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IDENTITY AND GENETIC STRUCTURE OF THE PHOTOBIONT OF THE EPIPHYTIC LICHEN *RAMALINA MENZIESII* ON THREE OAK SPECIES IN SOUTHERN CALIFORNIA[1]

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Lichens, a classic example of an obligate symbiosis between fungi and photobionts (which could be algae or cyanobacteria), are abundant in many terrestrial ecosystems. The genetic structure of the photobiont population found in association with a lichen-forming fungal species could be affected by fungal reproductive mode and by the spatial extent of gene flow in the photobiont. Using DNA sequences from one nuclear ribosomal and two chloroplast loci, we analyzed the genetic structure of the photobiont associated with the fungus *Ramalina menziesii* at an oak woodland study site in southern California. We had previously shown that the fungus exhibited no genetic structure among four local sites or three phorophyte species. Our goals were to identify the photobiont species and assess its genetic structure. We found that *R. menziesii* was highly specific in its photobiont choice and associated with one alga, *Trebouxia decolorans.* In contrast to the fungal population, we found significant differentiation among the algae sampled on three oak species and little genetic structure among the sites for two of the three algal loci. We hypothesize that *R. menziesii* is locally adapted to the phorophyte species through habitat specialization in the algal partner of the symbiosis.

Key words: genetic structure; horizontal transmission; lichen-forming fungi; mycobiont; phorophyte; photobiont; population genetics; *Quercus*; *Ramalina menziesii*; symbiosis; *Trebouxia decolorans*.

Lichens represent a fascinating symbiotic association because two species—a lichen-forming fungus (mycobiont) and an alga and/or a cyanobacterium (photobiont)—unite to become a combined organism (Ahmadjian, 1993a). The fungus cannot survive long if it does not associate with a photobiont (Ahmadjian, 1993b), but, in some cases, the photobiont species may be free-living until incorporated by a fungus (Mukhtar et al., 1994). Presumably, this relationship is mutually beneficial in that the fungus gains photosynthetic products and the algae gain protection. However, some evidence suggests that the fungus "cultivates" the photobiont, which may receive no benefit from or even may be parasitized by its mycobiont (Richardson, 1999; Hyvärinen et al., 2002; Lücking et al., 2009). Moreover, in some cases, the lichen, which is comprised of only one lichen-forming fungal species, may be associated with more than one species of photobiont (Kroken and Taylor, 2000; Romeike et al., 2002; Stenroos et al., 2003). Regardless of whether this symbiosis is a mutualism, commensalism, or parasitism, one indicator of how tightly coupled they are will be their comparative genetic structures.

For the photobiont population, the genetic structure will be strongly influenced by the manner in which photobionts are transmitted to the fungus. Vertical transmission occurs when the photobiont disperses as part of the vegetative propagule of the lichen. This mechanism has been hypothesized for exclusively asexual lichen fungi (Cassie and Piercey-Normore, 2008) and also for lichen-forming fungi with a predominantly asexual mode of reproduction, but occasional outcrossing (one example would be the predominantly clonal *Lobaria pulmonaria*, see e.g., Wagner et al., 2005; Werth et al., 2006). In these studies, the exclusively or predominantly clonal dispersal of the fungus provides evidence for codispersal of the photobiont, implicating a vertical transmission mode. However, horizontal transmission of algae has also been shown in asexual (e.g., Nelsen and Gargas, 2008) or nearly asexual (Piercey-Normore, 2006) lichen fungi. In cases of gene flow in the photobiont that occurs primarily through vertical transmission, we would expect tightly matching genetic structures in both partners of the symbiosis.

Horizontal transmission of the photobiont to the mycobiont occurs when the fungus incorporates new photobionts by a germinating spore or vegetative structure. In sexual fungal species, the photobionts are hypothesized to be horizontally transmitted to the fungus (Honegger et al., 2004). Horizontal photobiont

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exchange has also been shown in vegetatively dispersed species (see Nelsen and Gargas, 2008). In fact, re-lichenization involving horizontal transmission of photobionts may be common (Beck et al., 2002; Sanders and Lücking, 2002; Sanders, 2005; Nelsen and Gargas, 2008; Werth and Sork, 2008), and this form of gene movement could uncouple fungal and algal genetic structures.

Perhaps a more critical factor shaping the genetic structure of photobionts is the ability of the photobiont to move within and among sites through gene flow. First, while embedded in the lichen thallus, green-algal photobionts involved in a lichen symbiosis do not usually disperse, asexually or sexually, from the thallus (Nash, 1996), which implies that their ability to move around is very restricted. Some studies have suggested that algae may occasionally get released from a thallus as it gets damaged by herbivores, and if conditions are moist enough for the algae to form zoospores, which are mobile, flagellate asexual cells that can move around locally within trees or other substrates occupied by the "mother" thallus (Friedl and Büdel, 1996; Richardson, 1999). This restricted dispersal ability would imply that the photobiont populations might exhibit considerable genetic structure among sites. However, many green-algal photobionts also occur in free-living populations on soil, rocks, or tree stems (Mukhtar et al., 1994; Friedl and Büdel, 1996), and even unlichenized airborne cells of *Trebouxia* spp. have been found (Handa et al., 2007), suggesting the potential for movement among sites, possibly even over large distances. Thus, if the photobiont is restricted to the symbiotic, lichenized phenotype, we would expect extensive genetic structure, but if it is also free-living with the potential for aerial, long-distance dispersal, then we would expect very little genetic structure within a site.

Finally, the genetic structure of the photobiont species could be shaped by other evolutionary forces. Specifically, if the photobiont species is adapted to a particular habitat, then dispersal may not result always in successful establishment in a different habitat. The opportunity for habitat specialization will be greatest if gene flow of the photobiont is restricted either because it does not possess dispersal capability on its own or if photobiont transmission occurs vertically and if the dispersal range of vegetative propagules is restricted. Genetic differentiation of green-algal photobionts associated with habitat has been found elsewhere (Yahr et al., 2006).

The combined impacts of fungal dispersal, algal transmission to fungi, algal dispersal, and algal phorophyte specialization make it difficult to predict the extent and patterns of genetic structure in photobiont populations. Only two studies have compared the genetic structure of a lichen-forming fungal species with that of its green-algal photobionts (Piercey-Normore, 2006; Yahr et al., 2006). One study contrasted the regional genetic structure of the lichen-forming fungus *Cladonia subtenuis* with that of its photobiont and found that the patterns of fungi and algae differed dramatically (Yahr et al., 2006). The algae showed strong geographic associations of haplotypes and high levels of genetic differentiation among populations, whereas the fungus exhibited far less regional genetic structure (Yahr et al., 2006). A second study compared the local genetic structure of *Evernia mesomorpha* with that of its green algal photobiont (*Trebouxia jamesii*). In this study, genetic structure depended on the spatial scale: no fungal genetic structure was found among phorophyte trees, but at that same level, there was significant structure in the photobiont (Piercey-Normore, 2006). However, at larger spatial scales (e.g., transects), genetic differentiation was low, which the author attributed to high gene flow. Results of these two studies suggest that gene flow may be more restricted in the green-algal photobiont populations than in their mycobiont species, but genetic differentiation may also be scale-dependent.

The question we want to explore here is the extent to which the genetic structure of a photobiont population reflects the genetic structure of the mycobiont population. The specific example is the photobiont associated with a widespread and abundant lichen-forming fungus of western North America, *Ramalina menziesii* Tayl., in a single site in the center of its distribution. Previously, we showed that the fungal species exhibited a complete lack of genetic structure among locations less than 2 km apart within a single California oak savanna study site and no tendency for genetic differentiation among oak phorophyte species (Werth and Sork, 2008). In the present study, we first wanted to identify the photobiont species for *Ramalina menziesii* and then evaluated the genetic structure using DNA sequence data from two chloroplast and one nuclear ribosomal genes. Specifically, we examined whether gene flow is extensive or restricted among the local sites for the photobiont population associated with *R. menziesii* and then tested for genetic differentiation among photobiont populations on three tree phorophyte species: *Quercus agrifolia*, *Q. douglasii*, and *Quercus lobata*. These species differ in habitat quality because the first is evergreen and the latter two are deciduous. The fact that *R. menziesii* occurs more abundantly on the latter two in the study site indicates that the environments might differ in quality for the lichen. This research not only sheds light on the nature of gene flow and phorophyte specialization in the photobiont, but it also provides the first stage of information on the nature of the association of these two taxa.

MATERIALS AND METHODS

Study area and sampling—Our study site is located in Sedgwick Reserve, which is part of the University of California Natural Reserve System. This 2388-ha reserve is located in the Santa Ynez Valley, 56 km north of the city of Santa Barbara in California. The predominant habitats are oak savanna or oak woodland characterized by three oak species, California valley oak (*Quercus lobata*), blue oak (*Q. douglasii*), and coastal live oak (*Q. agrifolia*). Our study populations were found in oak woodland habitat at an elevation of 300–350 m a.s.l.

Within each site, we collected lichen thalli in different parts of the canopy from two trees each of the three oak species (*Quercus lobata*, *Q. douglasii*, *Q. agrifolia*). The total sample size was 72, and within each site, 18 samples were collected—six from each *Quercus* species. This design was repeated in four sites located at distances up to 2 km within a southern Californian oak savanna landscape. The study area and sampling design have been described elsewhere in detail (Werth and Sork, 2008). The entire sample was considered as one population for the purpose of our analyses, and sites were regarded as subpopulations. This design enabled detection of genetic structure at the phorophyte species, site, and individual tree level. However, the present sampling would not allow an evaluation of fine-scale genetic structure within phorophyte trees (e.g., the spatial distribution of photobiont haplotypes within branches).

Laboratory procedures—We performed DNA extraction, PCR, and sequencing as described in Werth and Sork (2008). Three algal loci were amplified from total genomic DNA of the lichen thallus using algal-specific primers: two loci on the chloroplast (cp) DNA and one nuclear ribosomal locus. We chose to investigate loci on the nuclear and chloroplast genomes because they are inherited independently, and we wanted to compare several independently evolving loci. Two chloroplast genes were studied, including a protein-coding gene involved in photosynthesis (*rbcL*), which was assumed to be under balancing selection, and an intergenic spacer region (*psbJ-L*).

To amplify the cpDNA locus *psbJ-L*, we used primers psbF (5′-GTW GTW CCA GTA TTR GAC AT-3′) and psbR (5′-AAC CRA ATC CAN AYA AAC AA-3′) (Provan et al., 2004). Our locus-specific annealing temperature for *psbJ-L* was 50°C. For the cpDNA gene *rbcL*, we used the primers rbcL.fwd (5′GAM ACT GAT ATT CTT CTT GCA GC-3′) and rbcL.rev (5′-GCA GCT AAT TCA GGA CTC CA-3′) and an annealing temperature of 52°C; the primer rbcL1.fwd (5′-CGT GGT GGT TTA GAT TTT AC-3′) was used as sequencing primer (S. Nyati, S. Scherrer, R. Honegger, University of Zürich, unpublished manuscript). To sequence the algal nrDNA *ITS* region, we used primers ITS1.T (5′-GGA AGG ATC ATT GAA TCT ATC GT-3′) and ITS4.T (5′-GGT TCG CTC GCC GCT ACT A-3′) at an annealing temperature of 56°C (Kroken and Taylor, 2000). DNA sequences were edited and aligned as described in Werth and Sork (2008). Alignments were visually inspected and modified manually, if required for consistency of, e.g., gap opening sites. Haplotypes were mapped using the program SnapWorkbench (Price and Carbone, 2005; Aylor et al., 2006). Sequences were deposited in GenBank (accessions FJ705175–FJ705216, FJ716587–FJ716614; see Appendix 1)

Data analysis—Species of *Trebouxia* such as the photobiont of *Ramalina menziesii* are morphospecies—species identified exclusively based on their morphology, which is usually assessed through axenic cultures and light microscopy (Beck, 1999; Beck et al., 2002). However, from DNA sequences, species identity can be inferred indirectly by performing BLAST searches in GenBank (Altschul et al., 1997) and comparing the sequence of an unknown photobiont with the sequences of species deposited in GenBank; completely or very closely matching sequences indicate identical species. Therefore, we performed a BLAST search and included the species of *Trebouxia* closest to our sequences, as well as some outgroup species (Table 1).

To investigate the taxonomic range of the photobiont of *R. menziesii* at our study site, we constructed phylogenetic trees of the ITS and *rbcL* haplotypes, where available, including type specimens and reference material from culture collections to infer the identity of the photobiont. All phylogenetic analyses are based on alignments from which ambiguous sites and gaps were removed. To assess whether the two data sets ITS and *rbcL* could be combined in a single phylogenetic analysis, we ran an incongruence-length difference test (Farris et al., 1995) in the program PAUP* version 4 (Swofford, 1998). Because the two loci were compatible, trees were constructed using the combined data. First, we performed a Bayesian tree search using the program MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). We ran PAUP* version 4.0 beta (Swofford, 1998) to generate model scores and used the program MrModeltest version 2.2 (Posada and Crandall, 1998; Nylander, 2004) to infer the appropriate substitution model. The best model according to the Akaike information criterion (AIC) was selected for the analyses (GTR+I+G). The program Tracer version 1.4.1 (Rambaut and Drummond, 2007) was used to assess whether runs had reached convergence, as indicated by effective sample sizes >>100 for each parameter.

TABLE 1. Species of *Trebouxia* analyzed, strain number, GenBank accessions.

Species	Isolate	Isolated from species	Accession [a]
T. arboricola	SAG-219-1a	Unknown	FJ626725*, AM158960
T. arboricola	P-360-IIa	*Xanthoria* sp.	AJ969665, AJ969609
T. arboricola	P-3-I	*Xanthoria ligulata*	AJ969669, AJ969509
T. arboricola	P-53-Ia	*Xanthoria ligulata*	AJ969670, AJ969528
T. arboricola	P-7-Ia	*Xanthoria parietina*	AJ969646, AJ969512
T. decolorans	P-97-Ia	*Xanthoria ectaneoides*	AM158965, AJ969539
T. decolorans	P-120a-IIIb	*Xanthoria parietina*	AM158966, AJ969545
T. decolorans	P-121-IIcd	*Xanthoria parietina*	AM158967, AJ969550
T. decolorans	P-319-Ig	*Xanthoria parietina*	AM159504, AM159503
T. decolorans	P-320-IIb	*Xanthoria parietina*	AM158964, AJ969600
T. decolorans	P-320-IIf	*Xanthoria parietina*	AM158963, AJ969603
T. gelatinosa	P-270-Ia	*Teloschistes chrysopthalmus*	AJ969640, AJ969579
T. gelatinosa	P-57-Ia	*Xanthoria* sp.	AJ969642, AJ969532
T. potteri	P-330-Ib	*Xanthoria* sp.	AJ969665, AJ969609

[a] All sequences are from S. Nyati, S. Scherrer, and R. Honegger, University of Zürich, Switzerland; unpublished data, with the exception of one sequence marked by an asterisk (E. M. Del Campo, L. M. Casano, A. Del Hoyo, F. Gasulla, and E. Barreno, Universidad de Alcala, Madrid, Spain; unpublished data).

Four chains were run in parallel with a generation number of 5 000 000 in MrBayes, sampling trees every 100th generation. Of the 50 000 trees sampled, the first 5000 were discarded (burn in). Second, we performed maximum likelihood analysis in PAUP* using the best model inferred by the AIC. We used 10 replicates and 100 bootstraps in a heuristic search and constructed a majority-rule consensus tree. The trees were rooted on the midpoint and visualized in the program FigTree version 1.2.2 (Rambaut, 2008).

To provide background data on the genetic diversity of our loci, we calculated the number of polymorphic sites *s*, the number of haplotypes *H*, haplotype diversity H_d, and nucleotide diversity π in the program ARLEQUIN version 3.0 (Excoffier et al., 2005).

To analyze the genetic structure, we performed an analysis of molecular variance for each locus. Two analyses were performed—first, an analysis with ARLEQUIN yielding *F*-statistics. The estimator of genetic differentiation, F_{ST}, took into account the number of mutational steps between haplotypes (Schneider et al., 2000). This implies that F_{ST} would be greater in cases where haplotypes, which are restricted to different groups of populations, are many steps apart from each other. Significance of F_{ST} was calculated using 1000 permutations. Secondly, we calculated θ in the program TFPGA version 1.3 (Miller, 1997). This measure of genetic differentiation, θ, is based exclusively on the degree of haplotype sharing among subpopulations, irrespective of the genetic distance among haplotypes. Thus, in cases where groups of populations differ by genetically distant haplotypes, F_{ST} would be greater than θ. Slightly negative values may occur in variance components in absence of genetic structure, because the true value of the parameter is zero (ARLEQUIN homepage, FAQs; http://anthro.unige.ch/software/arlequin/software/2.000/doc/faq/faqlist.htm). Bootstraps were performed across loci to assess the significance of the overall θ, but this statistical package does not allow determination of the significance of θ for individual loci. Both analyses were performed for each locus separately, calculating the structure for phorophytes and sites.

RESULTS

Photobiont identity and diversity—In our study area, a single photobiont species was found in association with *R. menziesii*. Our BLAST searches and phylogenetic analysis of two independently evolving genes showed that this species was the green alga *Trebouxia decolorans* Ahmadjian (Fig. 1). We could not include the third locus, the *psbJ-L* intergenic spacer, in the phylogenetic analysis or use it to determine the photobiont species because this locus has rarely been used to study green algae; the relevant taxa (e.g., *T. arboricola*, *T. decolorans*) are not available from GenBank to date.

The diversity of the *T. decolorans* population was high. We found a high number of algal haplotypes for the three genes, with the fewest at *rbcL* (Table 2). The frequency of the most frequent algal haplotype was rather similar across sites, but it differed among phorophyte species (Table 3). The two genes that were not protein coding, the nuclear ribosomal ITS and chloroplast intergenic spacer *psbJ-L*, showed very high levels of nucleotide diversity (Table 2). In contrast, the protein-coding chloroplast gene *rbcL* exhibited much less variability. We did not find any trends of haplotype or nucleotide diversity across sites (results not shown).

Genetic differentiation according to phorophyte species—We found high and significant differentiation among populations of *T. decolorans* collected from different phorophytes, *Quercus lobata*, *Q. douglasii*, and *Q. agrifolia* (Table 4). This differentiation was detected using both the F_{ST} approach (0.060–0.169) for individual loci and the θ approach, which yielded a significant overall θ (0.0360).

Genetic differentiation among sites—For the ITS region, but not for the other two loci studied, we found significant genetic differentiation among subpopulations of the photobiont collected from different sites if our analysis took into account

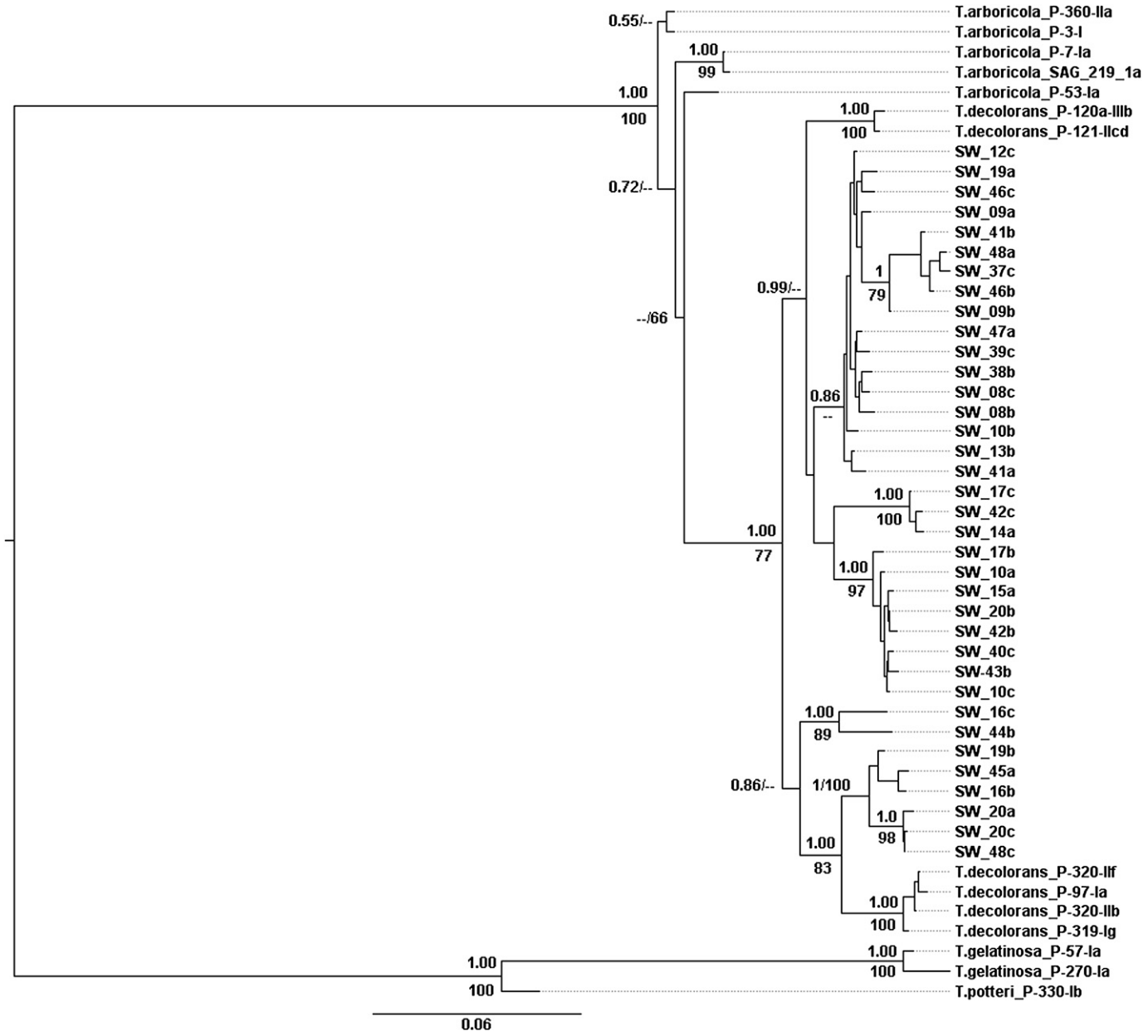

Fig. 1. Phylogenetic tree of the nuclear ITS region and the chloroplast *rbcL* gene, showing the species identity in photobionts of *Ramalina menziesii*. Photobionts associated with *R. menziesii* are labeled with "SW_". Trees were constructed excluding gaps and ambiguous sites. Support values indicate posterior probabilities from Bayesian analysis and bootstrap values from maximum likelihood analysis.

the genetic distance among haplotypes (F_{ST} = 0.047, Table 4). The other two loci provided no evidence of significant genetic differentiation among sites for either F_{ST} or θ. In the analysis where only the degree of haplotype sharing was considered (θ), the values were slightly negative. Such negative values occur if the true value is zero. Because F_{ST} was greater than θ, we interpret that the groups of populations included unique haplotypes that were genetically distant from one another.

DISCUSSION

The genetic structure of the algal symbiont of *Ramalina menziesii* provides useful insight about this lichen species, when combined with information on the genetic structure that we previously reported for its fungal symbiont (Werth and Sork, 2008). The first result is that the two symbionts have little similarity in their patterns of genetic structure. In contrast to the mycobiont where we found little structure, here we find evidence for significant genetic structure in the photobionts associated with exactly the same individuals. The patterns of genetic differentiation, which we will discuss below, are not due to multiple species of algae, but are due to genetic specialization of photobiont strains across phorophyte species.

Photobiont identity—We identified *Trebouxia decolorans* as the only photobiont species associated with *R. menziesii* in

TABLE 2. Diversity statistics for all algal and fungal loci.

Statistic	Algal locus	Algal estimate	Fungal locus [a]	Fungal estimate [a]
Sample size (N)		72		72
Number of haplotypes (H)				
	ITS	32	*bet*	6
	psbJ-L	28	*efa*	23
	rbcL	9	*gpd*	4
			uid	12
Nucleotide diversity (π)				
	ITS	0.02571 ± 0.01283	*bet*	0.00641 ± 0.00147
	psbJ-L	0.15389 ± 0.07393	*efa*	0.00630 ± 0.00144
	rbcL	0.00132 ± 0.00100	*gpd*	0.00154 ± 0.00035
			uid	0.00292 ± 0.00067
Number of polymorphic sites (s)				
	ITS	104	*bet*	13
	psbJ-L	1278	*efa*	26
	rbcL	11	*gpd*	4
			uid	15
Haplotype diversity (H_d)				
	ITS	0.839 ± 0.043	*bet*	0.593 ± 0.033
	psbJ-L	0.811 ± 0.050	*efa*	0.828 ± 0.040
	rbcL	0.557 ± 0.071	*gpd*	0.339 ± 0.067
			uid	0.528 ± 0.064

[a] From Werth and Sork (2008)

our study area, implying high fungal specificity. *Trebouxia decolorans* is a common lichen photobiont, which has been reported from several continents and was found in association with some widespread fungal species, such as *Xanthoria parietina* (Nyati et al., 2004).

The specificity of an association may partly be determined by the variability of the markers used, as well as their mode of inheritance. Here, we used two independently evolving genes to identify specificity, a nuclear and a chloroplast gene. The phylogeny of both genes provided strong evidence of the specificity of the fungal–algal association. Our results are similar to that of several other studies of lichens that have found high specificity and have determined a single green-algal species, or clade, in association with a particular fungal species (Beck et al., 2002; Yahr et al., 2004; Ohmura et al., 2006; Hauck et al., 2007). However, it is possible for some lichen-forming fungal taxa to use several green algal clades or species (Yahr et al., 2004; Doering and Piercey-Normore, 2009). While the association of *R. menziesii* and its photobiont seems to be highly specific in our southern Californian study area, it is important to bear in mind that the photobiont species could in principle vary among geographic areas, and only a range-wide study of photobiont associations could determine whether *R. menziesii* is associated with different algal species in other parts of its range. For instance, in areas where *R. menziesii* occurs on coniferous phorophyte trees in the northern part of its range or on cacti and other succulent plant taxa in the southern part of its range, given the phorophyte preferences of the algae, the fungus might be associated with other algal species. Here we show that, on a local scale, the association of both symbiosis partners is highly specific.

Some studies have found that the photobionts of a lichen symbiosis belonged to cryptic green algal species. For example, in western North American populations of *Letharia* spp., several cryptic algal species belonging to the morphospecies *Trebouxia jamesii* were found in association with *Letharia* spp. (Kroken and Taylor, 2000). One allopatric species of *Letharia* growing in the coastal ranges of western North America used a genetically distant cryptic photobiont species relative to the photobiont species used by five other *Letharia* species, which were distributed in mountain ranges farther inland. Kroken and Taylor (2000) concluded that biogeography or ecological differences among habitats might account for the pattern found in the photobionts of *Letharia* spp. In this study, the lichen involves only one species of photobiont; further studies will test whether this specialization is true throughout the range (S. Werth and V. Sork, unpublished data).

Patterns of genetic differentiation—The photobiont population associated with *Ramalina menziesii* shows significant genetic structure within our study area, but the differentiation is across the three oak phorophyte species, not among the four sites. The lack of genetic differentiation among sites with simultaneously significant structure among phorophyte species in the photobiont indicates extensive gene flow within our study area, but selection on genotypes on different oak species. Another study has also found genetic differentiation in photobiont populations collected from different habitats (Yahr et al., 2006).

Given that we detected genetic differences associated with phorophyte species, despite the disparate geographic location of the trees, we conclude that selective pressures associated with the oak species may lead to genetic differentiation. These three oak species provide very different environmental conditions. *Quercus agrifolia* is evergreen and year-round, its canopy is much more closed than that of the deciduous species *Q. douglasii* and *Q. lobata*; its evergreen leaves might provide a low-light environment relative to the deciduous oak species, on which *R. menziesii* is generally more abundant. Moreover, in winter, when the largest amount of precipitation is received in our study site and when *R. menziesii* generally reaches the highest growth rates in inland sites of California (Matthes-Sears and Nash, 1986; Matthes-Sears et al., 1986), the lack of leaves on the deciduous species would allow more light for photosynthesis by the algae. We presume that some photobiont strains might be better adapted to the microclimatic conditions in the canopy

TABLE 3. Distribution of haplotypes in *Trebouxia decolorans* associated with *R. menziesii* across four sites and three phorophyte tree species in a southern Californian oak savanna landscape. NA, missing data at a locus. For each locus, the most frequent haplotype is printed in boldface.

Locus	Haplotype	Site 1	Site 2	Site 3	Site 4	*Qlob*	*Qdou*	*Qagr*
ITS	ITS1	1	0	0	0	1	0	0
	ITS2	0	0	1	0	1	0	0
	ITS3	0	1	1	0	0	0	2
	ITS4	1	0	2	0	0	1	2
	ITS5	0	0	1	0	0	0	1
	ITS6	0	0	0	1	0	1	0
	ITS7	0	1	0	0	0	0	1
	ITS8	0	1	0	0	0	0	1
	ITS9	0	0	0	2	0	0	2
	ITS10	0	0	0	1	0	0	1
	ITS11	2	1	0	0	1	0	2
	ITS12	0	0	1	0	0	0	1
	ITS13	0	1	0	0	0	0	1
	ITS14	0	0	0	1	0	0	1
	ITS15	0	0	1	0	1	0	0
	ITS16	0	0	2	0	0	1	1
	ITS17	0	0	1	0	0	1	0
	ITS18	1	2	0	0	0	2	1
	ITS19	1	0	0	0	1	0	0
	ITS20	0	1	0	0	0	1	0
	ITS21	0	0	0	1	1	0	0
	ITS22	0	0	0	1	0	0	1
	ITS23	0	1	0	1	2	0	0
	ITS24	0	0	0	1	0	1	0
	ITS25	0	0	0	1	0	1	0
	ITS26	1	0	0	0	0	1	0
	ITS27	0	0	0	1	1	0	0
	ITS28	1	0	0	0	1	0	0
	ITS29	0	1	0	0	1	0	0
	ITS30	2	0	0	0	0	2	0
	ITS31	1	0	1	0	1	0	1
	ITS32	**7**	**8**	**7**	**7**	**12**	**12**	**5**
psbJ-L	psb1	0	1	0	0	0	0	1
	psb2	0	0	0	1	1	0	0
	psb3	0	0	0	3	0	2	1
	psb4	0	1	0	0	0	0	1
	psb5	0	0	1	0	0	0	1
	psb6	0	0	0	1	0	0	1
	psb7	0	0	1	0	0	0	1
	psb8	0	0	1	0	0	1	0
	psb9	0	0	0	1	0	0	1
	psb10	0	0	0	1	0	0	1
	psb11	0	0	0	1	0	1	0
	psb12	0	0	1	0	0	1	0
	psb13	1	0	1	0	0	0	2
	psb14	0	0	1	0	0	0	1
	psb15	0	1	1	0	2	0	0
	psb16	1	0	0	0	0	1	0
	psb17	0	1	0	0	0	1	0
	psb18	1	1	0	0	0	0	2
	psb19	**9**	**8**	**7**	**5**	**12**	**12**	**5**
	psb20	1	0	0	0	0	0	1
	psb21	1	0	0	0	0	1	0
	psb22	2	1	0	1	1	2	1
	psb23	0	0	0	1	1	0	0
	psb24	0	1	0	0	1	0	0
	psb25	0	1	0	0	1	0	0
	psb26	1	0	1	0	1	0	1
	psb27	1	0	0	0	1	0	0
	psb28	0	1	0	0	0	0	1
	NA	0	1	3	3	3	2	2
rbcL	rbcL1	1	0	0	0	1	0	0
	rbcL3	3	5	1	1	2	7	1
	rbcL4	0	0	0	1	0	0	1
	rbcL5	1	0	3	1	0	2	3
	rbcL6	0	0	0	2	0	0	2
	rbcL7	1	0	0	0	0	1	0
	rbcL8	1	0	0	0	0	0	1
	rbcL9	**9**	**9**	**12**	**12**	**18**	**12**	**12**
	rbcL10	1	0	0	0	0	0	1
	NA	1	4	2	1	3	2	3

of *Q. agrifolia*, while other strains might be better adapted to the canopy conditions of the deciduous species.

Other environmental differences among the tree species are the chemical and physical properties of the bark. The bark of *Q. lobata* and *Q. douglasii* is rugged and very coarsely structured with deep cracks, while it is smooth in *Q. agrifolia*. However, on twigs in the crown area where *R. menziesii* is growing, the structural differences in the bark do not seem to be as pronounced among the phorophyte species. Differences in bark pH or water retention capacity are not known, but could also potentially influence the photobionts of *R. menziesii* or a free-living algal population. Future studies should focus on isolating and identifying free-living algal populations from the same trees and check whether these match the haplotypes associated with *R. menziesii*. It would also be interesting to see whether the same algal haplotypes are shared by multiple lichen-forming fungal species. In this case, *R. menziesii* could in principle incorporate algae from the vegetative propagules dispersed by other fungal species. Also, the chemical and physical properties of the bark could be quantified for different phorophyte species. Regardless of whether they are caused by crown structure, bark pH, or other physical properties of the phorophyte species, the genetic patterns across species imply that ecology plays a major role in structuring the local genetic variability in the photobiont of *R. menziesii*.

The pattern of genetic differentiation in the photobiont among sites indicates that gene flow is extensive, as indicated by the results that F_{ST} and θ are so close to zero. The negative values for θ might be due to the high number of alleles or to our pooling of the data across species, but they are not unusual with extensive gene flow. This finding is a bit surprising because we had anticipated restricted dispersal of the algal species, which would lead to strong differences among sites. However, perhaps the algal species occasionally codisperses with the lichen fungus in thallus fragments, which would move the algal genotypes around in the landscape. We doubt that this form of algal gene flow is common at our study site because our previous study of genetic structure of the fungal partner of *R. menziesii* demonstrated very little vegetative dispersal. If it does take place intermittently, then vegetative dispersal could homogenize algal genotypes across the sites, while subsequent local selection pressures would maintain differentiation among algal subpopulations on different phorophyte species. *Trebouxia decolorans* may have other vectors for dispersal within a local site, but too little is known about algal movement to speculate.

In our previous analysis of genetic structure in the fungal genome (Werth and Sork, 2008), we found no genetic differentiation associated with phorophyte species or among sites and concluded that gene flow must be extensive. Similar to our results, the lichen-forming fungus *Evernia mesomorpha* exhibited

TABLE 4. Analysis of molecular variance for *Trebouxia decolorans*, the green-algal photobiont of *Ramalina menziesii* and its mycobiont (from Werth and Sork, 2008).

	Photobiont			Mycobiont		
Source	Locus	Algal F_{ST}	Algal θ	Locus	Fungal F_{ST}	Fungal θ
Among phorophyte species	ITS	0.1361***	0.0268	*bet*	−0.0240	−0.0116
	psbJ-L	0.1693***	0.0344	*efa*	−0.0048	−0.0074
	rbcL	0.0599*	0.0522	*gpd*	−0.0060	−0.0060
	Overall	—	0.0360*	*uid*	−0.0170	−0.0204
Among sites	ITS	0.0473*	−0.0098	*bet*	−0.0219	−0.0147
	psbJ-L	0.0090	−0.0095	*efa*	−0.0212	−0.0145
	rbcL	0.0279	−0.0001	*gpd*	−0.0475	−0.0475
	Overall	—	−0.0072*	*uid*	−0.0287	−0.0287

Notes: An asterisk indicates statistical significance at $P < 0.05$, three asterisks indicate significance at $P < 0.001$.

no fungal local genetic structure among trees, whereas the algae exhibited significant structure at this spatial scale; differences among phorophyte species were not investigated in this study (Piercey-Normore, 2006). Moreover, our results are consistent with the findings of a regional-scale study of genetic structure of *Cladonia subtenuis* by Yahr et al. (2006), who found weak structure in the fungus, but high genetic structure in the photobiont among habitats. This tendency of specialization of the algae on local environmental conditions and the extensive dispersal of the fungal spores allows the establishment of a lichen that can survive in a broad range of conditions and may account for the geographically wide distribution of *R. menziesii* across diverse ecosystems, including fog deserts in Baja California, oak woodland forests in California, and coniferous forests of Alaska.

Diversity patterns in photobiont and fungus—While the fungal and algal genetic structures differ from each other, it is questionable to which degree this pattern may be due to differences among individual fungal and algal loci. For instance, if the algal loci were generally more variable than the fungal loci, they could exhibit more genetic structure, e.g., due to rare alleles. However, this did not seem to be the case in our study. The number of haplotypes found in the photobiont appeared to be of a similar magnitude as that of its fungal partner (Table 2). Moreover, the total haplotype diversity estimates were rather similar between mycobiont and photobiont. Nucleotide diversity was similar in the algal *rbcL* and in the four fungal loci (Werth and Sork, 2008). For the hypervariable *psbJ-L* as well as for ITS, nucleotide diversity was about one or two orders of magnitude higher, which might be mostly due to the insertion/deletions in both alignments. However, all algal loci showed the same pattern, and also the least variable algal locus, *rbcL*, was significantly structured among phorophyte species. These results make it unlikely that the differences are merely due to the choice of markers.

We found high genetic variability in *T. decolorans* both in the nuclear ribosomal and chloroplast DNA. Future work should examine whether the fungus and its photobiont share a common regional biogeographic history. Based on our data, we would expect a large amount of genetic structure in the photobiont among different habitats, e.g., vegetation types, bioclimatic zones, or areas that harbor different phorophyte species.

Photobiont transmission mode—Our analysis of the genetic structure of *T. decolorans* in comparison to fungal genetic structure in *R. menziesii* provides some clues about the mode of symbiont transmission. Strictly vertical transmission generally leads to similar genetic structures among symbionts. For example, strict or predominant vertical transmission has been found in bacterial endosymbionts (Schröder et al., 1996; Clark et al., 2000; Dobson et al., 2002; Mira and Moran, 2002), termite-associated fungi (Johnson et al., 1981), coral-associated dinoflagellates (Loh et al., 2001; Rodriguez-Lanetty and Hoegh-Guldberg, 2003), and animal parasites (Smith and Dunn, 1991; Bigot et al., 1997; Terry et al., 2004; Conrad et al., 2005). However, asymmetries in gene flow between the symbiotic partners may lead to differential genetic structure, even in vertically transmitted systems (Sullivan and Faeth, 2004). In symbioses with horizontal transmission, the partners get uncoupled, leading to major differences in the genetic structure of symbiosis partners. In some systems, horizontal transmission of symbionts can predominate; for instance, it has been found in some microsporidian parasites (Mangin et al., 1995; Dunn and Smith, 2001), some species of termite-associated fungi (Aanen et al., 2002), and occasionally in endosymbiotic bacteria (Huigens et al., 2004). Because the mycobiont and photobiont genetic structures of *R. menziesii* are so different, our results indicate a predominantly horizontal transmission mode. In addition, we found little evidence for clonality in *R. menziesii* in our study area, which suggests that dispersal via ascospores is followed by germination and re-lichenization involving horizontal photobiont transmission (Werth and Sork, 2008). Only if photobionts are transmitted horizontally, would we predict to find any differences in the structure of mycobiont and photobiont. Horizontal transmission occurs usually when the fungus is reproducing sexually. However, some exceptions may occur: for instance, during juvenile development of thalli from soredia, the primary photobiont may be replaced with free-living algae or by algae taken up from another lichen thallus (Friedl, 1987; Ott, 1987; Ohmura et al., 2006). This could lead to an uncoupling of genetic structures in mycobionts and photobionts, even in predominantly vegetatively dispersing species.

Conclusions—We found that the local genetic structures of *R. menziesii* and its green-algal photobiont did not correspond to each other. Algal genetic structure was associated more with the species of phorophyte than with site. We speculate that specialization of the algal species to its phorophyte might allow the symbiotic phenotype of this widely dispersing fungal species to be adapted to local environmental conditions. This ecological specialization among lichen photobionts deserves further attention, and it may well be a key evolutionary process allowing the wide distribution and abundance of *Ramalina menziesii* in

western North America and a major theme driving evolution in the symbiotic association of lichen-forming fungi and their photobionts.

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APPENDIX 1. GenBank accessions for *Trebouxia decolorans*, the photobiont of the epiphytic lichen *Ramalina menziesii*, vouchers, and haplotype numbers of the nuclear rDNA gene cluster (ITS) and two chloroplast genes, *rbcL* and the *psbJ-L* intergenic spacer. Vouchers are deposited in the herbarium of V. L. Sork at the Department of Ecology and Evolutionary Biology, University of California at Los Angeles.

Locus	Haplotype	Voucher	Accession
ITS	H1_ITS	SW-08a	FJ705175
ITS	H2_ITS	SW-15a	FJ705176
ITS	H3_ITS	SW-14a	FJ705177
ITS	H4_ITS	SW-16b	FJ705178
ITS	H5_ITS	SW-45a	FJ705179
ITS	H6_ITS	SW-19b	FJ705180
ITS	H7_ITS	SW-14b	FJ705181
ITS	H8_ITS	SW-42c	FJ705182
ITS	H9_ITS	SW-20a	FJ705183
ITS	H10_ITS	SW-48c	FJ705184
ITS	H11_ITS	SW-10a	FJ705185
ITS	H12_ITS	SW-17b	FJ705186
ITS	H13_ITS	SW-42b	FJ705187
ITS	H14_ITS	SW-20b	FJ705188
ITS	H15_ITS	SW-43b	FJ705189
ITS	H16_ITS	SW-16c	FJ705190
ITS	H17_ITS	SW-44b	FJ705191
ITS	H18_ITS	SW-13b	FJ705192
ITS	H19_ITS	SW-37c	FJ705193
ITS	H20_ITS	SW-41b	FJ705194
ITS	H21_ITS	SW-46b	FJ705195
ITS	H22_ITS	SW-48a	FJ705196
ITS	H23_ITS	SW-12a	FJ705197
ITS	H24_ITS	SW-19a	FJ705198
ITS	H25_ITS	SW-47a	FJ705199
ITS	H26_ITS	SW-38b	FJ705200
ITS	H27_ITS	SW-18a	FJ705201
ITS	H28_ITS	SW-08b	FJ705202
ITS	H29_ITS	SW-12c	FJ705203
ITS	H30_ITS	SW-09b	FJ705204
ITS	H31_ITS	SW-10b	FJ705205
ITS	H32_ITS	SW-08c	FJ705206
rbcL	H1_rbcL	SW-08b	FJ705207
rbcL	H3_rbcL	SW-09a	FJ705209
rbcL	H4_rbcL	SW-20a	FJ705210
rbcL	H5_rbcL	SW-16b	FJ705211
rbcL	H6_rbcL	SW-20c	FJ705212
rbcL	H7_rbcL	SW-38b	FJ705213
rbcL	H8_rbcL	SW-10a	FJ705214
rbcL	H9_rbcL	SW-08c	FJ705215
rbcL	H10_rbcL	SW-39c	FJ705216
psbJ-L	H1_psbJ-L	SW-14a	FJ716587
psbJ-L	H2_psbJ-L	SW-46b	FJ716588
psbJ-L	H3_psbJ-L	SW-47b	FJ716589
psbJ-L	H4_psbJ-L	SW-42c	FJ716590
psbJ-L	H5_psbJ-L	SW-17c	FJ716591
psbJ-L	H6_psbJ-L	SW-20b	FJ716592
psbJ-L	H7_psbJ-L	SW-17a	FJ716593
psbJ-L	H8_psbJ-L	SW-44b	FJ716594
psbJ-L	H9_psbJ-L	SW-20c	FJ716595
psbJ-L	H10_psbJ-L	SW-48c	FJ716596
psbJ-L	H11_psbJ-L	SW-19b	FJ716597
psbJ-L	H12_psbJ-L	SW-16b	FJ716598
psbJ-L	H13_psbJ-L	SW-39b	FJ716599
psbJ-L	H14_psbJ-L	SW-45a	FJ716600
psbJ-L	H15_psbJ-L	SW-12a	FJ716601
psbJ-L	H16_psbJ-L	SW-38c	FJ716602
psbJ-L	H17_psbJ-L	SW-41c	FJ716603
psbJ-L	H18_psbJ-L	SW-10c	FJ716604
psbJ-L	H19_psbJ-L	SW-10b	FJ716605
psbJ-L	H20_psbJ-L	SW-39a	FJ716606
psbJ-L	H21_psbJ-L	SW-09b	FJ716607
psbJ-L	H22_psbJ-L	SW-09c	FJ716608
psbJ-L	H23_psbJ-L	SW-18b	FJ716609
psbJ-L	H24_psbJ-L	SW-40b	FJ716610
psbJ-L	H25_psbJ-L	SW-40a	FJ716611
psbJ-L	H26_psbJ-L	SW-10a	FJ716612
psbJ-L	H27_psbJ-L	SW-37a	FJ716613
psbJ-L	H28_psbJ-L	SW-14b	FJ716614