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A gene genealogical approach to recognize phylogenetic species boundaries in the lichenized fungus *Letharia*

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Abstract: *Letharia,* a genus of lichenized fungi described as a pair of sympatric species, one making abundant sexual structures and the other making few, was investigated as a model system in which to recognize species boundaries. Gene genealogies of 6 and 12 loci were used to estimate the evolutionary history of *Letharia,* based on the principles of lineage sorting of alleles in divergent lineages after genetic isolation. Instead of a species pair consisting of a putative clonal species derived from a progenitor sexual species, *Letharia* comprises at least six phylogenetic species. Judging by the presence of perennial apothecia and clonal reproductive structures, one species is exclusively sexual, three species are sexual and isidiate, and two species are sorediate and rarely sexual. Not all of these species would have been detected with a single gene genealogy, demonstrating the need for multiple independent loci in phylogenetic analysis to recognize recent speciation events. The results are concordant with aspects of both biological and phylogenetic species recognition. However, only phylogenetic species recognition can be applied to fungi like *Letharia* species that are difficult to cultivate and mate in the lab.

Key Words: clonal lichens, coalescence, congruence, cryptic species, Lecanorales, metaphyletic species, multiple loci, Parmeliaceae, sibling species, species pair, sympatry

INTRODUCTION

The lichenized fungus *Letharia* was chosen as a model genus to investigate species boundaries. *Letharia* has been thought to represent a classic example of a lichen species pair, defined as one progenitor sexual species and one descendant clonal species (Poelt 1970, 1972). Mature individuals of *L. columbiana* (Nutt.) Thomson always produce sexual structures (apothecia). Some individuals also produce clonal symbiotic propagules in the form of isidia, short lateral branches that break off easily for wind dispersal. Mature individuals of *Letharia vulpina* (L.) Hue rarely produce apothecia, whereas all individuals produce clonal propagules in the form of soredia consisting of ecorticate wefts of fungal hyphae, each surrounding a nest of algal cells.

The existence of lichen species pairs has been called into question, and it has been proposed that the clonal lichens are asexual individuals repeatedly arising from within a sexual species (Tehler 1982). This alternative hypothesis is represented diagrammatically by showing clonal individuals intermixed with sexual individuals throughout the phylogeny, rather than showing all the clonal individuals as a single lineage (FIG. 1). The disagreement between Poelt and Tehler turns on whether the sorediate individuals of a species pair represent a separate species or are conspecific with apotheciate forms (Mattsson and Lumbsch 1989). We wanted to know whether *Letharia vulpina* is a phylogenetic species or merely a collection of rarely apotheciate individuals of *Letharia columbiana.*

Until very recently, species recognition in most lichenized fungi was based on morphological, chemical and biogeographic characters (Purvis 1997). Recent published molecular studies have been based on a single locus, usually ITS (e.g., Groner and LaGreca 1997, Lohtander et al 1998a, b, Thell and Miao 1998). However, a single locus will not separate recently diverged species if the single gene tree does not reflect the species phylogeny (Avise and Wollenberg 1997, Maddison 1997). FIGURE 2 shows an ancestral species with two alleles (black and gray) for one locus. Genetic isolation results in two descendant species, both of which have inherited the two alleles. The process of lineage sorting eventually results in coalescence for one allele in first in species Y, and then the other in species X. Only when coalescence has occurred for different alleles between the two species can a single locus be used to separate species, and it cannot be known a priori if this has indeed occurred. If phylogenetic species recognition (PSR) were applied before coalescence occurred in both species, it would erroneously group the individuals

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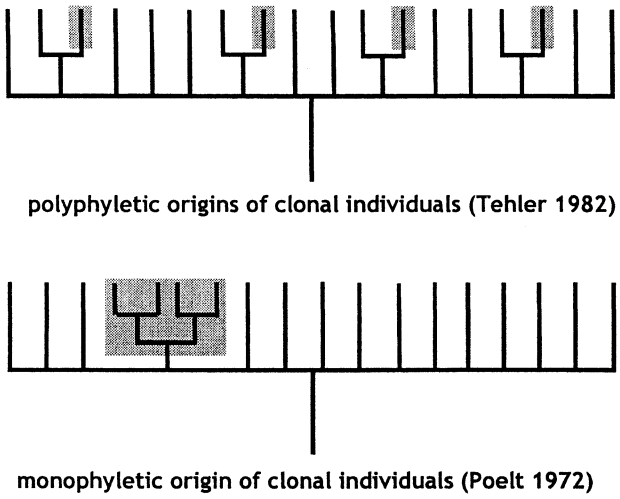

FIG. 1. Alternate hypotheses of the origin of clonal lichens from a sexual species.

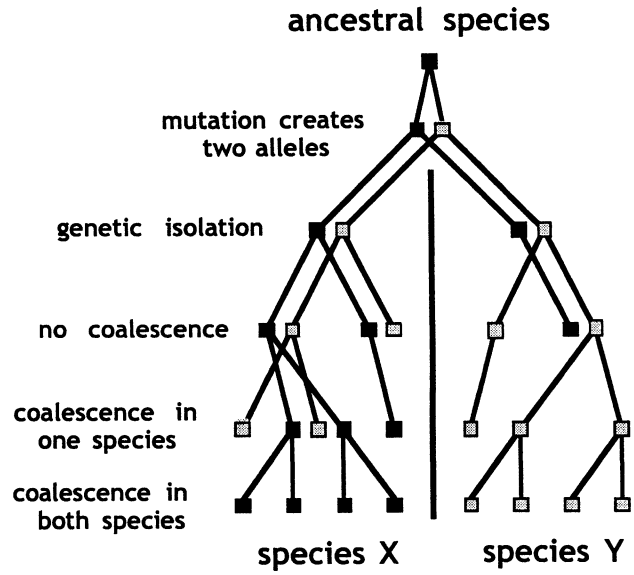

FIG. 2. Lineage sorting for a single locus (after Maddison and Avise).

containing the black allele or the grey allele as two separate clades and rank them as species.

The two described species of *Letharia* are sympatric, so there is the possibility of interbreeding. Reproductive isolation cannot be determined in lichenized fungi by the inability to interbreed, because, like many nonlichenized fungi, they have not yet been crossed in laboratory conditions. However, finding breeding compatibility would only demonstrate what can happen *in vitro,* and not what occurs in natural populations. Genetic isolation typically precedes the development of reproductive barriers, resulting in the delimitation of paraphyletic lineages larger than actual interbreeding species (Rosen 1979). Therefore, in order to determine breeding barriers in natural populations, we used a PSR based on the congruence of multiple gene genealogies to group genetically isolated clades that are ranked as phylogenetic species (Taylor et al 1999, 2000). FIGURE 3 summarizes this approach with a simplified example of recent genetic isolation and ongoing lineage sorting for three loci between two species.

No single gene genealogy reflects the species phylogeny, because no locus has completed the process of lineage sorting for both species. Locus 1 has coalesced in species X, locus 2 has coalesced in species Y, and locus 3 has coalesced in neither species. However, a combined 3-locus analysis will delimit two species, as each branch is supported by two characters. For example, species X is supported by allele a-b-c from locus 1, and allele f-b-c from locus 3 (the branch to individual 'a' in the combined phylogeny is scored as a homoplastic reversal.) In contrast, a strict consensus of the three single locus genealogies would not detect two species, as the result would be an unresolved tree (Barrett et al 1991). A strict consensus approach requires reciprocal fixation (exclusive sets of alleles) for all loci between the two species (Baum and Shaw 1995). Exclusivity is too strict a criterion given the long time interval between the beginning of genetic isolation and reciprocal fixation at all loci within those species. A combined analysis is more sensitive to uncovering recent phylogenetic divergences, and has uncovered morphologically cryptic species in *Coccidioides immitis* (Koufopanou et al 1997, 1998), *Aspergillus flavus* (Geiser et al 1998a, b), and *Histoplasma capsulatum* (Kasuga et al 1999). In this study, 12 loci were characterized, and the congruence of their genealogies was used to recognize phylogenetic species in *Letharia.*

MATERIALS AND METHODS

Lichen collection and preparation.—Letharia thalli were collected in North America, from California to British Columbia, and in Sweden and Italy. Collection information for *Letharia* is listed in TABLE I. Lichen thalli were removed from their substrate (usually conifer bark) and the limits of individuals were identified by locating their holdfasts. From each thallus, a ~10-mg branch was selected for DNA ex-

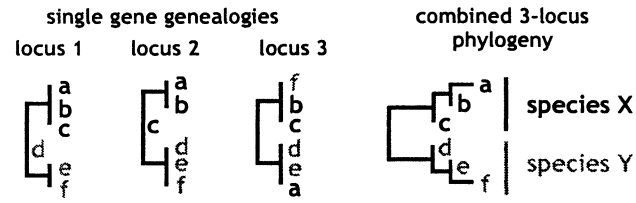

FIG. 3. Three single-gene genealogies, and the congruence of their gene genealogies.

TABLE I. *Letharia* specimens

ID	Location[a] and date	Collector
Letharia 'lupina'		
AP1	USA. WA: Armstrong Peak, Pasayten Wilderness, 7-1997	S. Kroken
BC1	CANADA. British Columbia (mycobiont culture)	Y. Yamamoto
CL1	USA. OR: Crater Lake Natl. park, Cleetwood trail, 7-1997	K. LoBuglio
DP1	USA. CA: Donner Pass, Nevada Co., 7-1994	K. LoBuglio
K2	USA. CA: Kennedy Meadow, Hwy. 108, 8-1994	S. Kroken
L22b	USA. CA: Liberty vista, Hwy. 4, Calaveras Co., 8-1994	T. Bale
MV1	USA. OR: Crate Lake Natl. Park, Mazama Village, 7-1997	K. LoBuglio
SG1	USA. WA: Snehumption Gap, Pasayten Wilderness, 7-1996	S. Kroken
SH1	USA. CA: Mt. Shasta, 3 km W of Panther Meadow, 9-1996	T. Morosco
ST2	USA. CA: Stanislaus Meadow trailhead, Hwy. 4, 8-1994	S. Kroken
T1, T4, T23a, T23b	USA. CA: Tamarack, Hwy. 4, Calaveras Co., 1-1994	T. Bale
TB1	USA. WA: Tatoosh Buttes, Pasayten Wilderness, 7-1996	S. Kroken
TC1	USA. WA: Tumwater Canyon, Hwy. 2, Chelan Co., 7-1995	L. Wilson
Letharia vulpina		
AV2, AV3	USA. CA: Anderson Valley, 3 km E of Yorkville, 11-1997	S. Kroken
CW8	CANADA. BC: 16 km N of Clearwater Village, 4-1998	T. Goward
H1	USA. CA: Henry Coe State Park, Middle Ridge trail, 5-1995	S. Kroken
IT1, IT2	ITALY. Between Pragser Wildsee and Grünwaldalm, 8-1998	H. Mayrhofer
SW1	SWEDEN. Dalarna, Pussmyr, near Falun, 7-1998	R. Lundquist
Letharia 'rugosa'		
CL1	USA. OR: Crater Lake Natl. Park, Cleetwood trail, 7-1997	K. LoBuglio
CP1, CP2	USA. CA: Carson Pass, trail to Round Lake, 8-1995	K. LoBuglio
PM1, PM2	USA. CA: Palomar Mt. State Park, Doane Valley, 11-1998	J. Platt
V1	USA. CA: Bear Canyon, Dick Smith Wilderness, 2-1996	S. Kroken
Letharia 'lucida'		
CL3	USA. OR: Crater Lake Natl. Park, Cleetwood trail, 7-1997	K. LoBuglio
DP2	USA. CA: Donner Pass, Nevada Co., 7-1994	K. LoBuglio
EP1	USA. CA: Ebbets Pass, Pacific Crest Trail, Hwy. 4, 8-1994	S. Kroken
L20a	USA. CA: Liberty vista, Hwy. 4, Calaveras Co., 8-1994	T. Bale
SG1	USA. WA: Snehumption Gap, Pasayten wilderness, 7-1996	S. Kroken
ST1	USA. CA: Stanislaus Meadow trailhead, Hwy. 4, 8-1994	S. Kroken
T3	USA. CA: Tamarack, Hwy. 4, Tuolomne Co., 1-1994	T. Bale
Letharia 'barbata'		
CL2	USA. OR: Crater Lake Natl. Park, Cleetwood Trail, 7-1997	K. LoBuglio
LP1	USA. CA: Lockwood Valley Rd., Ventura Co., 2-1996	S. Kroken
OC1	USA. CA: Overlook Camp, Los Padres Natl. Forest, 2-1996	S. Kroken
ST1	USA. CA: Stanislaus Meadow trailhead, Hwy. 4, 8-1994	S. Kroken
TB1, TB2	USA. WA: Tatoosh Buttes, Pasayten Wilderness, 7-1996	S. Kroken
TC1	USA. WA: Tumwater Canyon, Hwy. 2, Chelan Co., 7-1995	L. Wilson
Letharia 'gracilis'		
B1, B2, B3, B4	USA. CA: Baker Station, Hwy. 108, 10-1998	S. Kroken
K1, K141, K148	USA. CA: Kennedy Meadow, Hwy. 108, 8-1994	S. Kroken

[a] WA = Washington (state); OR = Oregon; CA = California.

traction. Any apothecia present were removed, because they might contain the DNA of a fertilizing partner, and were stored with the remainder of the thallus, leaving behind a single haploid vegetative individual for DNA extraction. Each branch was divided in two, and one half was used for DNA extraction, and the other half stored at −20 C as a DNA voucher. The remainder of each vegetative thallus was stored as a voucher specimen and deposited in the University of California (UC) Herbarium.

DNA extraction.—A version of the Lee and Taylor (1990) mini-prep protocol incorporating modifications from other protocols (Barns et al 1994, Crespo et al 1998, O. F. Cubero unpubl) was developed to deal with the difficulties of extracting DNA from lichenized fungi. Individual thalli were cleaned with a jet of pressurized air. Plastic pestles and 2-mL Eppendorf-style tubes were chilled with liquid nitrogen in a tube rack. In each tube, ~5 mg of material was ground with a plastic pestle and placed on ice to warm to 0 C.

(Tubes must not be closed until warm, due to the danger of explosion.) To each tube, 50 μL of 0.1-mm-diam zirconium/silica beads (BioSpec, Bartlesville, Oklahoma), 50 μL of PVPP powder (polyvinyl-polypyrrolidone, Sigma #P-6755, St. Louis, Missouri), and 1 mL of ice-chilled 1% SDS extraction buffer were added. The large amount of PVPP binds polysaccharides and other secondary metabolites. Tubes were mixed by hand, incubated at 65 C for 1 h, agitated on a Mini Bead-Beater (BioSpec) for 2 min at low speed, centrifuged for 3 min at 4 C at 12 000 *g*, and the supernatant was removed to a clean tube. To each tube, 500 μL each of chloroform and Tris-saturated phenol were added, mixed by inversion, and centrifuged for 2 min at 12 000 *g* at room temperature. The aqueous supernatant, 800 μL, was removed to a clean tube, and an equal vol of isopropanol and 0.1 vol of 8 M sodium acetate, pH 5.2, was added and mixed by inversion. Tubes were put on ice for 20 min, and centrifuged for 20 min at 4 C at 12 000 *g*. The supernatant was decanted and the tubes containing the DNA pellet were inverted on paper towels for 2 min. The DNA pellets were dried in a Speed Vac Concentrator (Savant Instruments, Holbrook, New York) for 5 min. The pellets were resusupended in 500 μL TE at 65 C for 20 min. To assay for DNA, a 10-μL sample was electrophoresed in 1X TAE on a 1.5% SeaKem agarose gel (FMC Bioproducts, Rockland, Maine) and stained with ethidium bromide.

The remaining solution containing DNA was further purified by being transferred to clean tubes containing 133 μL of 7.5 M ammonium acetate, mixed by inversion, and centrifuged for 30 min at 12 000 *g* at room temperature. The supernatant was transferred to a fresh tube and the DNA precipitated with an equal vol, 600 μL, of isopropanol. Tubes were put on ice for 20 min, and centrifuged for 20 min at 4 C at 12 000 *g*, the supernatant was decanted and the tubes were inverted on paper towels for 2 min. The pellet was rinsed with 1 mL of 70% ethanol, and dried in a Speed Vac Concentrator for 5 min. The pellets were resusupended in 500 μL TE in a 65 C heating block for 20 min. The DNA purified by both alcohol precipitations could be used as PCR template at 5 times the concentration of that purified by a single precipitation. Presumably, the concentration of PCR inhibitors was reduced by the double precipitation, allowing for improved amplification of single-copy loci.

Characterization of loci.—Twelve variable loci, comprising nuclear ribosomal DNA (rDNA), chitin synthase I, and 10 anonymous loci, were characterized as regions of DNA sequence with polymorphic nucleotide substitutions. Fungal specific primers ITSIF (Gardes and Bruns 1996) and ITS4A (D. L. Taylor unpubl) were used to amplify by polymerase chain reaction (PCR) the fungal ITS region of rDNA, and NS26 (B. H. Bowman unpubl) and NS18 (Gargas and Taylor 1992) were used to amplify an insert at position 287 in the fungal SSU rDNA (Gargas et al 1995). A fragment of chitin synthase was amplified with degenerate primers designed from published sequences in GenBank, based on regions conserved among chitin synthase class I from filamentous ascomycetes, and not among classes II and III (Bowen et al 1992). The 10 anonymous loci were developed by performing PCR with nonsense combinations of primers to obtain arbitrary PCR products (Burt et al 1994). Locus 2 was developed with primers NS3 and NS7 (White et al 1990), locus 4 with primers NS7 and ITS3 ITS 3 (White et al 1990), locus 11 from primers NS7 and CNL12 (T. Szaro unpubl), locus 12 from primers NS17 (Gargas and Taylor 1992) and 28kj (Cullings 1992), locus 13 from primers NS23 (Gargas and Taylor 1992) and 28kj, locus 14 from primers NS25 (B. H. Bowman unpubl) and 28kj, locus BA from primers benA1 (Geiser et al 1998a) and benA6 (Geiser et al 1998b), loci CS, CT, and DO from primers CS2 and CS4A, CT31 and CT6, and DO3 and DO6 (Koufopanou et al 1997).

PCR reactions on whole lichen DNA extracts were performed using 1 μL of 2-fold diluted genomic DNA template in 50-μL reactions. The mycobiont cultures T1 and BC1 served as positive controls to verify that any PCR product was fungal and not algal. Each reaction contained 0.5 unit AmpliTaq DNA polymerase LD (#N808-0107, Perkin Elmer, Mountain View, California), 0.2 mM deoxynucleotide triphosphates, 0.2 μM of each primer, and 5% glycerol in PCR buffer containing 2.5 μM MgCl, 50 μM KCl, 10 μM Tris HCl, and 0.1 μg/mL gelatin. PCR was performed on an PTC-100 thermocycler (MJ Research, Watertown, Massachusetts) with the following conditions: 2 min at 94 C, followed by 35 cycles of 1 min at 94 C, 1 min at the appropriate annealing temperature (TABLE II), and 1 min at 72 C, followed by one final treatment at 7 min at 72 C. This program was used to amplify the initial PCR products for loci BA and DO (56 C annealing temperature), CS (56 C), and CT (50 C). The SWAPP (Sequencing With Arbitrary Primer Pairs, Burt et al 1994) PCR program was used to amplify the initial PCR products for loci 2, 4, 11, 12, 13, and 14, as described in Burt et al (1996).

These 10 amplicons were gel purified as described in Burt et al (1994). Recovery of the purified products was confirmed by a second round of PCR and electrophoresis of the amplification on a mini-gel to check for a single PCR product. To determine the variability of each locus, and also the homology of each anonymous locus, all loci were screened with Single Strand Conformation Polymorphism (SSCP) gels (Lessa and Applebaum 1993) according to the protocol of Burt et al (1996). Representatives of the loci found to be polymorphic were sequenced to identify variable nucleotide positions. The gel-purified amplicon was subjected to a second round of PCR and was cleaned with a QiaQuick PCR purification kit (#28106, Qiagen, Valencia, California). The cleaned PCR product served as template for sequencing reactions on both strands using a Thermosequenase Dye Terminator cycle sequencing kit #US70754 (Amersham Pharmacia, Piscataway, New Jersey). The PCR primers also served as sequencing primers for all loci except ITS, where the internal primers ITS 1, 2, 3, and 4 were used (White et al 1990). The sequence reactions were cleaned by ethanol precipitation and sequenced with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, California).

Using the nucleotide sequences obtained for the arbitrarily amplified DNA fragments, internal specific PCR primers nested within the original primers were designed

TABLE II. Loci and their *Letharia*-specific primers

Locus	[a]Accession #	5′ Primer	3′ Primer	[b]Annealing T	Length
rDNA:ITS	AF228470	ITS1F ctt ggt cat tta gag gaa gta a	ITS4A att tga gct gtt gcc gct tca	60	681
rDNA:SSU intron	AF228471	NS26 ctg ccc tat caa ctt tcg a	NS18 ctc att cca att aca aga cc	60	428
chitin synthase I	AF228469	chs5 cag caa gac atg ggg aaa aga g	chs6 cca cag gtg gta gat aga atc ctt	46	402
2 (anonymous)	AF228463	2a gct gcc ctt aga ttc gta tgt ttg	2b gga cga cgc ggc agg gta tg	TD70[c]	187
4 (anonymous)	AF228464	4a ctg cgg gct gtt gag acg ag	4b gca agc ggg cgg aga aga tg	TD70	240
11 (anonymous)	AF228465	11a cag aag atg gcg tga gaa gg	CNL12 ctg aac gcc tct aag tca g	TD70	414
12 (anonymous)	AF228466	12a cga aga ccc cga aga aag ag	12b agc ggg acg agc rag ata tt	56	180
13 (anonymous)	AF228467	13c tga gga gct aag aag tga gca g	13d cga cgc cca tct act tga gat	50	367
14 (anonymous)	AF228468	14a tgt acc cgt aaa ctt tct tg	14b att atc taa ctc ttt acc ag	56	197
BA (anonymous)	AF228459	BAa gga gac aag gaa gga tgg ag	BAb agg gag gag gta gtg aag tc	TD70	434
CS (anonymous)	AF228460	CSa caa gtg gat cgt cgg gaa ga	CSb gcc ttc aag gtt tat atg ac	56	372
CT (anonymous)	AF228461	CT31 ctc caa act ctt gtc cag gc	CTb ggg gtg tag tgc cat aac ga	62	515
DO (anonymous)	AF228462	DOa tga aga gtc ggc aa gta gca	DO6 tca tca acg gac cag aaa cg	60	233

[a] Genbank sumission are the alleles from mycobiont BC1, sequenced with the original, non-specific primers.
[b] Temperatures in C
[c] Touchdown PCR program described in Materials and Methods.

to amplify the anonymous loci from all individuals in the study (TABLE II). Four of the primer pairs required the initial high stringency conditions of a touchdown (TD) PCR program (MJ Research, Watertown, Massachusetts) to amplify the desired band alone.

Data analysis.—The sequencing gels were analyzed with Sequencing Analysis 3.0 (Applied Biosystems, Foster City, California) to generate chromatograms from which DNA sequences were inferred. The sequences were assembled and aligned in Sequence Navigator 1.0.1 (Applied Biosystems, Foster City, California) and corrected by eye using both strands. The aligned sequences were exported to a NEXUS file and analyzed using PAUP 4.02ba (Swofford 1998). The 12-locus NEXUS file has been deposited in TreeBASE (SN376-1131).

Parsimony (MP) was the primary method of phylogenetic analysis, and all final results were compared to neighbor joining (NJ) and maximum likelihood (ML) to detect any algorithm-specific results. 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 analysis, which does not allow gaps as character states. ML analysis was done with the following parameters to reflect the values calculated for the data sets: the transition/transversion ratio was set to 2:1 for the 12-locus data set and 3:1 for the 6-locus data set, the ratio of A/C/G/T was set to 0.2455/0.2457/0.2699/0.2389 for the 12-locus data set and 0.2311/0.2630/0.2685/0.2374 for the 6-locus data set. The gamma distribution was set to 0.5. The resulting phylogenies were tested for robustness with 1000 bootstrap replicates. Bootstrap analysis was conducted with MaxTrees set to 1000 to conserve memory; bootstrap estimates with this restriction were not obviously different from those allowing larger settings for MaxTrees.

Alternative phylogenetic hypotheses were tested for significance with a Kishino-Hasegawa test (Kishino and Hasegawa 1989). Each hypothesis was stated as a tree with a topology constraining the taxon in question as a monophyletic group. The constrained tree was imported as a PAUP file and analyzed by maximum likelihood to find the best tree given the constraint. The likelihood of the resulting tree was compared to that of the most likely tree given no constraint. The hypothesis underlying the constraint tree is rejected if the difference in log likelihood divided by the standard deviation (Ln L/SD) is greater than 1.96 (Felsenstein 1990).

Congruence among data sets was determined with Partition Homogeneity Test (Incongruence Length Difference test, Farris et al 1994) as implemented in PAUP 4.0b2. A score of $P \leq 0.001$ indicates significant conflict between data sets, rejecting the null hypothesis of congruence (Cunningham 1997).

RESULTS

12-locus analysis of 17 individuals.—Twelve polymorphic loci were characterized for 17 individual lichens. Individual thalli had only one allele at each locus, suggesting that *Letharia* individuals are haploid. Of

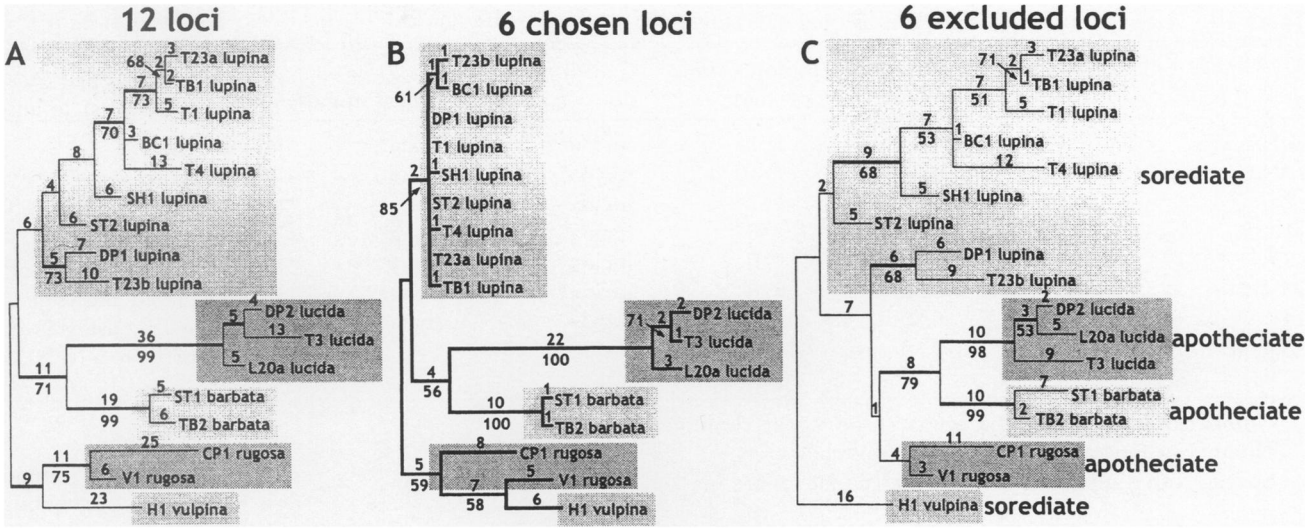

FIG. 4. Phylogeny of 17 individuals. Trees are unrooted. A. 1 of 11 MP trees for 12 locus data set. Branches that appear in a strict consensus of all MP trees are noted in bold. Bootstrap values >50% are shown below the supporting branches, number of steps are shown above branches. 3846 characters, 113 informative, 61 uninformative. 272 steps. RC = 0.47. B. Single MP tree for six chosen loci. Bootstrap values >50% are shown below the supporting branches, number of steps are shown above branches. 1976 characters, 49 informative, 21 uninformative. 85 steps. RC = 0.76. C. 1 of 39 MP trees for six excluded loci. Branches that appear in a strict consensus are in bold. Bootstrap values >50% are shown below the supporting branches, number of steps are shown above branches. 1872 characters, 64 informative, 40 uninformative. 178 steps. RC = 0.40.

3848 nucleotides sequenced in the 12 loci for each individual, 174 were polymorphic, of which 113 were phylogenetically informative (3.5%) and 61 were unique mutations. Most polymorphic sites had only two alternate nucleotides. Four of the 174 sites had three alternate nucleotides present: site 675 in the rDNA intron, site 1450 in locus 2, site 1584 in locus 13, and site 3536 in locus CS. The rDNA ITS locus included three indels, which were not homoplastic when mapped on the single gene genealogy.

The protein-coding frames for each anonymous locus (4, 11, 12, 13, 14, BA, CS, CT, DO, and the last 109 bases of locus 2) were inferred by selecting the one of six possible ORF maps that had no stop codons. However, the identity of these loci is unknown, because BLAST searches did not reveal homologues in sequence databanks. The polymorphic sites in the chitin synthase I and the anonymous, putative protein-coding loci translate as synonymous substitutions in 53% of the codons, and none result in a stop codon. Ten of the twelve single gene genealogies have rescaled consistency indices (RC) = 1.0. One locus, CS, had a RC = 0.91 due to one homoplastic site, likely due to a convergent evolutionary event in the allele found in individual H1. A second locus, 12, which was the only noncoding anonymous locus, had a RC = 0.66 due to six homoplastic sites. Locus 12 can be partitioned into three blocks of adjacent data, each containing one, three, or five polymorphic sites. Each block of data produces a genealogy of RC = 1.0, which suggests that the partitions were created by intra-locus recombination. Where used, locus 12 was treated as three separate loci.

To recognize phylogenetic species by concordance of DNA sequence genealogies, phylogenetic analysis was performed on a data set combining all 12 sequences for all 17 individuals. Using the logic that strongly supported branches should separate genetically isolated species due to coalescence of different alleles in their members, while poorly supported branches should be found within recombining species, putative species could be seen as in FIG. 4a. Two well supported branches grouped individuals that produced abundant apothecia were nicknamed *Letharia* '*barbata*' and *L.* '*lucida*.' A weakly supported branch supported a third group of individuals, again with abundant apothecia, nicknamed *L.* '*rugosa*.' All but one sorediate individual formed a monophyletic group with no support, nicknamed *L.* '*lupina*.' The remaining sorediate individual was nicknamed *L. vulpina*. The phylogeny based on all 12 loci had a much lower RC (0.47) than the RC of the individual genealogies, due to lack of coalescence among species, or due to recombination within species, or both.

6-locus analysis of 51 individuals.—To improve species recognition, the number of individuals in was

TABLE III. Selected loci from the 17 individual data set

Locus	RC gene	RC 6-locus/	#Homoplasies[a] /#chars[b]	Coalescence[c] in *Letharia* species:			
rDNA: ITS	1.0	0.96	1/11	lucida*	barbata*	lupina*	
rDNA: intron	1.0	0.95	1/10	lucida*	barbata	lupina	
2	1.0	0.93	1/7	lucida*	barbata*	lupina	
11	1.0	0.83	2/6	lucida*	barbata*	lupina*	
DO	1.0	0.75	3/6	lucida*	barbata*	lupina	
13	1.0	0.52	6/8	lucida*	barbata*	lupina	rugosa
14	1.0	0.50	1/1	lucida	barbata	lupina	rugosa
Average	1.0	0.83	15/43 (39%)				

[a] Number of homoplastic characters in the 6-gene combined phylogeny.
[b] Number of phylogenetically informative characters.
[c] Species with a unique clade of coalesced alleles are marked with an asterisk.

tripled from 17 to 51. This addition necessitated reducing the number of sequences per individual, so the six loci with the highest levels of phylogenetic signal were selected from the original 12 loci. For each locus, the amount of homoplasy in the individual gene genealogy was compared to the amount of homoplasy for that locus when constrained to include the well supported branches in the combined 12-locus phylogeny (TABLES III, IV). In general, the six loci with the smallest change in consistency indices and the smallest number of homoplastic characters were selected. These six chosen loci are rDNA (including ITS and SSU intron 287), and anonymous loci 2, 11, 13, 14, and DO.

The concern that excluding loci would discard information contradictory to the remaining loci was checked by comparing the phylogenies of all 12 loci (FIG. 4a), the six chosen loci (FIG. 4b) and the six excluded loci (FIG. 4c). The topologies of the trees produced by the six chosen loci and the six excluded loci were not in conflict as assessed by the PHT, and they did not contain conflicting branches with strong bootstrap support. Apotheciate *L.* '*lucida*' and *L.* '*barbata*' were well supported in all three trees. However, there were differences among the three phylogenies. Using just the selected loci, the previously unsupported sorediate *L.* '*lupina*' clade was now well supported, but the sorediate *L. vulpina* and apotheciate *L.* '*rugosa*' groups were no longer separable. Using just the excluded loci, only apotheciate *L.* '*lucida*' and apotheciate *L.* '*barbata*' were well supported.

The resulting single gene genealogies for five of the six chosen loci for 51 individuals are shown in FIG. 5 (locus 14 is not displayed, as it has only one polymorphic site). The single gene genealogies based on the larger sampling showed less coalescence among species, as more alleles were found in each species, as might be expected (FIG. 2) when more individuals were examined (TABLE V). In addition, more homoplastic sites were found within loci. One homoplastic site was found in locus 2 at site 1440,

TABLE IV. Excluded loci from the 17 individual data set

Locus	RC gene	RC 6-locus/	#Homoplasies[a] /#chars[b]	Coalescence[c] in *Letharia* species:		
BA	1.0	0.89	5/24	lucida*	barbata*	
CT	1.0	0.55	9/10	lucida	barbata	rugosa
chs I	1.0	0.50	5/7	lucida	barbata*	
CS	0.91 (1[d])	0.50	7/8			rugosa*
4	1.0	0.40	6/6		barbata	rugosa
12	0.66 (6[d])	0.25	9/9			
Average	0.94	0.52	41/64 (64%)			

[a] Number of homoplastic characters in the 6-gene combined phylogeny.
[b] Number of phylogenetically informative characters.
[c] Species with a unique clade of coalesced alleles are marked with an asterisk.
[d] Number of homoplastic characters in the single gene genealogy.

and another in locus 13 at site 1674. Four homoplastic sites were found in the rDNA: one in the SSUrDNA intron at site 747, and three in the ITS at site 100, 450, and at 41 (or 171, as mapped onto a parsimonious tree with a different topology). The rDNA locus also has homoplastic sites when ITS and SSU intron loci are analyzed as a single linked locus. A gene genealogy for the ITS and SSU intron together has a RC of 0.70, much lower than the RC values of the gene trees of either the ITS (0.91) or the SSU intron (0.94). When the ITS and SSU introns are treated as separate loci, they are significantly incongruent as established by the PHT ($P = 0.001$), suggesting that recombination has occurred between the ITS and SSU intron. This incongruence is seen by comparing the ITS tree, where *L.* '*gracilis*' groups next to *L.* '*lucida*' (FIG. 5a), and the SSU intron tree, where *L.* '*gracilis*' is mixed in with *L.* '*rugosa*' and *L. vulpina* (FIG. 5b). This is the second case of intralocus recombination inferred in this study, the first case being locus 12.

The six loci were combined into a single phylogenetic analysis. Of 1976 nucleotides sequenced in the 6 loci, 113 were polymorphic, of which 80 were phylogenetically informative (4.0%) and 33 were unique mutations. The six homoplastic sites present in the single gene genealogies were excluded from the combined analysis, which reduced the number of MP trees generated from >20 000 to 4416 and allowed the heuristic search to be completed. This exclusion did not produce topological changes nor did it improve bootstrap support for the phylogeny. The resulting 6-locus phylogeny for 51 individuals (FIG. 6) is congruent with the phylogeny for 17 individuals (FIG. 4a) in recognizing the same five species. The results produced by parsimony analysis are concordant with results obtained by neighbor joining and maximum likelihood analyses (results not shown) in recognizing these five species. The 6-locus phylogeny also recognizes a sixth species, *L.* '*gracilis*,' which had not been sampled previously.

The sorediate individuals appear in two monophyletic species: *Letharia vulpina* and *L.* '*lupina*.' A Kishino-Hasegawa test was performed to test an alternative hypothesis of a monophyletic clade of sorediate *Letharia*. The log likelihood (Ln L) of the best tree supporting this hypothesis is −4143.22, which is not significantly worse (1.02 Ln L/SD) than the Ln L for the best phylogeny that suggests two origins of soredia (−4112.22). However, the phylogeny constrained to a single origin of soredia still resolves two well supported branches indicating that *Letharia vulpina* and *L.* '*lupina*' are separate phylogenetic species, even if they are sister species.

Most of the apotheciate individuals appear in three monophyletic species: *Letharia* '*lucida*,' L. '*barbata*,' and *L.* '*gracilis*.' The five monophyletic species are moderately to well supported by bootstrap analysis (values 76–100%), which correlates with the number of nonhomoplastic characters on each supporting branch. The remainder of the apotheciate individuals, nicknamed *L.* '*rugosa*,' form a paraphyletic grade to sorediate *L. vulpina*. A Kishino-Hasegawa test was performed to test an alternative hypothesis of a monophyletic *L.* '*rugosa*.' The Ln L of the best tree supporting this hypothesis is −4118.51, is not significantly worse (0.47 Ln L/SD) than the Ln L of the best phylogeny that suggests a paraphyletic *L.* '*rugosa*' (−4112.22).

Attempts to root the phylogeny.—Attempts to root the *Letharia* tree with putative close relatives did not produce statistically significant results. In an ITS phylogeny of the Parmeliaceae, *Letharia* does not group together with any genus tested (*Ahtiana, Allocetraria, Bryoria, Cavernularia, Cetraria, Cetrelia, Cornicularia, Dactylina, Esslingeria, Evernia, Flavocetraria, Hypogymnia, Kaernfeltia, Lethariella, Melanelia, Neofuscelia, Nodobryoria, Platismatia, Pleurosticta, Pseudevernia, Tuckermanopsis, Usnea, Vulpicida,* and *Xanthoparmelia*) with significant bootstrap support (results not shown). This result is similar to those of published studies that indicate that, while ITS is sufficient to group and rank genera within the Parmeliaceae, it is insufficient to resolve relationships among most of those genera due to high levels of homoplasy (Mattsson and Wedin 1998, Crespo and Cubero 1998). The Parmeliaceae ITS sequences root the *Letharia* phylogeny in a way suggesting that *L.* '*rugosa*' and *L. vulpina* are basal (FIG. 5a). However, there are only enough non-homoplastic characters to provide weak to moderate bootstrap support of this root. There may be a more closely related genus that awaits discovery, or perhaps all closely related genera have gone extinct.

Other genera of the Parmeliaceae were investigated to find additional loci to provide a multi-locus rooting of *Letharia*. Unfortunately, these attempts were not successful, as each locus rooted *Letharia* on different branches in the gene genealogies. The SSU intron we found in *Letharia* at position 287 was also found in *Pseudevernia cladoniae* (P. T. DePriest pers comm) and closely related *P. concosians* and *P. intensa* (but not more distantly related European species *P. furfuracea*), in *Bryoria tortuosa,* and in *Nodobryoria abbreviata* and *N. oregana*. These introns rooted *Letharia* to suggest that *L.* '*gracilis*,' *L.* '*rugosa*,' and *L. vulpina* are basal (FIG. 5b). Thus, *L.* '*rugosa*' and *L. vulpina* are present in the basal clades of the ITS tree and the SSU intron tree.

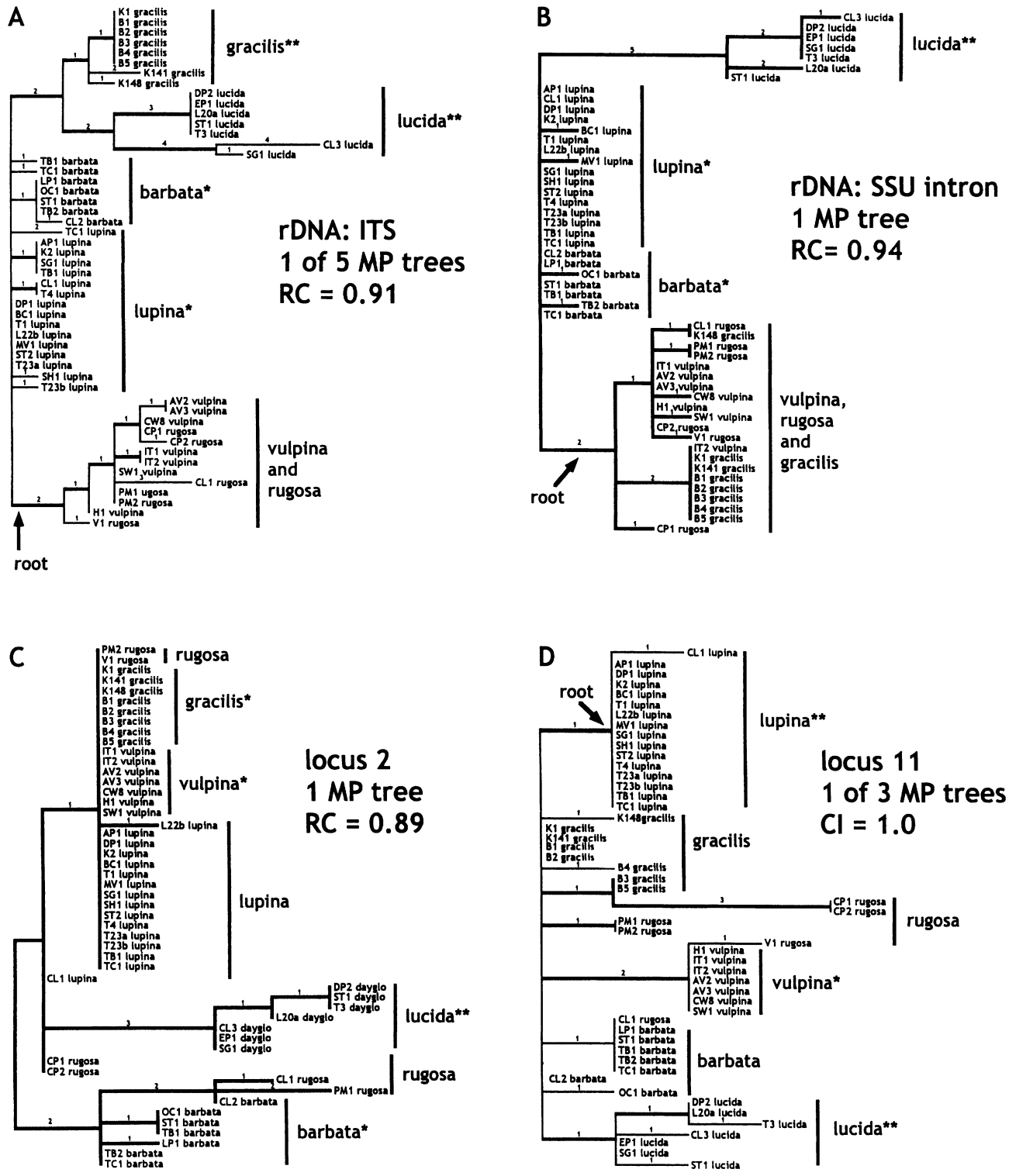

FIG. 5. Single gene genealogies of six chosen loci. Species that have coalesced to a unique clade of alleles are marked with a double asterisk. Species that are fixed for an allelic clade that is shared by another species are marked with a single asterisk. A. ITS. 1 of 5 MP trees, branches present in a strict consensus are in bold, RC = 0.91. Tree is rooted with Parmeliaceae. B. SSU intron. 1 MP tree, RC = 0.94. Tree is rooted with *Bryoria tortuosa, Nodobryoria abbreviata, N. oregana, Pseudevernia cladoniae, P. concosians,* and *P. intensa.* C. Locus 2. 1 MP tree, RC = 0.89. D. Locus 11. 1 of 3 MP trees, branches present in a strict consensus are in bold, CI = 1.0. Tree is rooted with *Nodobryoria abbreviata, N. oregana, Pseudevernia cladoniae, P. concosians,* and *P. furfuracea.* E. Locus 13. 1 MP tree, RC = 0.95. Tree is rooted with *Bryoria tortuosa, Pseudevernia cladoniae* and *P. concosians.* F. Locus DO. 1 MP tree, CI = 1.0.

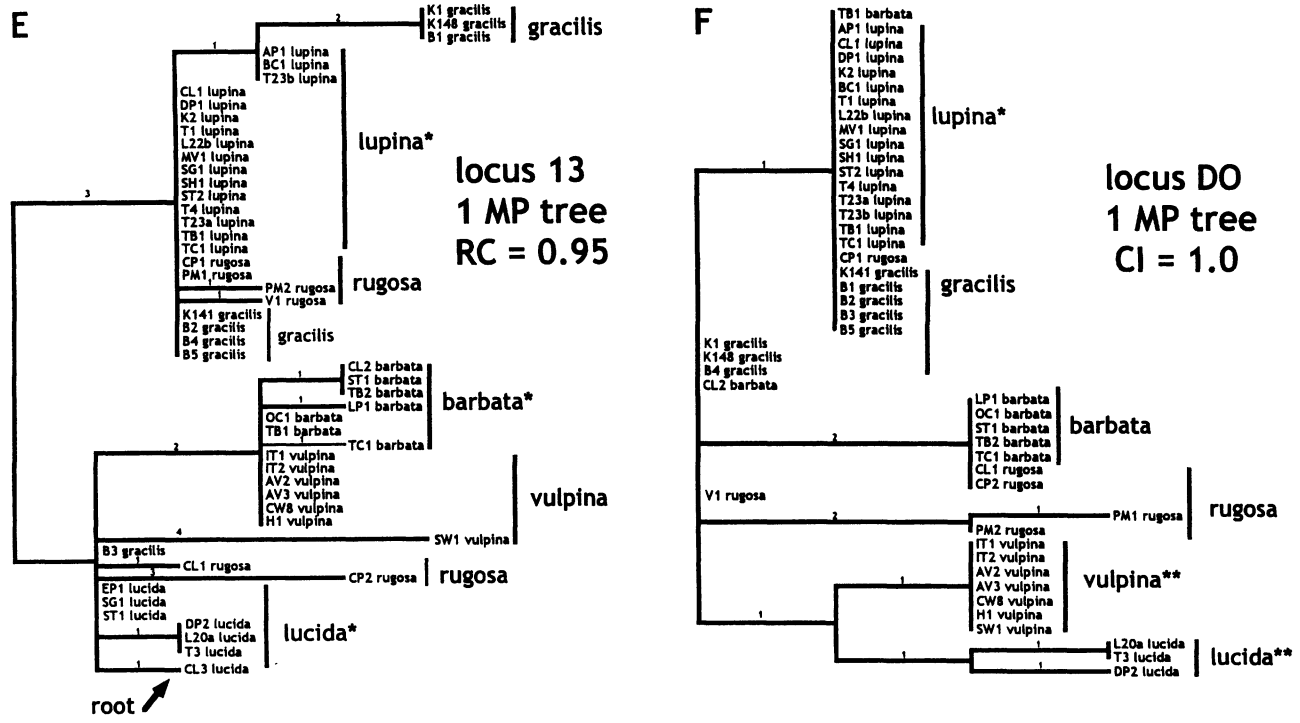

FIG. 5. Continued

The four anonymous loci with the highest levels of coalescence (2, 11, 13, and DO) were also investigated for homologues in other genera of Parmeliaceae. PCR amplification and gel purification of single amplicons were successful only for locus 11 in *Nodobryoria* and *Pseudevernia,* and for locus 13 in *Bryoria* and *Pseudevernia.* These outgroup sequences root the locus 11 tree for *Letharia* with *L.* '*lupina*' as the basal clade (FIG. 5d), and the locus 13 tree for *Letharia* within *L.* '*lucida*' (FIG. 5e). A combined analysis of the three loci (rDNA, locus 11, and locus 13) suggested that *Letharia* '*rugosa*' and *L. vulpina* are basal. Thus, the *Letharia* 6-locus phylogeny has been drawn as such, but there is no statistical support for this root (FIG. 6).

DISCUSSION

The use of multi-locus data to rank phylogenetic species.—We wanted to know whether *Letharia vulpina* is a phylogenetic species or if it is a form of *Letharia columbiana.* To do so, we developed a set of 12 sequence-based loci that were used to determine the phylogeny of the genus based on the concordance of

TABLE V. Selected loci for the 51 individual data set

Locus	RC gene	RC 6-locus	#Homoplasies[a] /#chars[b]	Coalescence[c] in *Letharia* species					
rDNA: ITS	0.87 (3[d])	0.81	9/24	lucida*	barbata	lupina			gracilis*
rDNA: intron	0.94 (1)	0.72	7/15	lucida*	barbata	lupina			
2	0.89 (1)	0.46	7/9	lucida*				vulpina	gracilis
11	1.0	0.72	5/11	lucida*		lupina*		vulpina	
DO	1.0	0.70	3/9	lucida				vulpina*	
13	0.95 (1)	0.46	9/11	lucida	barbata	lupina			
14	1.0	0.20	1/1	lucida	barbata	lupina	rugosa	vulpina	
Average	0.95	0.58	41/80 (51%)						

[a] Number of homoplastic characters in the 6-gene combined phylogeny.
[b] Number of phylogenetically informative characters.
[c] Species with a unique clade of coalesced alleles are marked with *.
[d] Number of homoplastic characters in the single gene genealogy.

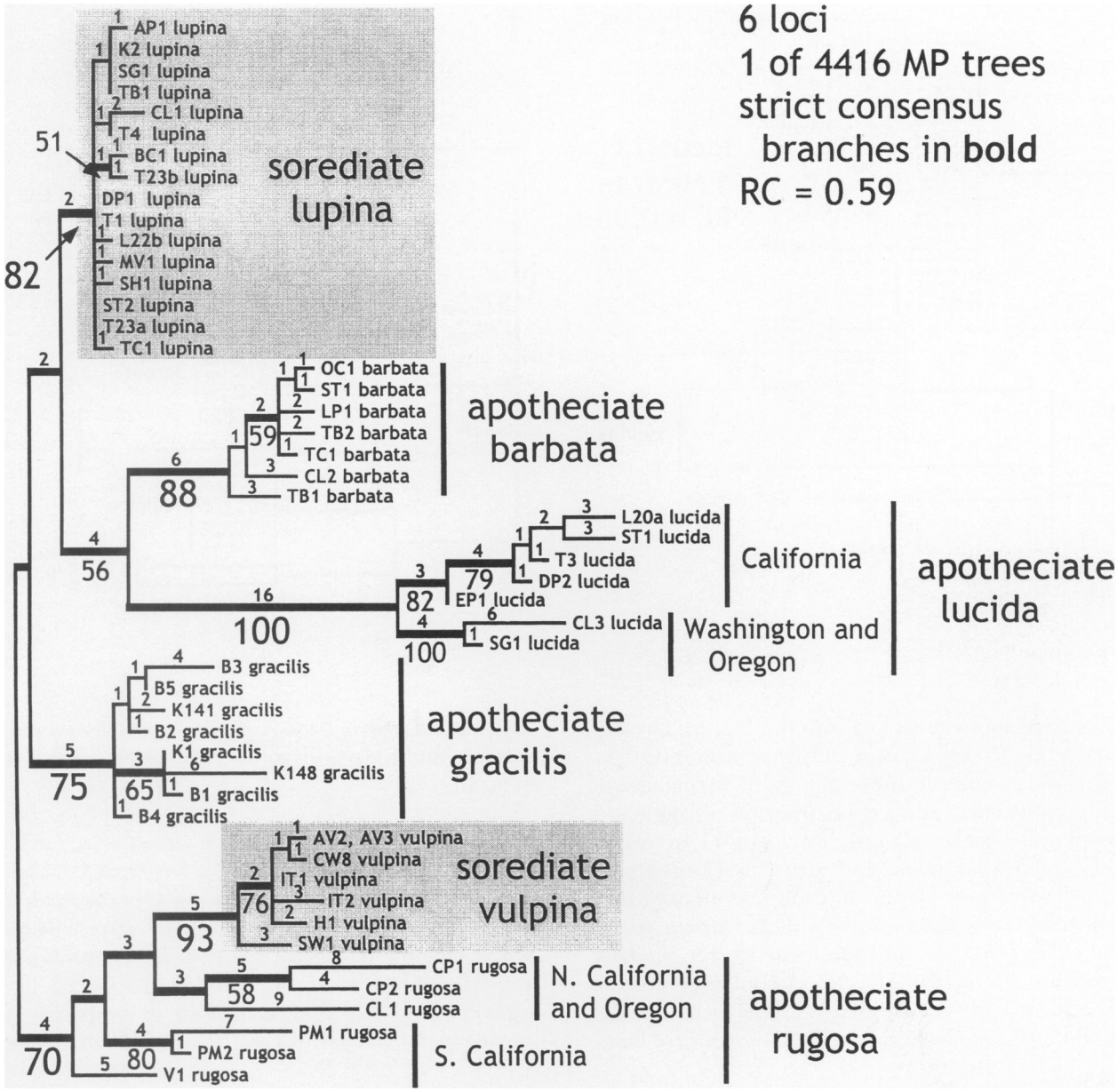

FIG. 6. One of 4416 MP trees inferred from sequence data of 6 loci: rDNA, and loci 2, 11, 13, 14 and DO. Branches that appear in a strict consensus of all MP trees are noted in bold, and are also found by NJ and ML. Bootstrap values >50% are displayed below the supporting branch. Tree is unrooted. 1970 characters (6 characters that are homoplastic in the six single-locus genealogies have been excluded from the initial 1976 characters), 74 informative, 48 uninformative, 186 steps. RC = 0.59.

gene genealogies, using individual lichen thalli as operational taxonomic units. Each individual was found to contain only one allele for each locus, indicating that each has a single haploid genotype. This result corroborates developmental studies of fruticose (shrubby) lichens demonstrating that each thallus developed from a single meiospore or clonal propagule and was not the result of fusion of fungi of different genotypes (Honegger 1993). This result indicates that it is appropriate to use thalli as genetic individuals in phylogenetic analyses.

In order for the loci to be useful in recognizing species, it is necessary that they have low levels of homoplastic mutation that will not be confused with either homoplasy due to a lack of lineage sorting above the species level, or with homoplasy due to recombination below the species level. Each locus except rDNA and locus 12 has a genealogy with RC

close to or at 1.0, indicating that few back-mutations have occurred at variable nucleotide sites. Locus 12 and rDNA have lower RC, apparently due to intralocus recombination. In the 12-locus analysis of 17 individuals, there were 104 phylogenetically informative characters present among the single gene genealogies, of which only one was homoplastic. In the 6-locus analysis of 51 individuals, there were 80 informative characters among the single gene genealogies, of which six were homoplastic. Therefore the main sources of homoplasy present in the combined analyses are shared polymorphisms among species due to a lack of lineage sorting among phylogenetic species, and incongruence due to recombination within phylogenetic species. The well supported branches in the combined phylogeny were used to rank phylogenetic species, suggesting the level at which genetic reticulation ends and genetic isolation of species begins (Taylor et al 1999).

Is Letharia *a species pair?*—The results suggest that *Letharia* is not just a species pair, but rather consists of six genetically isolated species, each marked by predominant reproductive mode as inferred from morphology (FIG. 6). There are two sorediate species and four apotheciate species. The two sorediate species produce isidoid soredia (Büdel and Scheidegger 1996) on older individuals, but these are not the true isidia found on mature specimens of three of the four apotheciate species (*L.* '*lucida*' apparently does not produce isidia). Rarely do individuals of the two sorediate species produce apothecia, which are usually small in size and few in number on a reproductive thallus. These results support the hypothesis of lichen species pairs (Poelt 1970, 1972), with two complications. First, the sorediate species are not strictly clonal, but are rather mostly clonal. Evidence for recombination in both a predominantly and a rarely apotheciate species will be presented in a companion paper (S. Kroken and J. W. Taylor unpubl). And second, cryptic speciation has made the taxonomy of *Letharia* more complicated than a species pair.

We wanted to know if the two sorediate species evolved from apotheciate ancestors as predicted by the concept of species pairs (Poelt 1970, 1972). However, attempts to root the *Letharia* phylogeny with significant support were not successful, and so it is not possible to assess the ordering of character states among the species. This result highlights the difficulty in finding a suitably close outgroup to serve as a phylogenetic root. A similar study of the *Histoplasma capsulatum* species complex encountered the same problem (Kasuga et al 1999).

Diagnostic characters and taxonomic implications.—The *Letharia* species are cryptic or sibling species (Culberson 1986) in that they were not previously recognized by morphological characters. The genus is subject to environmentally-induced variation that obscures species-specific morphological characters. Individuals of two species from a dry habitat resemble each other more than individuals from the same species growing in dry and moist habitats.

However, a post hoc examination of the species revealed subtle morphological differences that appear to be diagnostic. The species' nicknames are based on these differences. The vegetative thalli of *L.* '*rugosa*' have a rugose or veined surface. The thalli of *L.* '*gracilis*' are elongate and infrequently branched. The apothecia of *L.* '*barbata*' are more heavily ringed with short branches (cilia). And, the vegetative thalli of *L.* '*lucida*' are more brightly pigmented and never produce isidia. Some of these morphotypes may correspond to the different forms of *L. columbiana* and *L. vulpina* described by Schade, which he claimed were all developmental variants of one species (Schade 1954, 1955). However, several of his forms are environmentally induced, such as *L. gigantea, L. pygmea, L. densa,* and L. *pusilla.* Schade also described transitional forms such as *L. composita* and *L. intermedia* that consist of an apotheciate form growing out of a sorediate form. This study does not support those transitional taxa, as careful dissection of entangled thalli revealed that different morphotypes were attached to the substrate by separate holdfasts, and were found to be members of separate phylogenetic species. Schade's work has been largely ignored because of these confusions. However, it is cited by the Dictionary of the Fungi (Hawksworth et al 1995), which lists these 10 forms as 10 species of *Letharia.*

It is not yet known which of these four apotheciate species correspond to the holotypes of *L. columbiana* and its synonymized form *L. californica* (Thomson 1969), or to the holotypes of Schade's forms. Formal taxonomic description of the four apotheciate species can only be done after available holotypes are investigated. The Academy of Natural Sciences in Philadelphia has located the holotype of *L. columbiana,* and will send it to UC Herbarium for the authors to identify which phylogenetic species corresponds to this name. Unfortunately, the holotype of *L. californica* in the Museum of Natural History in Paris is currently unavailable. Loan requests will also be made to herbaria that house the holotypes of Schade's 10 forms (Leiden, Kew, Budapest, and Vienna). Any holotypes that cannot be obtained may have to be abandoned and new types and names chosen for the phylogenetic species of *Letharia.*

The two sorediate species also revealed morphological differences (T. Goward pers comm). *Letharia*

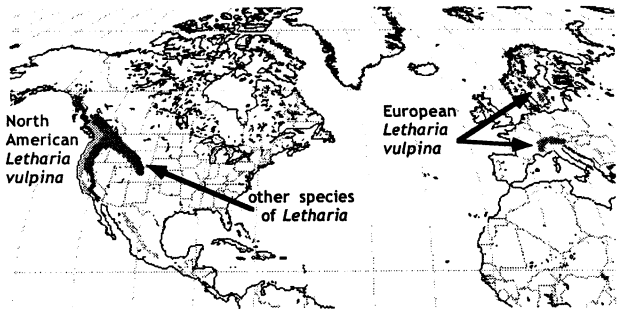

FIG. 7. Map showing worldwide distribution of *Letharia* species.

vulpina is greener in color, less branched, and has soralia (regions producing soredia) covering more limited areas of the vegetative thallus (usually on tops of ridges). *Letharia* '*lupina*' is more yellow, more highly branched, and has larger soralia. These are qualitative differences that mark all individuals included in the study. *Letharia vulpina* is also allopatric from the other *Letharia* species, including *L.* '*lupina*' (FIG. 7), corroborating the phylogenetic and morphological evidence. In addition, the two sorediate species are specifically associated with phylogenetically distant species of the green algal symbiont *Trebouxia* (Kroken and Taylor 2000). *Letharia vulpina* is the only species that occurs in Europe and thus retains the name based on a Swedish holotype first described by Linnaeus. *Letharia* '*lupina*' will be formally described as a new species (T. Goward and S. Kroken unpubl).

A diagnostic chemical difference was also detected in one species. Culberson (1969) reported that the apothecia of some individuals of *Letharia* contain norstictic acid, and others do not. An HPLC test revealed that apothecia of all species except *L.* '*lucida*' contain this acid. This result is concordant with the view that chemical characters are best examined a posteriori in the light of independent lines of phylogenetic evidence (Lumbsch 1998). In this case, chemical variation occurs among species and not among individuals of a species.

Recognizing cryptic species is important for future studies because the phylogenetic differences may correlate with phenotypic differences that are not yet known. This awareness is import to all areas of study, including those that make use of herbarium specimens. This phylogenetic picture of *Letharia* has already been used to investigate the structure of European and American populations of *L. vulpina,* and will have implications for conservation practices to protect that species where it is endangered or threatened (N. Högberg, S. Kroken, G. Thor, T. Bruns, J. W. Taylor unpubl) The topic of recognizing phylogenetic species of lichenized fungi and its implications on taxonomy is discussed at length in Grube and Kroken (2000).

Inadequacy of a single locus for species delimitation.— In the *Letharia* study, rDNA was not adequate for delimiting species. As seen in FIG. 5a, ITS only resolved *L.* '*lucida*' and *L.* '*gracilis,*' and did not resolve sorediate *L.* '*lupina*' from apotheciate *L.* '*barbata,*' and did not resolve sorediate *L. vulpina* from apotheciate *L.* '*rugosa.*' An analysis of ITS alone would group and rank *L.* '*lupina*' and *L.* '*barbata*' as one species, and *L. vulpina* and *L.* '*rugosa*' as one species. This result would support the hypothesis that sorediate individuals are not members of a separate phylogenetic species, and instead arise repeatedly within an apotheciate species (FIG. 1, Tehler 1982), a result that was suggested by three single-locus studies of putative lichen species pairs. ITS sequence did not separate the sorediate individuals of *Roccellina capensis* as a separate species from apotheciate individuals (Lohtander et al 1998a), nor the sorediate *Dendrographa minor* as separate from apotheciate *D. leucophea* (Lohtander et al 1998b). Sequence of ITS and four SSU introns similarly did not resolve sorediate *Roccella tuberculata* from apotheciate *R. canariensis* (Myllys et al 1999). The SSU introns found among various individuals of *Roccella* provided congruent data to ITS, indicating that they are part of one linked rDNA locus. The SSU intron did not provide any further resolution of sorediate individuals from apotheciate individuals in *Roccella* (Myllys et al 1999), as was also the case for *Letharia* (FIG. 5b).

The ITS sequences for *Roccellina, Dendrographa,* and *Roccella* obtained from Genbank suggest the presence of several species and not just one, as in each resulting phylogram, there are several groups of ITS alleles that vary within a group with as much variation within the groups as among them (analysis not shown). Some of these allelic groups are found in both sorediate and apotheciate individuals, and other allelic groups in individuals with only apothecia or only soredia. This interpretation is comparable to the ITS genealogy for *Letharia.*

In the *Letharia* study, rDNA and the five protein-coding loci provide different sets of coalesced alleles in two to five species (TABLE V) that lend support for the branches leading to phylogenetic species in the combined 6-locus analysis. Four of the six species (excluding apotheciate *L.* '*barbata*' and apotheciate *L.* '*rugosa*') have coalesced for a unique clade of alleles of at least one locus (TABLE V, marked by a double asterisk on the single gene genealogies in FIG. 5). Five of the six species (excluding *L.* '*rugosa*') have at least one locus that has coalesced for a clade of alleles

that are still shared with another species (marked with a single asterisk on the single gene genealogies in FIG. 5). Only apotheciate *L.* '*lucida*' has coalesced for all six loci. The other species have coalesced for different subsets of loci, a result consistent with the arbitrary nature of lineage sorting for different loci among closely related species.

Is Letharia 'rugosa' *a separate species?*—All species of *Letharia* are delimited as monophyletic in all 4416 most parsimonious trees, except for the genetically diverse *L.* '*rugosa,*' which is paraphyletic to *L. vulpina.* A Kishino-Hasegawa test showed that the Ln L of a monophyletic *L.* '*rugosa*' was not significantly worse than the best phylogeny. *Letharia* '*rugosa*' may be viewed as a metaphyletic species (a paraphyletic species that cannot be ranked into smaller monophyletic species, Donoghue 1985), as the branching structure within *L.* '*rugosa*' is based on homoplastic sites and therefore is not truly structured into two species. Rather, the branching structure appears to represent populations with reduced gene flow between southern California, and northern California and Oregon (FIG. 6). A similar north-south geographic division is found between the two clades of *L.* '*lucida*' (but is not found *in L.* '*barbata,*' *L.* '*lupina*' or *L. vulpina,* which were also sampled from California to the Pacific Northwest).

Letharia '*rugosa*' has unique alleles for each of the six chosen loci (FIG. 5a–f), which suggests that the species is genetically isolated from other species (Hare and Avise 1998). The sympatry of *L.* '*rugosa*' with *L.* '*lucida,*' *L.* '*barbata,*' and *L.* '*lupina*' also suggests that gene flow is not occurring, even though individuals of *L.* '*rugosa*' have been found next to individuals of these other species. In contrast, *L.* '*rugosa*' shares many alleles with allopatric *L. vulpina,* the species to which it is paraphyletic (FIG. 6).

We hypothesize that *L. vulpina* represents a disjunct species that has recently descended from the genetically diverse progenitor species *L.* '*rugosa*' (FIG. 7). In this scenario, the less genetically diverse *L. vulpina* has coalesced for different loci faster than has *L.* '*rugosa.*' The phylogenetic status of *L.* '*rugosa*' may be clarified by the addition of more individuals and more loci, including the previously excluded loci 4, CT, and CS that were coalesced for this species in the 17-individual data set (TABLE IV).

Conclusion.—Using a gene genealogical approach, recent speciation can be detected before genomes have diverged to the point of coalescence for all alleles, before obvious phenotypic differences have developed, and before breeding barriers have evolved between genetically isolated species (Avise and Wollenberg 1997). It was found that each species of *Letharia* is marked by a predominant morphologically inferred reproductive mode—sexuality or clonality (FIG. 1). The results support the concept of lichen species pairs, and provide another example of previously undetected fungal species that were not evident by a priori examination of phenotypic characters. The phenomena of species pairs and cryptic species have both played a role, as *Letharia* comprises not one or two, but at least six phylogenetic species.

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