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Outcrossing and Recombination in the Lichenized Fungus *Letharia*

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Kroken, S., and Taylor, J. W. 2001. Outcrossing and recombination in the lichenized fungus *Letharia. Fungal Genetics and Biology* We report evidence for recombination in lichenized fungi based on sequenced nuclear DNA markers, judging from the incongruence of their gene genealogies. Recombining population structures were found in two phylogenetic species of *Letharia,* one species that is observed in nature to produce abundant sexual structures (ascomata) and another species that produces abundant clonal reproductive structures (soredia) and only rarely produces ascomata. To determine whether sexual reproduction was the cause of recombination in both species, we compared several variable loci in the ascomata and maternal tissue for evidence of outcrossing. All ascomata of both species were heterozygous for at least one locus, as would be expected to result from outcrossing and not from selling. Therefore, it appears that even in the sorediate species, rare sexual reproduction results in recombination.

Index Descriptors: ascomycete; clonal reproduction; congruence of gene genealogies; heterozygosity; incongruence length difference/partition homogeneity test; Kishino–Hasegawa test; permutation tail probability test; phylogenetic species; sexual reproduction.

The effectiveness of sexual reproduction in creating genetic diversity through outcrossing and recombination in lichenized fungi has not been well documented at the genetic level, despite their production of sexual reproductive structures (ascomata) in nature. No lichenized fungus has yet proven to be amenable to mating experiments, nor has any physical evidence of recombination, such as microscopic observation of karyogamy and meiosis, been reported in these fungi. Given these difficulties, the most tractable approach to detecting recombination has been to analyze genetic variation present in field-collected specimens.

Lichenized fungi have advantages for field-based studies. Because they are symbiotic with algae, they need to be exposed to light and thus are easy to collect. Individuals are often obvious, defining the borders between potentially different haplotypes. Their reproductive structures are perennial and are easily collected for genetic analysis. One such study used lichen acids as markers and implicated recombination in the distribution of chemotypes in natural populations of the *Cladonia chlorophaea* lichen species complex (Culberson *et al.,* 1988). Two of the four chemotypes appeared to be interfertile where they cooccurred because of single maternal thallus of one chemotype produced ascospores that germinated *in vitro* into progeny of both chemotypes.

In this study, we investigated *Letharia* species for the presence of clonal or recombining populations. We selected this genus because it had been described as a species pair consisting of one sexual species and one asexual species (Poelt, 1970, 1972). In a previous study, we tested Poelt's taxonomic hypothesis by using the congruence of sequence-based loci to delimit phylogenetic species (Taylor *et al.,* 2000; Grube and Kroken, 2000) and found not just two, but six phylogenetic species that differ in their reproductive mor-

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phologies (Kroken and Taylor, 2001). The mature thalli of four of these species produce abundant ascomata and ascospores and would be expected to have recombined population structures if ascospores are the result of outcrossing. In contrast, the mature thalli of two of these species produce abundant soredia (symbiotic clonal propagules). However, these two sorediate species may not be strictly clonal because they also produce ascomata, albeit so rarely that they are difficult to find and collect in nature.

We looked for evidence of recombination in one putatively sexual species (provisionally named *L.* 'gracilis') and one putatively asexual species (*L.* 'lupina'). To do so, we used subsets of unmapped loci that were variable within each species. Sequence-based loci allowed us to compare gene genealogies within each species. Gene genealogies should be congruent in asexual species, in which all loci are effectively linked as one locus by clonal reproduction. In contrast, gene genealogies should be incongruent in sexual species, in which unlinked loci are recombined by independent assortment of chromosomes or by chromosomal crossing-over during sexual reproduction. We assessed congruence of gene genealogies using maximum-parsimony (MP)[2] analyses, the permutation tail probability (PTP) test, the ILD/PHT (incongruence length difference (Farris *et al.*, 1994); partition homogeneity test (Huelsenbeck *et al.*, 1996)) and the KH test (Kishino and Hasegawa, 1989). For both species, the data sets were consistent with recombination, regardless of scarcity of ascomata in the sorediate species.

Ascomata may be the result of outcrossing or the result of selfing. Selfing in haploid organisms produces meiospores that are clonal propagules (Anderson and Kohn, 1995). Therefore, to determine whether ascomata are the result of outcrossing and thus the likely site of observed recombination, we compared haplotypes of maternal thalli with the diplotypes of the ascomata that each thallus produced for evidence of heterozygosity. For both species, all comparisons showed the contribution of paternal haplotypes, consistent with outcrossing.

[2] Abbreviations used: MP, maximum-parsimony; PTP, permutation tail probability; ILD, incongruence length difference; PHT, partition homogeneity test; KH, Kishino–Hasegawa.

MATERIALS AND METHODS

Determination of Reproductive Mode

Thalli of *L.* 'gracilis' were collected in Kennedy Meadow Resort, Hwy. 128, 6 miles E of Sonora Pass, Tuolumne Co., California (37° 18′ N, 119° 45′ W), and thalli of *L.* 'lupina' were collected from California and British Columbia (Kroken and Taylor, 2001). DNA was extracted from whole lichens, and PCR and sequencing were performed with fungal-specific primers (Kroken and Taylor, 2001). The 12 loci characterized previously and used to recognize phylogenetic species were investigated to find informative loci within species. Two of these loci are known genes (*nrDNA* and *chs1* encoding chitin synthase I) and 10 of these loci are anonymous. The data set for *L.* 'gracilis' consists of eight individual thalli that were sequenced for 6 loci in Kroken and Taylor (2001) and for an additional 6 loci in this report. The data set for *L.* 'lupina' consists of nine individual thalli that were sequenced for the same 12 loci (Kroken and Taylor, 2001). The data sets for *Letharia* 'gracilis' and *L.* 'lupina' have been deposited in TreeBASE database (Submission No. SN376-1131). A representative sequence for each of the 12 loci has been deposited in GenBank database (Accession Nos. AF228459–471).

Reproductive mode was inferred by comparison of gene genealogies for congruence or incongruity. These comparisons were done by maximum-parsimony analyses and by applications of the permutation tail probability test, the incongruence length difference/partition homogeneity test, and the Kishino–Hasegawa test, using algorithms available in PAUP* 4.0b6. MP analyses were performed on each species with individual loci and all loci as data sets. Heuristic searches were performed with random addition, steepest descent, and gaps treated as new states. The sum of the number of steps present in the single-gene genealogies provides the minimum number of steps expected with clonality. This sum is compared to the number of steps observed in a combined analysis. The extra steps suggest homoplastic distribution of character states due to recombination (Burt *et al.*, 1996). The PTP test was used to compare the minimum and observed tree lengths of the combined data sets to a distribution of tree lengths based on 100,000 artificially recombined data sets, in which the alleles of each informative site had been sampled with replacement. The probability represents the number of randomly generated trees that are as short or shorter than the observed tree. A score of $P < 0.05$ is taken to indicate

significant structure in the data set, rejecting a null hypothesis of panmixia (Burt *et al.*, 1996). The ILD/PHT was designed to assess congruity among data sets. In our case, each locus was considered one data set and we assumed that the several loci would have congruent trees under clonality and incongruent trees under recombination. The test compares the sum of the most parsimonious tree lengths for the observed data to the distribution of sums of tree lengths of 10,000 data sets for which the parsimony-informative sites have been swapped among data sets. If the loci are congruent, swapping of the informative nucleotide positions among loci will have little or no effect (as the loci are effectively linked), and the artificial data sets will produce sums of tree lengths similar to those for the original data set. However, if the loci are incongruent, swapping of sites among loci will introduce homoplasy among loci and result in longer trees (note that in the ILD/PHT, shorter observed trees are consistent with recombination, whereas in the PTP test, shorter observed trees are consistent with clonality). A score of $P < 0.001$ (Cunningham, 1997) is taken to indicate significant conflict among loci, rejecting a null hypothesis of clonality (Taylor *et al.*, 1999a). The Kishino–Hasegawa test was designed to assess the fit of a data set to different topologies (Kishino and Hasegawa, 1989). In this context, the KH test was used to assess the congruity of pairwise combinations of loci by comparing the likelihoods of obtaining the observed data for each locus, given the most likely topology of the locus and that of the other locus. Each pairwise comparison was done symmetrically. Only pairs of loci with at least two informative characters each were compared. Likelihoods were calculated with both informative and uninformative characters. The substitution model used an estimated transition/transversion (Ti/Tv) rate, except for those loci with only transitions, in which case all rates were equal. Base frequencies were estimated with the Felsenstein two-parameter model variant for unequal base frequencies. The among-site variation was described by an estimated proportion of invariable sites and an estimated gamma distribution. The starting parameter values used the Rogers–Swofford method for starting branch lengths. A value of $P < 0.05$ is taken to indicate a significant difference in likelihoods, rejecting a null hypothesis of clonality.

Determination of Outcrossing

Individual lichen thalli with at least six ascomata were selected. For *L.* 'gracilis,' four thalli were investigated: B2, B3, B4, and B5, all from Baker campground, Hwy. 108, Stanislaus National Forest, California (37° 19′ N, 119° 45′ W). For *L.* 'lupina,' two thalli were investigated: N1, collected at the Niagra campground, Hwy. 108, Stanislaus National Forest, California (37° 19′ N, 119° 54′ W), and SH1, collected 3 km W of Panther Meadow campground, Everitt Memorial Hwy., Shasta Trinity National Forest, California (41° 24′ N, 122° 6′ W). For each thallus, six ascomata and the maternal thallus were extracted separately for DNA (Kroken and Taylor, 2001). Single ascomata were trimmed of their stipes and surrounding cilia to reduce the proportion of maternal haploid tissue. PCR amplifications were performed (Kroken and Taylor, 2001) with 4- to 32-fold dilutions of genomic DNA as template because ascomata contain higher levels of PCR inhibitors than vegetative thalli (Wolinski *et al.*, 1999). Younger ascomata as small as 2 mm in diameter gave DNA yields comparable to larger ascomata and contained lower levels of PCR inhibitors.

The loci that were found to be variable within *L.* 'gracilis' and *L.* 'lupina' were used to detect heterozygosity in individual ascomata. For each of these previously sequenced loci, the program DNA Strider 1.2 (Christian Marck, Gif-sur-Yvette, France) was used to find variable sites that lay in the recognition sequence of commercially available restriction enzymes. Restriction enzyme sites were selected to maximize the balance of allele frequencies and minimize the cost of the restriction enzyme. Restriction digests were performed with enzymes from New England Biolabs, with the exceptions of *Tai* I and *Eco57* I, both from MBI Fermentas. Each reaction contained 10 μl of PCR template, 3 μl of 10$\times$ buffer, 5 U of restriction enzyme, 100 μg/μl of bovine serum albumin and/or 1 μg/μl of S-adenosylmethionine if recommended, and distilled water to a total volume of 30 μl. Digests were incubated at the manufacturer's recommended temperature and time on a computer-controlled heating block to provide a constant temperature to avoid partial digestion, which could falsely indicate the presence of the uncut allele. Digests were loaded on agarose TAE minigels, electrophoresed at 80 V for 2 h, stained with EtBr for 30 min, destained with TAE for 30 min, and imaged with an Eagle Eye system (Stratagene). Digests were scored for the presence of the alternate allele in each ascoma compared to the allele found in the maternal thallus. The PCR amplifications for all loci of one ascoma were performed with the same PCR extract as template, so that the same relative proportions of maternal and paternal DNA were present for amplification and subsequent restriction en-

TABLE 1
Informative Nucleotide Sites for 11 Loci in Eight Individual Lichen Thalli of *L.* 'gracilis'

Locus	*ITS*	*11*	*13*	*DO*	*14*	*CT*	*BA*	*12*	*chs*	*4*	*CS*
Weight	22	22	7	22	22	2	3	4	7	11	7
Position	1	1	1 1 1	1	1	2 2 2 2 2 2 2 2 2 2	2 2 2 2 2 2 2	2 2 2 2 2	3 3 3	3 3	3 3 3
	0	0	4 6 7	9	9	0 0 1 2 3 3 3 3 3 3	5 6 6 6 7 7 7	8 8 8 8 8	1 2 2	3 4	5 7 7
	0	1	9 1 1	3	7	0 5 9 4 0 1 1 2 2 4	6 7 3 5 7 4 4	4 2 3 4 6	7 5 6	7 5	9 2 3
		7	8 7 3	5	6	3 5 6 0 6 2 5 1 4 0	2 2 3 9 1 4 6	7 2 5 0 5	1 2 7	6 9 8	9 8 7
	G	G	G T C	C	C	T C T T C T T A T C	G A G T C C T	G T A G C	G T C C	C G T	G A T
K1	·	·	A C T	T	·	· · · · · T · · · C	· · · · · · C	· - G A ·	· A · T	· C	· C C
K141	-	·	· · ·	·	·	· · · · C · C C G ·	G A G · C · T	· C · · ·	· · · ·	· C	· · ·
K148	-	·	A C T	T	·	C A C · T · · · C ·	· G · C · T · C	· · · T A	· · · ·	A ·	· · ·
B1	·	·	A C T	·	·	· · · · · · · C ·	· · · A · T · ·	· · · · ·	· T ·	A ·	A · ·
B2	·	·	· · ·	·	G	· · · · · · · C ·	· · · A · · · C	· - G A ·	· T ·	· ·	· C C
B3	·	A	· · ·	·	G	· · · · · · · C ·	· · · A · · · C	· · · · ·	· T ·	· ·	· · ·
B4	·	·	· · ·	T	·	C A C · T · · · C ·	· · · A · T · ·	· · · T A	· · ·	· C	A · ·
B5	·	A	· · ·	·	·	· · · · C · C C G ·	G A G · C · · A	· - G A ·	· A · T	A ·	· C C

Note. Dots represent no change from the most frequently occurring nucleotide for that position sequence. Dashes represent deletions.

zyme digest. This procedure allowed us to attribute the different diplotypes among the ascomata of one maternal thallus to different contributing paternal genotypes.

RESULTS AND DISCUSSION

Recombination in Letharia 'gracilis'

The gene genealogies of the variable loci found in *L.* 'gracilis' were investigated for congruity that would suggest clonality or for incongruity that would suggest recombination. *L.* 'gracilis' was known to be variable for 11 of 12 loci; no variation was found in locus *2*, so it was removed from the data set. Locus *12* has a history of intralocus recombination, so linkage partition *12a* was selected to represent this locus. Uninformative characters were removed (those that were invariant or that were present in only one individual thallus), leaving 41 informative characters in the initial data set. MP analyses of each single locus showed three homoplastic nucleotide positions in the single-gene genealogies of locus *CT* (2222), locus *BA* (2719), and locus *chs* (3212). These positions containing the homoplastic characters were removed from the alignment to insure that incongruity detected in the combined data could be attributed to recombination and not to nucleotide reversals (Burt *et al.*, 1996). To give each locus equal weight, the 38 remaining characters present among the 11 loci were weighted reciprocally to the total informative characters in each locus (Slowinsky and Page,

TABLE 2
Kishino–Hasegawa Test *P* Values for Pairwise Combinations of Informative Loci of *L.* 'gracilis'

	CT^{11}	BA^{7}	12^{5}	13^{3}	CS^{3}	chs^{3}	4^{2}
CT^{16}	—	**0.0004**[a]	**0.0003**	**0.01**	**0.0005**	**0.0003**	**0.006**
BA^{7}	**0.004**	—	**0.03**	**0.004**	0.3	**0.006**	**0.008**
12^{4}[b]	**0.03**	**0.03**	—	**0.03**	0.1	**0.04**	**0.04**
13^{3}	0.06	**0.04**	0.07	—	**0.03**	0.08	**0.03**
CS^{3}	**0.03**	0.2	0.2	**0.02**	—	**0.03**	**0.04**
chs^{3}	1.0	1.0	0.4	1.0	0.06	—	1.0
4^{2}	0.1	0.1	0.1	0.1	0.09	0.09	—

Note. For each pair, the locus listed in the first row is constrained by the topology of the locus listed in the first column. The number of informative sites is given in superscript after the locus name.

[a] Significant values ($P < 0.05$) are highlighted in boldface.

[b] Informative character at position 2822 was excluded, as maximum-likelihood does not accept gaps as character states.

TABLE 3
Informative Nucleotide Sites for Six Loci in Nine Individual Lichen Thalli of *L.* 'lupina'

Locus	*13*	*BA*	*12*	*chs*	*4*	*CS*
Weight	16	1	3	16	5	8
Position	1	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	2 2 2	3	3 3 3 3 3	3 3
	4	4 4 4 5 5 5 6 6 6 6 7 7 7 7 7 7	8 8 8	2	3 4 4 4 4	5 6
	9	3 6 8 1 3 7 5 7 7 7 1 4 4 4 4 8	2 3 4	1	5 4 5 6 9	3 8
	8	7 6 1 3 7 2 9 1 2 3 3 0 1 4 7 3	2 5 0	6	2 6 8 6 0	6 7
	G	G T A C C A T T A A A A G T C G A	- G A	G	G T C T T	A A
DP1	·	C A T T G G C C G C G T C T C G	T A G	·	A C T A A	· ·
BC1	A	· · · · · · · · · · · · · · · ·	· · ·	·	· · · · ·	G G
T1	·	· · · · · · · · · · · · · · · ·	T A G	·	· · · · ·	G G
SH1	·	· A T · G · · · · · · · · · · ·	· · ·	·	· · T · A	· ·
ST2	·	C A T T G · · C G C G T · · · G	· · ·	·	A C T A A	· ·
T4	·	· · · · · · · · · · · · · · · ·	· · ·	A	· · · · ·	G G
T23a	·	· · · · · · · · · · · · · · · ·	T A G	·	· · · · ·	· ·
T23b	A	C A T T G G C C G C G T C T C G	· · ·	A	· · T ·A	G G
TB1	·	· · · · · · · · · · · · · · · ·	T A G	·	· · · · ·	· ·

Note. Dots represent no change from the most frequently occurring nucleotide for that position sequence. Dashes represent deletions.

1999), with approximate whole integer numbers as required by PAUP (Table 1). For example, the one informative character of locus *11* was weighted with a value of 22, and the 11 informative characters of locus *CT* were each weighted with a value of 2.

MP analyses of the 11 individual loci gave their gene genealogies (each with a CI = 1.0), which had a sum of 239 steps. MP analysis of the combined data gave a most parsimonious tree with 442 steps (RC = 0.36), 203 steps more than the sum of the 11 single-gene genealogies, suggesting recombination. To assess the statistical significance of this incongruence of gene genealogies, we analyzed the data set with the PTP test, the ILD/PHT, and the KH test.

MP analysis of the 100,000 randomized data sets produced by the PTP test resulted in trees ranging from 382 to 503 steps. The observed tree length (442 steps) fell within the range of tree lengths for randomized data sets ($P = 0.245$), not allowing us to reject the null hypothesis of panmixia. The application of the PTP test was the only combined-data analysis whose scores differ for unweighted and equally weighted loci. With unweighted loci, MP analyses of randomized data sets generated trees ranging from 68 to 82 steps. The observed tree length (68 steps) fell at the lower tail of tree lengths for randomized data sets ($P = 0.00002$), suggesting that the structure observed in the unweighted data set was not due to congruity among loci, but to character-heavy loci such as *CT* and *BA*.

MP analysis of the 10,000 randomized data sets produced by the ILD/PHT resulted in trees ranging from 280 to 383 steps, all longer than the sum of the original tree lengths for each locus (239 steps), allowing us to reject the null hypothesis of clonality ($P < 0.0001$). This test may spuriously reject homogeneity among loci if high levels of homoplasy are present within loci ($\alpha \cong 0.01$; Barker and Lutzoni, 2000), particularly when levels of homoplasy vary among loci due to processes such as rate heterogeneity (Sullivan, 1996; Cunningham, 1997). However, the loci used here had no homoplasy that could cause a false rejection of the null hypothesis.

The KH test was used to determine which pairs of loci are in significant conflict. For each locus, the like-

TABLE 4
Kishino–Hasegawa Test *P* Values for Pairwise Combinations of Informative Loci of *L.* 'lupina'

	BA^{16}	4^4	12^3	CS^2
BA^{16}	—	**0.04**[a]	**0.0001**	**0.0001**
4^4	**0.03**	—	**0.005**	0.08
$12^{2\,b}$	0.15	0.15	—	0.15
CS^2	0.06	0.06	0.06	—

Note. For each pair, the locus listed in the first row is constrained by the topology of the locus listed in the first column. The number of informative sites is given in superscript after the locus name.

[a] Significant values ($P < 0.05$) are highlighted in boldface.

[b] Informative character at position 2822 was excluded, as maximum-likelihood does not accept gaps as character states.

TABLE 5
Restriction Enzyme Sites for Variable Loci in *L.* 'gracilis'

Locus	RE	RE site +	RE site −	Size +	Size −
SSU[1]	*Tai* I	acgt/	c[3] (785)	252 + 216	428
chs	*Alw* I	ggatc(n)$_4$/	c (3267)	339 + 61	400
4	*Eco*57 I	ctgaag(n)$_{16}$/	a (3379)	109 + 131	240
11	*Tsp*509 I	/aatt	g (1017)	95 + 319	414
12[2]	*Afl* III	a/crygt	a (2936)	155 + 35	180
13	*Nci* I	cc/sgg	a (1596)	135 + 232	367
14	*Dde* I	c/tnag	c (1976)	65 + 132	197
BA	*Hga* I	gacgc(n)$_5$/	t (2659)	239 + 176	408
CS	*Hha* I	gcg/c	t (3737)	227 + 142	379
CT	*Bbs* I	gaagac(n)$_2$/	g (2222)	245 + 290	515
DO	*Dde* I	c/tnag	c (1935)	170 + 63	233

[1] *SSU rDNA intron* used as ribosomal locus in *L.* 'gracilis.'
[2] Linkage partition *12b* was selected to represent locus *12.*
[3] Underlined nucleotide in the RE site is biallelic, and the number in parentheses is the position of that site as ordered in the 12-locus, 3846-nucleotide data set.

lihood of the best tree was compared to the resulting likelihood when constrained by the topology of each other locus in turn (Table 2). All 21 pairs of loci compared were incongruent (17 of them significantly so) and represent incompatibility, as evidenced by the existence of all four pairwise combinations of alleles at the pairs of biallelic loci (Anderson and Kohn, 1998). Frequently, the incongruity was significant for only one of the two KH tests performed on each pair of loci, due to one locus having more informative nucleotide positions than the other. However, if just one of the two tests shows significant conflict, we consider the loci to be incongruent. The results of the KH test highlight which pairs of loci contribute to the incongruity noted in the analyses in which the data is combined, such as MP, the PTP test, and the ILD/PHT. Together, these analyses suggest that recombination has occurred to produce the reticulate evolutionary history of these eight individual thalli collected from one population in Kennedy Meadow, California.

TABLE 6
Restriction Enzyme Sites for Variable Loci in *L.* 'lupina'

Locus	RE	Re site +	RE site −	Size +	Size −
ITS[1]	*Sac* II	ccgc/gc	c (37)	38 + 278	316
chs	*Fok* I	ggatg(n)$_9$/	c (3087)	164 + 191	355
4	*Tsp*509 I	/aatt	a (3458)	37 + 44	81
11	[2]				
12[3]	*Afl* III	a/crygt	a (2936)	155 + 35	180
13	*Taq* I	t/cga	a (1499)	38 + 105	142
14	[2]				
BA	*Nde* I	ca/tatg	a (2481)	58 + 376	434
CS	*Ear* I	/(n)$_1$gaagag	a (3536)	49 + 330	379
CT	*Dpn* II	/gatc	c (2379)	28 + 46	74
DO	[2]				

[1] *ITS1F/2* used as ribosomal locus in *L.* 'lupina.'
[2] Locus is invariant in *L.* 'lupina.'
[3] Linkage partition *12b* was selected to represent locus *12.*
[4] Underlined nucleotide in the RE site is biallelic, and the number in parentheses is the position of that site as ordered in the 12-locus, 3846-nucleotide data set.

TABLE 7
Allele in Maternal Thallus and Contributing Paternal Allele in Each Ascoma of *L.* 'gracilis'

	Locus										
	SSU	*chs*	*4*	*11*	*12*	*13*	*14*	*BA*	*CS*	*CT*	*DO*
B2 maternal	+	+	+	−	+	+	+	+	+	+	−
B2-1 paternal		−			−	−			−	−	
B2-2 paternal		−			−				−	−	
B2-3 paternal		−			−				−	−	
B2-4 paternal		−							−	−	
B2-5 paternal		−							−	−	
B2-6 paternal		−							−	−	
B-3 maternal	+	+	+	−	−	−	+	+	−	+	−
B3-1 paternal		−		+						−	
B3-2 paternal				+	+						
B3-3 paternal				+	+						
B3-4 paternal				+	+						
B3-5 paternal		−		+	+					−	
B3-6 paternal				+	+						
B4 maternal	+	−	+	−	−	+	−	+	−	−	+
B4-1 paternal											−
B4-2 paternal								−			−
B4-3 paternal								−	+		
B4-4 paternal											−
B4-5 paternal											−
B4-6 paternal					+			−	+		
B5 maternal	+	−	−	+	+	+	−	−	+	+	−
B5-1 paternal	−			−	−				−	−	
B5-2 paternal	−			−	−				−	−	
B5-3 paternal					−	−			−	−	
B5-4 paternal	−			−	−				−	−	
B5-5 paternal	−			−	−				−	−	
B5-6 paternal	−			−	−				−	−	

Recombination in Putatively Clonal Letharia 'lupina'

Only 8 of the 12 loci for *L.* 'lupina' were known to be variable; no variation was found in loci *2, 11, 14,* and *DO.* Two of these 8 variable loci (*CT* and *nrDNA*) had one unique allele present in only one individual. Therefore, only the 6 informative loci were used in this analysis. The uninformative characters were removed from these 6 loci. MP analyses showed no homoplasy in their single-gene genealogies (CI = 1.0). The resulting nine-individual data set is composed of 28 informative characters, which were weighted inversely to the number of informative characters present in that locus (Table 3).

These six single-gene genealogies had a sum of 94 steps, whereas combined MP analysis produced a longer most parsimonious tree with 162 steps, a result consistent with recombination among these six loci (RC = 0.36). A distribution of tree lengths produced by the PTP test from 100,000 randomized data sets ranged from 148 to 216 steps. The observed tree length (162 steps) fell within the low tail end of this range of randomized data sets ($P = 0.0054$), suggesting that, even with equally weighted loci, there is more structure in the data set than would be found with panmixia. This observed structure could be due to the fact that these individuals were collected from a wide geographic area, within which there might be several populations with reduced gene flow among them. MP analysis of the 10,000 randomized data sets produced by the ILD/PHT resulted in trees ranging from 104 to 155 steps, all longer than the sum of the original tree lengths for each locus (94 steps), allowing us to reject the null hypothesis of clonality ($P < 0.0001$). Each of six pairs of loci compared by the KH test were incongruent, four of which could be scored as significantly incongruent in at least one direction of comparison ($P < 0.05$; Table 4).

These analyses suggest that recombination has occurred to produce the reticulate evolutionary history of the nine *L.* 'lupina' individuals collected from California to British Columbia. We propose that long-distance dispersal of clonal soredia and their establishment and rare develop-

TABLE 8
Allele in Maternal Thallus and Contributing Paternal Allele in Each Ascoma of *L.* 'lupina'

	Locus							
	ITS	*chs*	*4*	*12*	*13*	*BA*	*CS*	*CT*
N1 maternal	−	+	+	−	+	−	+	−
N1-1 paternal							−	
N1-2 paternal							−	
N1-3 paternal							−	
N1-4 paternal							−	
N1-5 paternal							−	
N1-6 paternal							−	
SH1 maternal	+	−	+	−	+	−	−	−
SH1-1 paternal	−	+		+				
SH1-2 paternal	−	+		+				
SH1-3 paternal	−	+		+				
SH1-4 paternal	−	+		+				
SH1-5 paternal	−	+		+				
SH1-6 paternal		+		+				

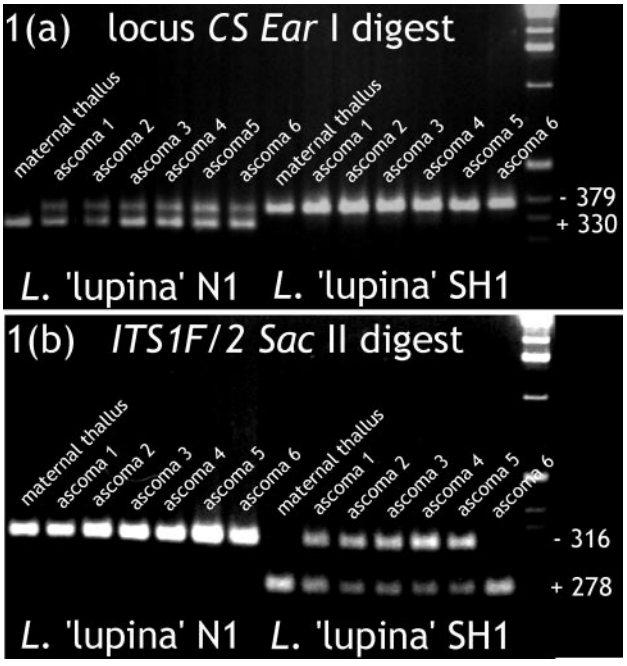

FIG. 1. (a) *Ear* I restriction enzyme digest of locus *CS* for two maternal thalli of *L.* 'lupina' and six ascomata of each thallus. The digests were electrophoresed with the allele from the maternal thallus in the first lane and the allele(s) from the six ascomata in the next six lanes. All six ascomata of thallus N1 were heterozygous, indicated by the presence of the cut allele from the maternal haplotype and the uncut allele from the fertilizing paternal haplotype. In contrast, no ascomata of SH1 were heterozygous for locus *CS,* as only the uncut allele was present, a result that emphasizes the need to survey multiple loci to determine selfing, cf. outcrossing. Outcrossing for five of the six loci from thallus SH1 was demonstrated by locus *ITS1F/2.* (b) *Sac* II restriction enzyme digest of locus *ITS1F/2* of the same maternal thalli and ascomata as in (a). No ascomata of thallus N1 were heterozygous for this locus, as only the uncut allele was present. In contrast, five of the six ascomata of thallus SH1 are heterozygous, indicated by the presence of the uncut allele from the maternal haplotype and the cut allele from the fertilizing paternal haplotype. The sixth ascoma does not have the uncut allele, showing that it was fertilized by a paternal haplotype different from that of the other five ascomata. Outcrossing for the sixth ascoma was supported by heterozygosity at locus *12* and locus *chs* (Table 4).

ment into sexually reproductive thalli has resulted in gene flow and recombination among the North American populations investigated here.

For both *Letharia* species, incongruence among gene genealogies provided evidence for recombination, but not about the frequency of recombination, how recently it has occurred, or whether it occurs by sexual or parasexual means (Anderson and Kohn, 1998). Our finding of recombination by comparison of gene genealogies is similar to those demonstrating recombination in bacteria (Dykhuizen and Green, 1991) in putatively clonal species of fungi where ascomata have never been observed (*Coccidioides immitis,* Koufopanou *et al.,* 1997, 1998; *Aspergillus flavus,* Geiser *et al.,* 1998) and in fungi that are known to produce ascomata (*Sclerotinia sclerotiorum,* Carbone *et al.,* 1999). The presence of ascomata in both *Letharia* species suggests the simple explanation that we are detecting current recombination resulting from sexual reproduction and not just the footprint of ancient recombination in currently clonal species (Taylor *et al.,* 1999a).

Determination of Outcrossing in Letharia

To test the idea that ascomata are the site of recombination, we used restriction enzyme digests to characterize the alleles at all variable loci for individual ascomata and the maternal thallus of both *L.* 'gracilis' and *L.* 'lupina.' Finding two alleles at any of the variable loci among DNA extracts taken from single ascomata was taken as evidence that outcrossing was occurring. Each paternal haplotype was deduced by comparison of the ascomatal diplotype and the maternal haplotype. Four sexually mature thalli of *L.* 'gracilis' and two sexually mature thalli of *L.* 'lupina' were investigated. Six ascomata were studied from each thallus, for a total of 36 ascomata. For each thallus of *L.* 'gracilis' and its ascomata, digests were performed on 11 variable loci (Table 5). For each thallus of *L.* 'lupina' and its ascomata, digests were performed on 8 variable loci (Table 6).

All 24 ascomata of *L.* 'gracilis' investigated were heterozygous for at least one locus and at most five loci (Table 7). Similarly, all 12 ascomata of *L.* 'lupina' investigated were heterozygous for at least one locus and at most three loci (Table 8), suggesting that all ascomata are the result of outcrossing. Two examples of loci that showed outcrossing in *L.* 'lupina' are shown in Figs. 1a and 1b. Each of the six maternal thalli appeared to be fertilized by one common paternal haplotype, presuming that the eight loci used are sufficient to distinguish different haplotypes. In one thallus (*L.* 'lupina' N1; Table 8), all 6 ascomata had the same paternal haplotype. In thallus SH1 of *L.* 'lupina,' and in each of the four thalli of *L.* 'gracilis,' we detected at least one additional paternal haplotype.

In lichenized fungi, ascomata result from fertilization of a maternal receptive hypha (trichogyne) by windborne spermatia produced in the spermagonia found on mature hermaphroditic thalli (Bellemére and Letrouit-Galinou, 1988). Our results suggest that fertilization of a single thallus primarily is due to spermatial rain from a single paternal haplotype that is different from the maternal haplotype. However, our data indicate that more than one paternal haplotype can fertilize a thallus and suggest that each ascoma is the result of a separate fertilization event.

The finding of outcrossing suggests that ascomata are the site of recombination and thus provide the best explanation for the recombined population structures observed in both species. We presume that *Letharia* and other lichenized ascomycete fungi that outcross have the mating type gene *MAT* that has been shown to prevent selfing in many other classes of ascomycete fungi (Glass and Lorimer, 1991; Turgeon, 1998). Both *Letharia* species outcross to produce recombined, sexual progeny and reproduce by clonal means. All adult thalli of *L*. 'gracilis' produce ascomata and isidia (large branch-like clonal propagules). In contrast, all adult thalli of *L*. 'lupina' produce abundant soredia, but thalli that produce ascomata are exceedingly rare. However, we cannot say much about the relative contributions of sexual and clonal reproduction in these two lichenized fungi, because a few recombination events in a population can result in mixis (Burt *et al.*, 1996).

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