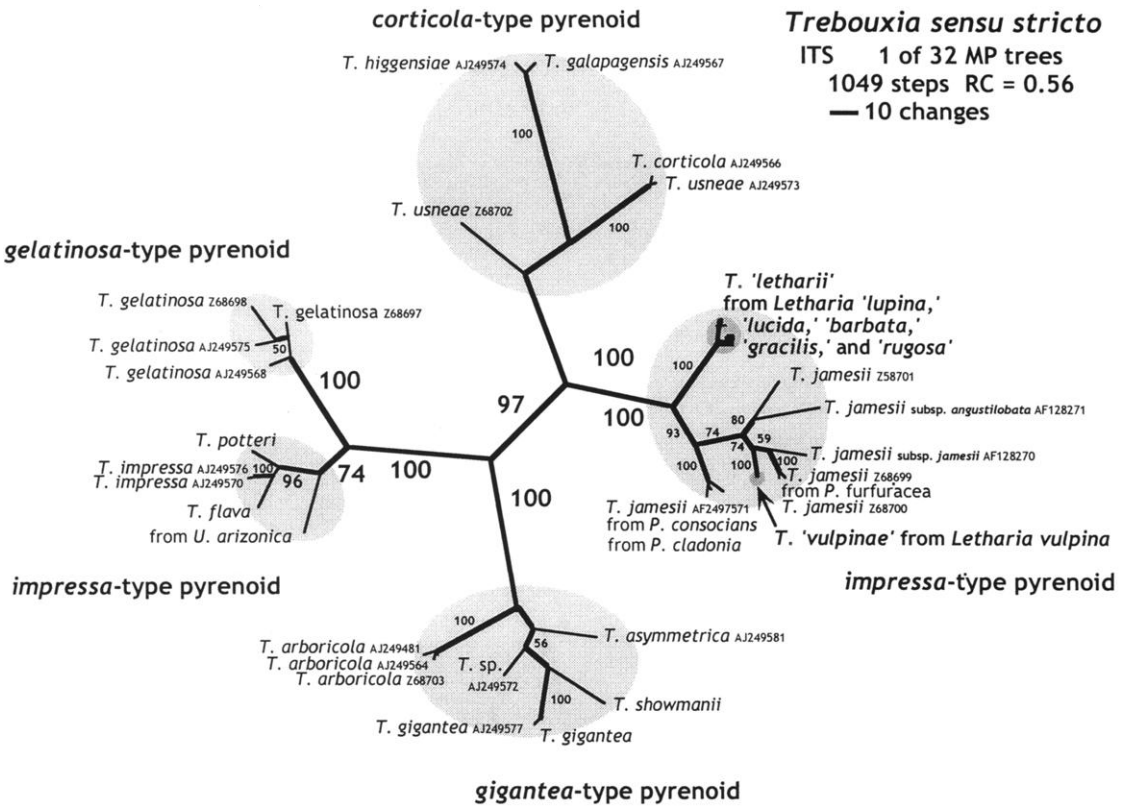

FIGURE 2. Phylogeny of *Trebouxia sensu stricto* based on ITS. One of 16 MP trees. Branches present in a strict consensus are in bold. Number along branch is the bootstrap value of that branch. 769 characters, 330 phylogenetically informative, 54 uninformative. 1,034 steps. RC = 0.57. Tree is unrooted.

in *Letharia,* their ITS sequences were aligned with the ITS sequences from the taxa listed in Table 1, and were submitted to parsimony analysis. Of the 1,085 nucleotides in the final alignment, a 220 base insert in *T. gelatinosa* and an 93 base insert in *T. gigantea* were excluded, leaving 772 bases that were used in the phylogenetic analysis (TreeBASE SN420–1168).

The resulting unrooted phylogeny in Figure 2 is one of 64 most parsimonious (MP) trees with 1,043 steps, and rescaled consistency indices (RC) = 0.56. The strict consensus of 64 MP trees (consensus branches are in bold line) is congruent to topologies obtained from maximum likelihood and neighbor joining. The tree indicates the same five clades of *Trebouxia* species reported by Beck et al. (1998) and Friedl et al. (2000), with average divergence of 128 among them at the ITS locus. All clades have moderate to strong bootstrap support, and each clade corresponds to one of the several pyrenoid types described by Friedl (1989), except for the *impressa*-type, which is found in two non-sister clades represented by *T. impressa* and *T. jamesii.*

The *gigantea*-type pyrenoid clade is composed of *Trebouxia arboricola* (three isolates with an average of seven steps apart), *T. showmanii, T. gigantea, T. asymmetrica,* and *T.* sp., five lineages with an average of 67 steps among them. The *gelatinosa*-type pyrenoid clade is composed of four isolates of *T. gelatinosa* with an average of 27 steps among them. The sister clade to the *gelatinosa* clade is the first *impressa*-type pyrenoid clade, which comprises *T. impressa, T. potteri, T. flava,* and *T.* sp., with an average distance of 31 steps among them. The *corticola*-type pyrenoid clade is composed of a branch with *T. higgensiae* and *T. galapagensis* (four steps apart), another branch with *T. cortiola* and *T. usneae* AF249573 (five steps apart), and a third branch with another morphospecies of *T. usneae* Z68702, the three branches have an average of 106 steps among them.

The second clade with an *impressa*-type pyrenoid is composed of many isolates of the *Trebouxia jamesii* morphospecies (Fig. 2, Fig. 3 for expanded view). *Trebouxia jamesii* has a chloroplast and pyrenoid shape like that of *T. impressa* (Friedl 1989). However, *T. jamesii* and *T. impressa* did not group

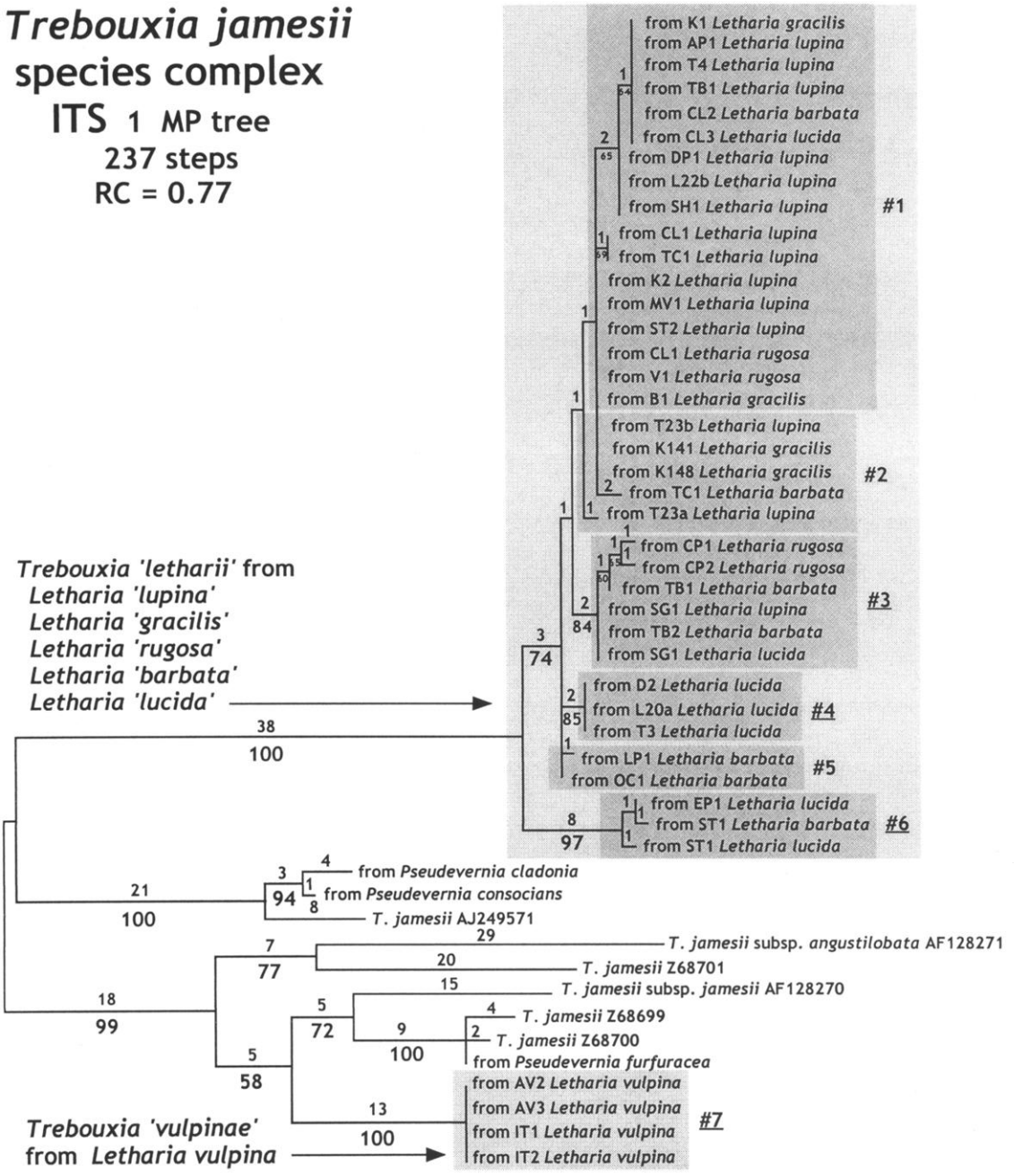

FIGURE 3. Phylogeny of *T. jamesii* species complex based on ITS. One MP tree. Number above branch is number of nucleotide changes, number below is the bootstrap value. 769 characters, 112 phylogenetically informative, 51 uninformative. 237 steps. RC = 0.77. Tree is unrooted. Algae from *Letharia* are labelled by the collection name.

as sister taxa. This *T. jamesii* clade has all of the *Letharia* phycobionts. The basal branch is composed of all the phycobionts from *Letharia 'lupina,' L. 'gracilis,' L. 'rugosa,' L. 'barbata,'* and *L. 'lucida,'* and comprises several branches that are divergent by 19 steps at most. The *Trebouxia jamesii* lineages from these five species of *Letharia* have a highly lobed chloroplast shape that resembles the holotype for *T. jamesii* (Beck 1999; Hildreth & Ahmadjian 1981). For convenience, we will refer to the *T. jamesii* found in these five species of *Letharia* as *Trebouxia 'letharii.'*

The next branch to diverge in the *Trebouxia jamesii* clade is composed of the phycobionts from *Pseudevernia cladonia* and *P. consocians* (five steps apart), and *T. jamesii* AF24974 which is di-

vergent by 12–16 steps. This branch is divergent from *T. 'letharii'* by 64–76 steps.

The remaining branch of the *Trebouxia jamesii* clade is composed of seven taxa, including the phycobionts from *Letharia vulpina* collected in California and Italy, all of which have identical ITS sequences. For convenience, we will refer to the *T. jamesii* found in *Letharia vulpina* as *Trebouxia 'vulpinae.'* Freshly collected material of *T. 'vulpinae'* has a chloroplast shape that resembles the more finely lobed (crenulate) chloroplast of *T. jamesii* subsp. *angustilobata* (Beck 1999) compared to the more broadly lobed chloroplast of the holotype for *T. jamesii.* However, *T. 'vulpinae'* is divergent by 28–56 steps from the six other isolates of *T. jamesii* within this clade, including isolate AF128271 representing *T. jamesii* subsp. *angustilobata,* its sister isolate Z58701, isolates Z68699 and Z68700 and *T. jamesii* from *Pseudevernia furfuracea,* and isolate AF128270 representing *T. jamesii* subsp. *jamesii.*

Addition of actin as a second locus.—The initial set of algal-specific primers were designed to amplify a ca 400 nucleotide fragment of the actin exon. The primers amplified PCR products whose sequences grouped with other green algal actin sequences with moderate bootstrap support, showing that the primers favored green algae over fungi (Fig. 4, TreeBASE SN420–1166). However, there appeared to be two types of algal actin sequences in *Trebouxia,* as had also been found in the Trebouxiophycean alga *Chlorella vulgaris* (Chow & Chan 1997). Type I actin from *C. vulgaris* groups with the majority of actin sequences from green algae and plants, and with the *Trebouxia* actin sequences from the thalli *Letharia 'barbata'* LP1 (Genbank AF242472), *L. 'lucida'* EP1 and *L. vulpina* IT1 and AV3. Type II actin from *C. vulgaris* groups with the *Trebouxia* actin sequences from thalli *L. 'barbata'* OC1 (Genbank AF242473) and *L. 'rugosa'* CP1, as well as with an actin sequence from *Nannochloris bacillaris* (Arai et al. 1998).

In order to compare only orthologous sequences, the initial primer pairs were redesigned to amplify only type I actin. The primer pair ACT3T/4T amplified a fragment of actin type I from *Trebouxia 'letharii'* and *T. 'vulpinae'* found in thalli of *Letharia,* and from *T. jamesii* found in thalli of *Pseudevernia furfuracea, P. cladonia,* and *P. consocians.* The ACT3T/4T PCR product of ca 400 bases contains an intron of 192–202 bases in *T. 'letharii'* and 268–274 bases in *T. 'vulpinae.'* This intron is located at nucleotide position 738 of the actin exon, and is also present in the actin genes of *Chlamydomonas* and *Chlorella.* The ACT3T/4T fragment provided almost twice the variation as the ITS provided—37 cf. 22 phylogenetically informative characters within *T. 'letharii'.* All informative characters from this actin fragment are in intron 738.

The actin intron 738 phylogeny for the *Trebouxia jamesii* species complex shown in Figure 5 is one of 351 MP trees, has 216 steps, and its RC = 0.77 (TreeBASE SN420–1169). The topology is congruent to the ITS phylogeny (Fig. 3) in resolving *T. 'letharii'* and its sister clade *T. jamesii* found in *Pseudevernia cladonia* and *P. consocians,* and *T. 'vulpinae'* and its sister clade *T. jamesii* found in *Pseudevernia furfuracea.* The intron sequences were difficult to align across *T. 'letharii'* and *T. 'vulpinae'* due to their divergence at ca 70% of nucleotide sites. *Trebouxia 'letharii'* has six clades that vary by one to five steps within clades, and are separated six to 19 steps among clades. *Trebouxia 'vulpinae'* has two clades separated by 24–26 steps.

In order to add more data to resolve the large *Trebouxia 'letharii'* clade, an additional intron-containing actin fragment was sequenced. The primer pair ACT1T/2T was used to specifically amplify a ca 280 base fragment of actin type I that contains an intron of 131–136 bases in *T. 'letharii.'* This intron is located at exon position 569, and is not present in *Chlorella* or *Chlamydomonas.* The ACT1T/2T fragment contains 46 phylogenetically informative characters within *T. 'letharii',* all but two of which are in intron 569.

The actin intron 569 phylogeny for *Trebouxia 'letharii'* in Figure 6 is one of eight MP trees, has 51 steps, and the RC = 0.95 (TreeBASE SN420–1170). ITS resolves five of the six clades found by intron 738 as monophyletic with moderate to strong bootstrap support. Clade #2 of the intron 738 tree is not resolved in the intron 569 tree, where its members are paraphyletic to clades #1, 2, 3, and 4. The PHT was used to determine that actin introns 738 and 569 are congruent (p = 0.473), suggesting that they have the same evolutionary history. A combined analysis of the actin introns 738 and 569 resolve clades #1–6 as monophyletic (not shown).

The two phylogenies for ITS and actin from *Trebouxia 'letharii'* were tested for congruence (Maddison 1997) using the PHT. Incongruity between the two loci were found to be significant at the p = 0.001 level (p = 0.0006) (Cunningham 1997). By successively removing individuals (OTU's) from the test, the largest source of incongruity was found to be the branching order among individuals of clade #1 in both the ITS and actin phylogenies (Table 2). Incongruity would be expected if recombination was occurring among individuals of this clade (Taylor et al. 1999). The remaining clades have an insignificant amount of incongruity (p = 0.0753).

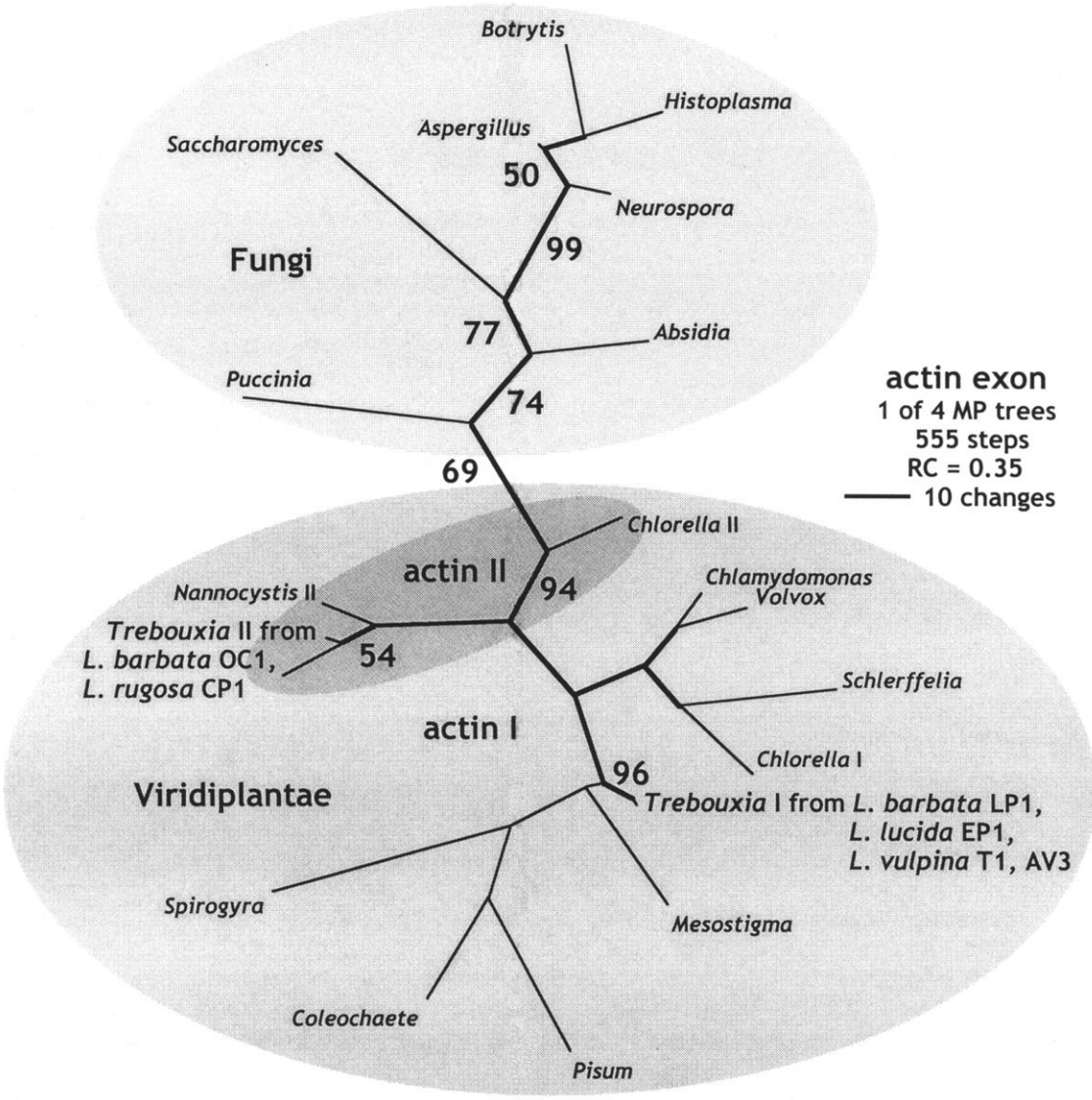

FIGURE 4. Actin phylogeny (exon only, introns excluded) for partial actin sequences from *Trebouxia,* aligned with representative actin sequences from the Fungi and the Viridiplantae. One of four MP trees. Branches present in a strict consensus are in bold. 754 characters included, 374 third position of codon excluded from analysis, unsequenced portion of *Trebouxia* actin treated as missing characters. 152 informative characters, 84 uninformative characters. 555 steps. RC = 0.35. Tree is unrooted.

The two phylogenies were also tested for congruence by using the Kishino-Hasegawa (KH) test (1989). In this test, the best tree generated by the actin data set was used as a constraint topology on which the ITS data was forced to construct the most likely gene tree (Table 3). This constraint tree was significantly worse than the best tree (Δ3.82 ln L/SD). A repeat of the test with the removal of the clade #1 resulted in a constraint tree that was still significantly worse. Although the Δln L declined by 0.63, it was still above the threshold of significance (Δ1.96 ln L/SD, Felsenstein 1990), due to smaller amounts of incongruity within clades #2–5. The KH test appears to be more stringent than the PHT in assessing levels of incongruity among data sets.

However, most of the incongruity detected by the KH is within the six clades and not among them. Incongruity is expected within phylogenetic species, as their loci have different histories due to recombination. The only difference in the actin and ITS data sets in resolving clades 1–6 is that the members of clades one and two in the actin tree appear as one clade in the ITS tree. This difference is probably due to differing rates of lineage sorting

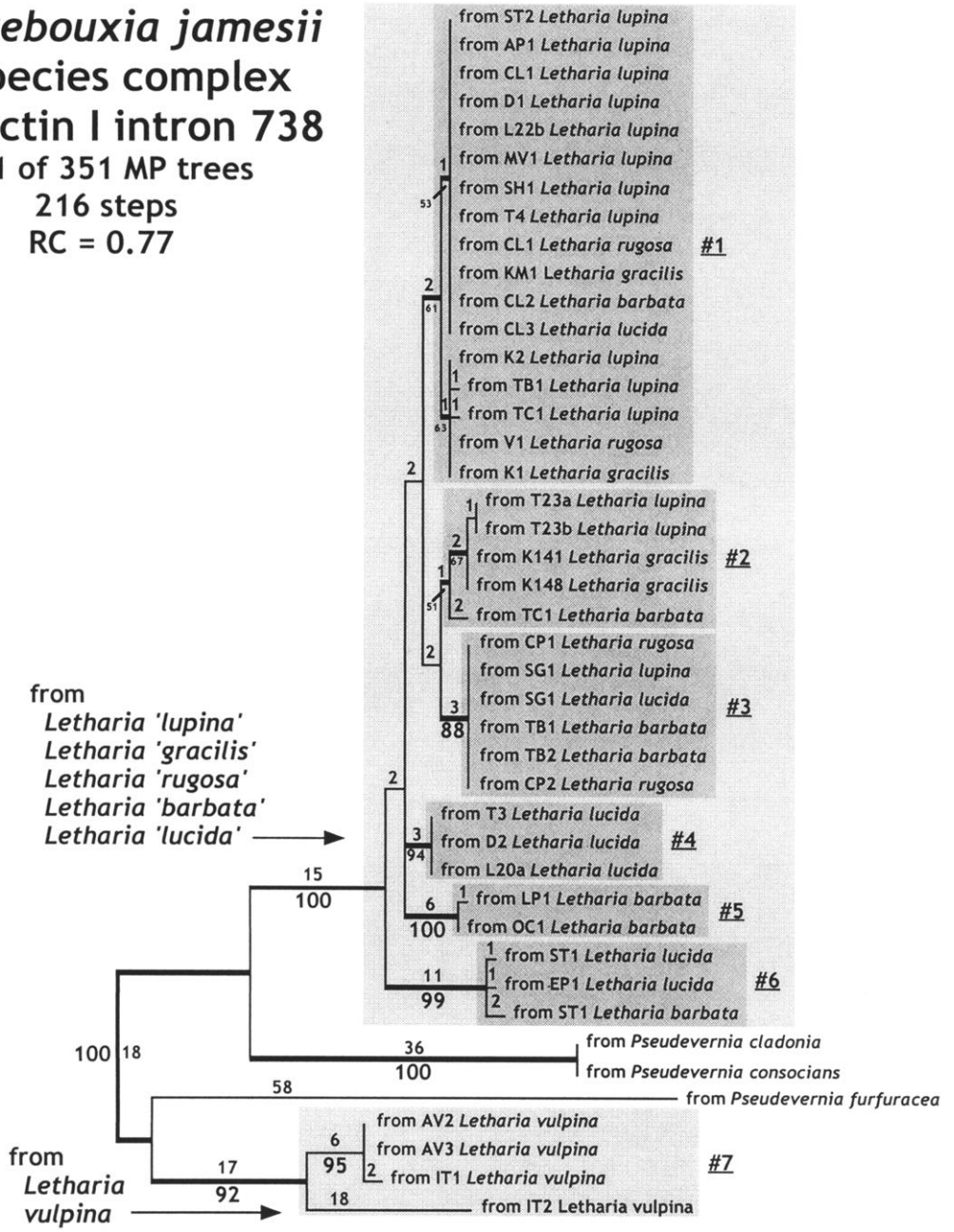

FIGURE 5. Phylogeny of *T. jamesii* species complex based on actin I intron at exon position 738. One of 351 MP trees. Branches present in a strict consensus are in bold. Number above branch is number of nucleotide changes, number below is bootstrap value. 360 characters, 107 informative, 47 uninformative. 216 steps. RC = 0.77. Tree is unrooted.

between two genetically isolated lineages that have coalesced to unique clade of alleles for actin, but have not done so for ITS. Therefore, the two data sets were combined in one analysis to delimit species by the concordance of their gene genealogies (Avise & Wollenberg 1997).

The resulting 2-locus phylogeny of *Trebouxia 'letharii'* has six clades with moderate to strong bootstrap support (Fig. 7 left, TreeBASE SN420–1168). Clade #1 is composed of 17 of the 36 genotypes examined. The photobiont found in *Letharia 'barbata'* TC1 from southeastern Washington may represent another clade, as it does not group with clade #2 in all MP trees, and bootstrap tests

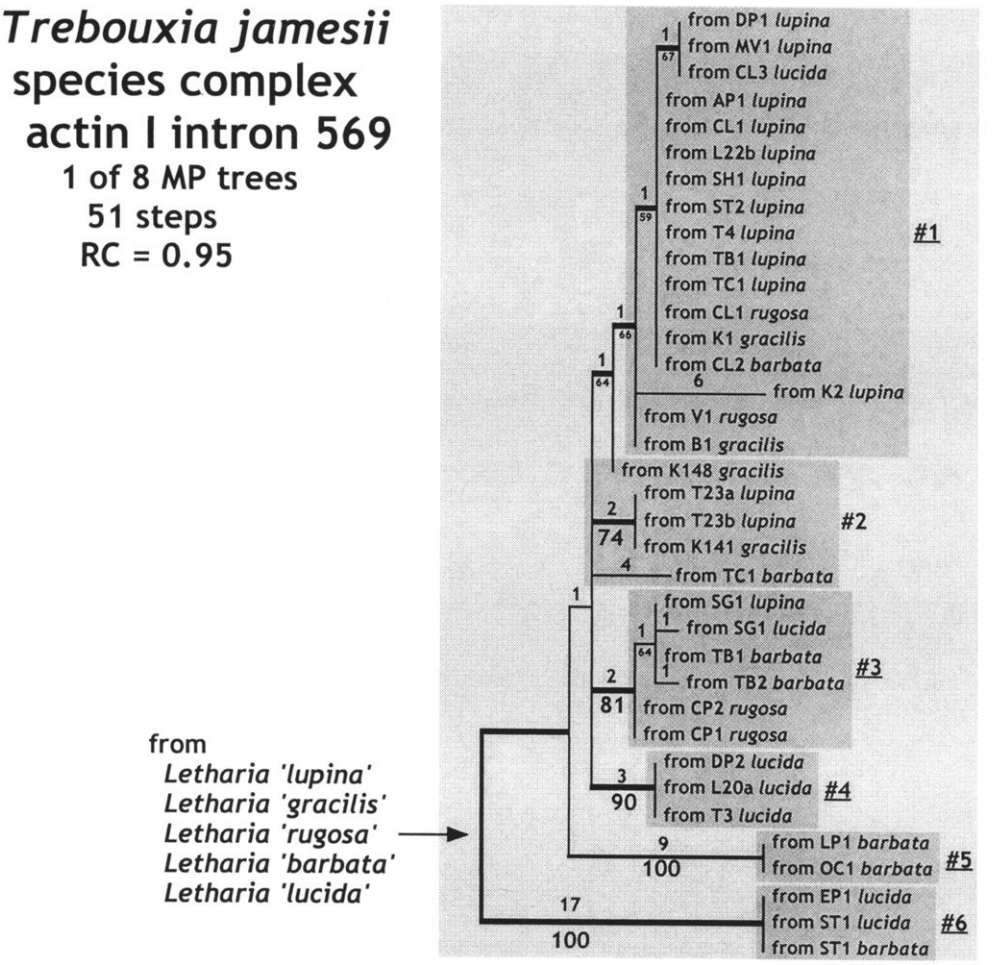

FIGURE 6. Phylogeny of *T. 'letharii'* based on actin I intron at exon position 569. One of eight MP trees. Branches present in a strict consensus are in bold. Number above branch is number nucleotide changes, number below is bootstrap value. 277 characters, 37 informative, 10 uninformative. 51 steps. RC = 0.95. Tree is unrooted.

do not support this grouping. However, for convenience, it will be treated as a member of clade #2.

In order to compare specificity of each *Letharia* species for its symbiont, the 2-locus *Trebouxia 'letharii'* phylogeny is presented side by side with the 6-locus phylogeny of *Letharia* (Fig. 7 right, TreeBASE SN420–1170). Although there is a strong correspondence between several clades, there are also many cases where there is no clear correspondence between fungus and alga. *Letharia 'lucida'* and *L. 'barbata'* were found primarily with *Trebouxia 'letharii'* clades # 4, 5, and 6. *Letharia 'lupina'* was found primarily with *Trebouxia 'letharii'* clades #1 and 2. The significance of this congruity was tested with the PHT, in which the data sets for *Trebouxia* and *Letharia* were combined in a single analysis to see if the histories of the two taxa were incongruent. The analysis indicated significant incongruity ($p = 0.001$), which is visually evident in Figure 7.

TABLE 2. Partition homogeneity tests for *Trebouxia 'letharii'* species complex. [[1]Significant at the level of $p = 0.001$ for conflict between loci (Cunningham 1997).]

OTU's excluded	(*P*)	sig. incongruent?[1]
none	0.0003	yes
species # 1	0.0778	no
species # 2	0.0037	yes
species # 3	0.0006	yes
species # 4	0.0006	yes
species # 5	0.0024	yes
species # 6	0.0006	yes

Reproductive mode.—Reproductive mode was assessed by observing two alleles for the two genes with the expectation that all four possible pairs would result from recombination, whereas as at most three would be expected under clonality. *Tre-*

TABLE 3. Kishino-Hasegawa tests for *Trebouxia 'letharii'* species complex. [[a]Determined by MP, with indel characters excluded. [b]Difference in log likelihood compared to that of the best tree. [c]The standard deviation of log likelihood. [d]Δ ln L/S.D. [e]The tree is considered significantly worse if the Δ ln L/S.D ≥ 1.96 (Felsenstein 1990)].

constraint tree	# steps[a]	ln L	Δ ln L[b]	S.D.[c]	T[d]	Sig. less likely?[e]
including species # 1						
(best actin tree)	82	−1262.17	—	—	—	—
actin data, ITS topology	103	−1355.93	93.82	26.47	3.54	yes
excluding species # 1						
(best actin tree)	67	−1166.24	—	—	—	—
actin data, ITS topology	77	−1202.59	43.86	11.76	3.09	yes

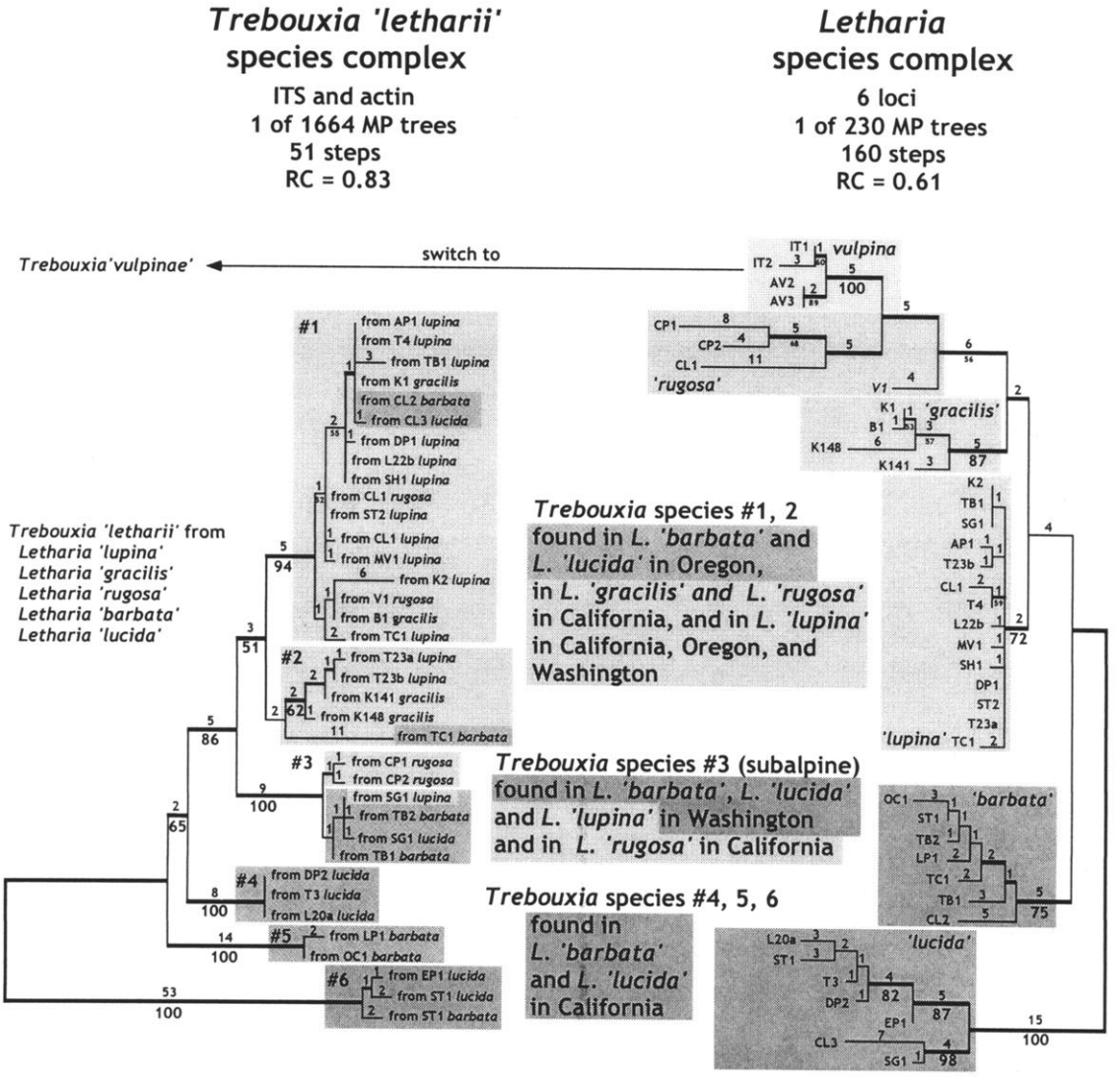

FIGURE 7. Left. Phylogeny of *T. 'letharii'* based on ITS and actin introns. One of 1,664 MP trees. Branches present in a strict consensus are in bold. Number above branch is number of nucleotide changes, number below is bootstrap value. 1,227 characters, 105 informative, 10 uninformative. 155 steps. RC = 0.83. Tree is unrooted. Right. Phylogeny of *Letharia* species complex based on six loci. One of 230 MP trees. Branches present in a strict consensus are in bold. Number above branch is number of nucleotide changes, number below is bootstrap value. 1,970 characters, 70 informative, 36 uninformative. 160 steps. RC = 0.61. Tree is unrooted.

bouxia 'letharii' clade #1 was chosen because it had the most members ($n = 17$). The sequence data for ITS (Fig. 8, upper left) and fragment of actin (Fig. 8, upper right) were used to define two alleles for each locus. The two variable bases for the ITS alleles are at position 65 (C↔T transition) and position 158 (C↔T transition). ITS allele P = C/T, and allele p = T/C. The two bases for the actin alleles are at position 739 (A↔T transition) and 836 (G↔C transversion). Actin allele Q = A/G, and allele q = T/C. The gene genealogies for both ITS and actin have consistency index (CI) values = 1.0, indicating that no homoplasy has occurred in the form of back mutations. The two alleles of ITS and actin appear in all four pairwise combinations among different individuals: PQ ($n = 8$), pQ ($n = 4$), pq ($n = 4$), and Pq ($n = 1$), consistent with recombination in this species (right column of Fig. 8, bottom). Given the observed allelic frequency, the distribution of all four pairwise combinations is consistent with random association of alleles ($\chi^2 = 3$, df = 3, $p = 0.40$, Hartl & Clark 1997). Because the alleles are characterized by two polymorphic sites, it is less likely that the fourth genotype could arise by back mutation alone.

Incongruity between the two loci can also be shown by comparing the length of the individual gene trees with the length of a combined tree. The ITS tree is four steps long (CI = 1.0) and the actin tree is 12 steps long (CI = 1.0). A combined data set produces 26 MP trees of 21 steps (RC = 0.64), five steps longer than the sum of the single locus trees. A strict consensus of the two loci produces a poorly resolved and statistically unsupported phylogeny (Fig. 8, bottom).

The null hypothesis of clonality was also tested phylogenetically by using the PHT to assess congruity between the ITS and actin data sets. If the species is clonal, the two loci should have the same phylogenetic history, and the PHT will show them to be congruent. If the species is sexual, the two loci will have different histories, as their alleles sort randomly within a recombining population, and the PHT will show them to be incongruent. This species has a PHT score of $p = 0.00035$, so the two loci are in significant incongruence, rejecting the null hypothesis of clonality.

Discussion

1) What is the identity of the photobionts of Letharia*, based on molecular characters?*—The mycobionts of *Letharia 'lupina,' L. 'gracilis,' L. 'rugosa,' L. 'barbata,'* and *L. 'lucida'* are specifically associated with a clade of closely related *Trebouxia* species that is provisionally nicknamed *Trebouxia 'letharii.'* This species complex forms the basal clade of the *Trebouxia jamesii* morphospecies (Fig. 5), based on rooting with the ITS sequences of other *Trebouxia* morphospecies. The chloroplasts of *Trebouxia 'letharii'* resemble the holotype of *T. jamesii* with the diffuse pyrenoids and large chloroplast lobes characteristic of that species (Beck 1999).

Letharia vulpina is specifically associated with a clade that is provisionally nicknamed *Trebouxia 'vulpinae'* and is the sister clade of the *T. jamesii* found in *Pseudevernia furfuracea.* Based on the observation of algal cells from intact thalli, the chloroplasts of *Trebouxia 'vulpinae'* appear to be different from the holotype of *Trebouxia jamesii,* and resemble the chloroplasts from *T. jamesii* subsp. *angustilobata.* However, cultured material of *T. 'vulpinae'* must be examined before it can be assigned to the *T. jamesii* subsp. *angustilobata* morphospecies.

Trebouxia 'letharii' is not the sister group to *T. 'vulpinae.'* It seems most likely that *L. vulpina* switched algae, although other scenarios are possible. Sorediate *Letharia vulpina* is found in North America in warmer coastal mountains near the Pacific and is also present in Scandinavia and the Alps (Fig. 9). The thalli IT1 and IT2 from Trentino-Alto Adige (South Tyrol) in Italy, and thalli AV2 and AV3 from the north coast ranges of California have *Trebouxia 'vulpinae'* as their phycobiont, and have identical phycobiont ITS sequences. *Letharia vulpina* is allopatric to sorediate *L. 'lupina,'* which is present in the colder, interior western mountains—the Sierra Nevada, the Cascades, and the Rockies. Their specificities for different *Trebouxia* species may be genetic, or may be due to biogeography. *Letharia vulpina* and *L. 'lupina'* become sympatric at the northern end of their ranges in British Columbia, where they occur in the same valleys, with *L. vulpina* favoring the lower elevations (Goward 1999). Such sites should be investigated to find if the two species maintain their phycobiont specificity even when they are sympatric, a finding that would support the hypothesis of genetic-based specificity.

The morphospecies *Trebouxia jamesii* is known from ten lichen genera (Ahmadjian 1993*b*). However, the ITS data suggest *T. jamesii* represents many phylogenetic species, because their ITS sequences show far more divergence (up to 73 steps) than is known to exist in a phylogenetic species of fungi delimited by multiple gene genealogies (Geiser et al. 1998; Kasuga et al. 1999; Koufopanou et al. 1997, 1998; Kroken & Taylor 2001). The *Trebouxia* phylogeny (Fig. 3) also shows the lack of correlation between morphological species and genetic divergence. *Trebouxia higgensiae* and *T. galapagensis* are different morphospecies that differ

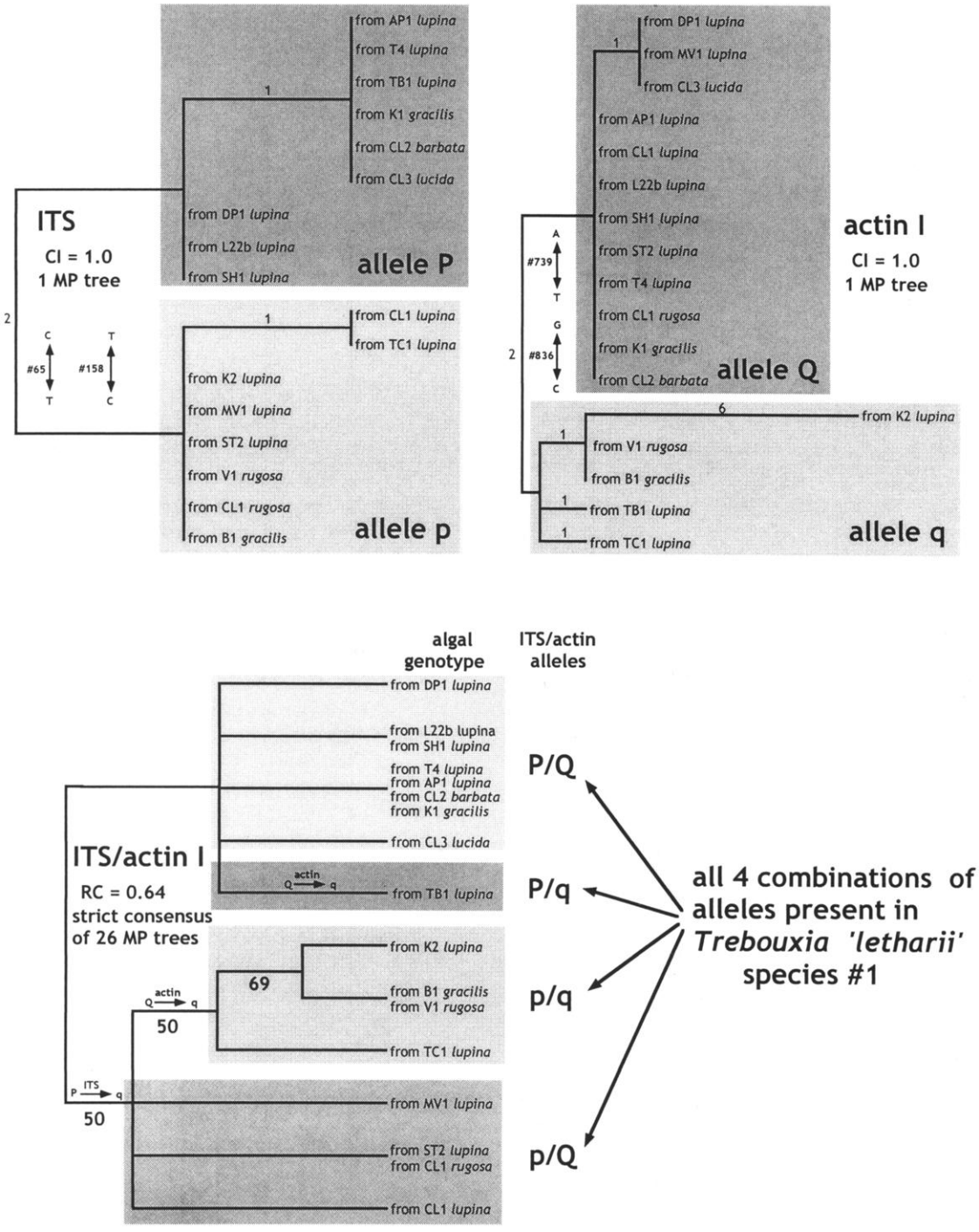

FIGURE 8. *T. 'letharii'* species #1. Upper left. ITS phylogeny showing two classes of alleles. I MP tree, 701 characters, of which four are phylogenetically informative, four steps, RC = 1.0. Upper right. Actin phylogeny showing two classes of alleles. One MP tree, 239 characters, four phylogenetically informative, 12 steps, RC = 1.0. Bottom. Combined ITS and actin phylogeny, duplicate genotypes removed. 1,227 characters, of which 16 are phylogenetically informative, 21 steps, RC = 0.64. Strict consensus of 26 MP parsimonious trees. There is only weak significant bootstrap support (≥ 50%) for any clade.

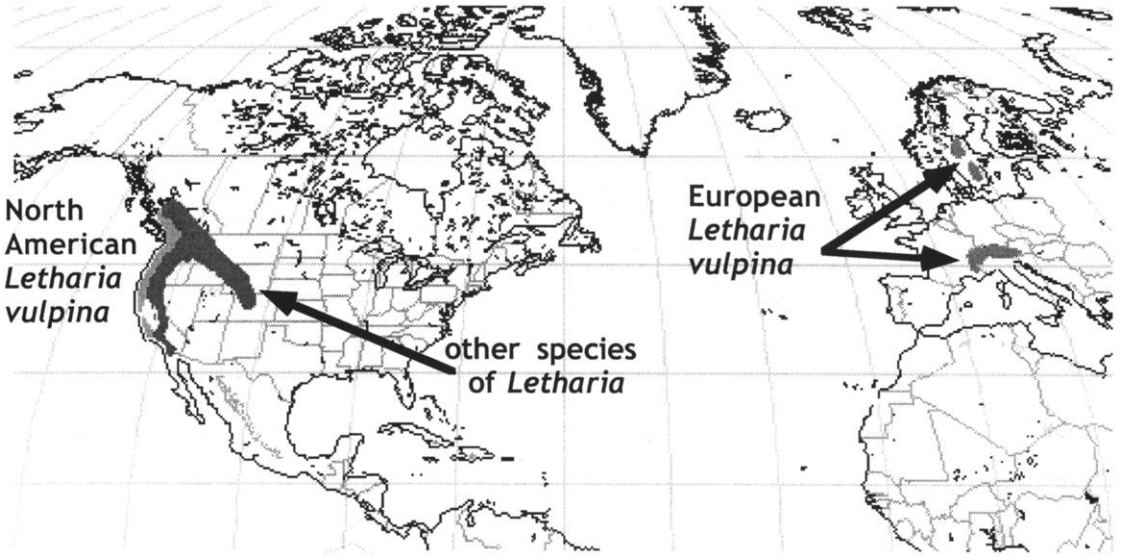

FIGURE 9. Global distribution of *Letharia.* Sorediate *L. vulpina* occurs in the Pacific coast ranges of western North America (Kroken & Taylor 2001), and in the Alps and Scandinavia, and more rarely in the Pyrenees, the Canary Islands, northwestern Africa, and southeastern Europe to the Caucasus (Schade 1954). Sorediate *L. 'lupina,'* and nonsorediate *L. 'gracilis,' L. 'rugosa,' L. 'barbata,'* and *L. 'lucida'* occur only in the interior mountains of western North America. Small populations of sorediate *Letharia* also occur in the mountains of Mexico and the Caribbean. Map obtained from the XEROX Park web viewer (http://mapweb.parc.xerox.com/map/).

by only four bases, and *T. potteri, T. impressa,* and *T. flava* are morphospecies that vary by 21 bases from each other.

The ITS sequence for *Trebouxia 'letharii'* suggests the presence of at least two and probably more species. However, a single locus (i.e., ITS) cannot accurately resolve closely related species because a single locus phylogeny may not reflect the organismal phylogeny (Avise and Wollenberg 1997; Maddison 1997). Thus, a second locus, an intron-containing fragment of the type I actin gene, was selected to test the ITS phylogeny for *Trebouxia 'letharii'* and *T. 'vulpinae,'* and to further resolve the number of phylogenetic species within the *T. 'letharii'* species complex. The initial green algal-specific actin primers amplified two types of actin from *Trebouxia*–type I, which is orthologous to the actin present in representatives of the Trebouxiophyceae, Chlorophyceae, Charophyceae, and land plants, and type II, a paralog that has been found only in representatives of the Trebouxiophyceae. Type II actin was suggested to be a pseudogene (Chow & Chan 1997), however, the type II actin sequences have no mutations that would prevent their being translated to proteins. They may represent a duplication event during the evolution of the Trebouxiophyceae.

The type I actin phylogeny was congruent with the ITS phylogeny in resolving *Trebouxia 'letharii'* and *T. 'vulpinae'* and their phylogenetic placement within the *T. jamesii* species complex. The actin phylogeny (Fig. 5) also resolved six clades within the *T. 'letharii'* clade, with clade #2 being resolved as monophyletic and not paraphyletic as in the ITS phylogeny (Fig. 3). The ITS and actin phylogenies were incongruent within the *T. 'letharii'* species complex, as shown by the PHT. However, by successively removing individual species from the PHT, the significant conflict was identified to be among genotypes of large clade #1 of this species complex. Our interpretation is that this clade represents a single phylogenetic species, in which recombination may be expected to result in incongruity between the two loci. The combined 2-locus tree revolves six clades that we interpret as six phylogenetic species in which lineage sorting among these reproductively isolated species has gone to completion for both loci in all species, except for ITS in species #1 and 2. This result highlights the potential fallacy of species delimitations that are based on a single locus (Taylor et al. 1999). The alternative interpretation is that the six clades represent populations. However, the term population implies a group with reduced gene flow and differences in allelic frequencies. In this case, most alleles are not shared among clades, which suggests that they represent reproductively isolated species. Within each clade, the two loci are incongruent, as would be expected within a phylogenetic species where recombination is occurring, but the only clade within which the conflict is significant is the aforementioned clade #1, which is discussed next.

2) Are the photobionts clonal or cryptically sexual?—One species of *Trebouxia 'letharii'* included enough individuals to test for evidence of recombination. Within species #1, all four pairwise combinations of two alleles of two loci were found among these individuals. The first three combinations may be explained by clonal evolution. The fourth can only occur by recombination or reverse mutation. Since each pair of alleles differs by two nucleotide bases, reverse mutation is unlikely, and recombination is the best explanation for the data. A phylogenetic analysis comparing the summed tree lengths of individual loci to the tree length of a combined data set suggest that the loci are in conflict, consistent with a scenario of recombination. A phylogenetic analysis of the same data using the PHT indicated that the phylogenies of the two loci are significantly incongruent, rejecting the null hypothesis of clonality.

Trebouxia has been considered to be clonal because sexual structures have never been observed. However, it appears that at least one species of *Trebouxia* has a recombining population structure, suggesting that sexual reproduction is taking place. This result emphasizes the difficulty of observing sex directly in these symbiotic green algae. The problem may be one of looking at the right time and place, perhaps only when the algae occur in a free-living state. It may also be that sex is rare in *Trebouxia.* Still, rare events of recombination have a large effect in producing a recombinant population structure (Burt et al. 1996). The results suggest that the Trebouxiophyceae are not wholly clonal, supporting the dogma that recombination is a prerequisite for long term evolutionary success.

3) Is Letharia *specific for its phycobiont?—Letharia vulpina* is specifically associated with the phylogenetic species *Trebouxia 'vulpinae,'* and the other five *Letharia* species are specifically associated with the species complex *Trebouxia 'letharii',* which represents six phylogenetic species. The maintenance of this specificity is easy to comprehend for *Letharia vulpina* and *L. 'lupina',* because they carry their algae with them in soredia and avoid the need to encounter a compatible photobiont (Beck et al. 1998). This co-dispersal may allow the sorediate species to establish broader biogeographical ranges compared to the non-sorediate species of *Letharia.*

However, when *Letharia vulpina* or *L. 'lupina'* reproduce by meiospores, they must acquire their specific alga from the environment, from neighboring thalli or from their own soredia. *Letharia 'gracilis,' L. 'rugosa,'* and *L. 'barbata'* often produce isidia and behave similarly to sorediate species. However, *Letharia 'lucida'* does not produce isidia or soredia and appears to only produce by meiospores. For this species in particular, the question arises—where does the fungus find its specific alga? A germinating fungal meiospore must find a compatible photobiont in order to grow into a new lichen. It may find its photobiont by taking it from a free-living population of phycobionts, or picking it up from another lichen, or its dispersed soredium, isidium, or thallus fragment (Beck et al. 1998; Honegger 1993).

Free-living populations of *Trebouxia* as a source of phycobionts has been thought to be the less likely scenario, as the class Trebouxiophyceae is not frequently found in the free-living state, occurring in low numbers on lichen substrates such as tree bark and rock (Tschermak-Woess 1988). It has been argued that symbiotic algae found outside of lichens are liberated from decomposing lichens and their symbiotic propagules (Ahmadjian 1993*a*). However, one study found free-living *Trebouxia* on recently fire-sterilized rocks, long before lichens colonized the rocks (Mukhtar et al. 1994). The *Trebouxia* found was morphologically and immunologically identical to the *Trebouxia* found in the lichens *Xanthoria* and *Buellia,* which colonized the rocks at a later date. The apparent paucity of free-living *Trebouxia* may be an artifact of the daunting nature of finding and identifying them, and few investigators have reported on this topic.

The alternative source of phycobionts would be from other lichens, either *Letharia* or other genera. For example, species such as *Letharia 'lucida'* may depend on the isidia produced by *L. 'barbata,' L. 'gracilis,'* and *L. 'rugosa,'* and on the more abundant soredia produced by *L. 'lupina'* to disperse the specific photobionts. However, as discussed below in section four, *Letharia 'lucida'* does not indiscriminately use all of these sources. This species commonly occurs with sorediate *L. 'lupina,'* yet the two species do not share the same pool *of Trebouxia 'letharii'* species. A third means of vegetative propagation, thallus fragmentation, may play a role in dispersing *Trebouxia* associated *with Letharia 'lupina.'* For example, experiments on the fruticose lichens *Ramalina menziesii* and *Usnea barbata* showed that their thalli break into wind dispersed pieces in storms (Bailey 1976). Similarly, thallus fragments of *Letharia lupina* may serve as algal sources for germinating meiospores of the same species. Other lichen genera that occur with *Letharia* should be investigated for their phycobionts to see if they also harbor the same algal species and may thus serve as sources of algae for *Letharia.* Such a study may demonstrate that *Trebouxia 'letharii'* is specific to only *Letharia,* which would suggest that the specificity between fungus and alga is due to concomitant evolutionary descent (co-speciation). Alternatively, *Trebouxia 'letharii'*

may be shared among other genera of lichenized fungi, which would suggest that *Letharia* phylogenetically tracked onto a clade of algae that had previously diversified (co-evolution).

4) Are phylogenetic species of Letharia *specifically associated with phylogenetic species of* Trebouxia *'letharii'?*—At the higher taxonomic levels, the pattern of specificity is unambiguous in that a clade of closely related phylogenetic species of fungi is specific for a clade of closely related phylogenetic species of algae. The fungal and algal clades were investigated for specificity at the lower level of phylogenetic species (Fig. 7). To do so, the amount of congruity was determined between the phylogenies of *Letharia* spp. and *Trebouxia 'letharii.'* The PHT indicates that they are significantly incongruent, which is evident by visual inspection of the phylogenies that indicate a given fungal species associates with more than one species of *Trebouxia 'letharii.'* However, there is evidence for specificity of a given fungal species for an algal species. For example, *Letharia 'lupina'* and *L. 'gracilis'* are primarily symbiotic with *Trebouxia* sp. #1–2. *Letharia 'barbata'* and *L. 'lucida'* are primarily symbiotic with *Trebouxia* spp. #1–3 in Oregon and Washington, and with *Trebouxia* sp. #4–6 in California.

These specific associations may be explained by the biogeographical distributions of the species of *Trebouxia 'letharii.' Trebouxia* sp. #1 was found throughout California, Oregon, and Washington, whereas *Trebouxia* sp. #4–6 was found only in California. Similarly, the main branch of *Trebouxia* sp. #2 was found only in California (genotype TC1 from Tumwater Canyon, Washington may represent a different species). One branch of *Trebouxia* sp. #3 was found in Washington, and the other branch was found at California in a similar subalpine habitat. There are two plausible hypotheses to explain the biogeographical distribution of these *Trebouxia* species. They may have photosynthetic maxima under different environmental conditions influenced by latitude and elevation. This result would be similar to that found in morphologically cryptic species of the dinoflagellate *Symbiodinium* that are symbiotic with coralline animals, where different species are specialized in their adaptation to different ocean temperatures (Rowan 1998). Alternatively, the distributions of the *Trebouxia* species may be the result of their ability to disperse within symbiotic propagules. This hypothesis is supported by the broad distribution of *Trebouxia* sp. #1, which is easily dispersed by soredia of *Letharia 'lupina.'* The more narrowly distributed *Trebouxia* sp. #2–6 are less easily dispersed by the species of *Letharia* that produce only isidia or no specialized symbiotic propagules.

The phylogenetic species of *Trebouxia* may be morphologically cryptic, which can be determined only when the species are cultured and microscopically observed through their life cycle. However, the *Trebouxia* species appear to differ in specificity with phylogenetic species of *Letharia,* which suggests that morphologically cryptic species may not be physiologically cryptic.

Acknowledgments

The authors thank François Lutzoni, Tom Bruns, Ignacio Chapela, and two anonymous reviewers for their suggestions on previous versions of this manuscript, and Mat Fisher, Jamie Platt and Charles Delwiche for their helpful discussion. This paper is based on part of the Ph.D. dissertation of SK, and was supported by NSF and Novartis.

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ms. submitted March 17, 2000; accepted July 31 2000.