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Ecological specialization in *Trebouxia* (Trebouxiophyceae) photobionts of *Ramalina menziesii* (Ramalinaceae) across six range-covering ecoregions of western North America[1]

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- *Premise of the study:* Many lichens exhibit extensive ranges spanning several ecoregions. It has been hypothesized that this wide ecological amplitude is facilitated by fungal association with locally adapted photobiont strains.
- *Methods:* We studied the identity and geographic distribution of photobionts of the widely distributed North American lichen *Ramalina menziesii* based on *rbcL* (chloroplast DNA) and nuclear ribosomal ITS DNA sequences. To test for ecological specialization, we associate photobiont genotypes with local climate and phorophyte.
- *Key results:* Of the photobiont lineages of *R. menziesii*, 94% belong to a clade including *Trebouxia decolorans*. The remaining are related to *T. jamesii*. The photobionts showed (1) significant structure according to ecoregion and phorophyte species and (2) genetic associations with phorophyte species and climate.
- *Conclusions:* Geography, climate, and ecological specialization shape genetic differentiation of lichen photobionts. One great advantage of independent dispersal of the fungus is symbiotic association with locally adapted photobiont strains.

Key words: ecological specialization; genetic differentiation; host plant specificity; lichenized ascomycetes; phorophyte; photobiont; *Ramalina menziesii*; Ramalinaceae; symbiosis; *Trebouxia decolorans*; Trebouxiophyceae.

Lichens are mutualistic associations between a fungus (mycobiont) and photosynthetic green algae and/or cyanobacteria, the so-called photobionts (Richardson, 1999). The symbiosis is obligate for the fungus, but the green algal and cyanobacterial species participating in the lichen symbiosis are often able to persist outside of the lichen thallus (Bubrick et al., 1984; Mukhtar et al., 1994; Wirtz et al., 2003; O'Brien et al., 2005; Sanders, 2005). Given that lichens require a mutualistic associate to exist, one might think that this requirement for two compatible taxa to find each other would constrain their distribution geographically and to specific environmental conditions. Instead, many species are widespread on different continents, albeit in the same habitat type (Otte et al., 2002, 2005). For instance, the lichen *Cetraria aculeata* (Schreb.) Fr. occurs in temperate, arctic, and antarctic environments (Fernández-Mendoza et al., 2011), while *Cavernularia hultenii* Degel. occurs disjunctly on two continents in similar forest types (Printzen et al., 2003). Then again, other species range continuously across multiple climatic zones and ecosystems. For instance, *Niebla cephalota* (Tuck.) Rundel & Bowler ranges from the desert of southern Baja California to the coniferous forest in the State of Washington (Brodo et al., 2001), and *Ramalina menziesii* Taylor is distributed from coastal deserts of Baja California to temperate rain forests of Alaska (Sork and Werth, 2014).

The high dispersal potential of lichens is amplified by their ability to tolerate environmental stress. Because lichens are poikilohydric organisms, their metabolism approaches zero when they dry out, allowing them to tolerate extreme conditions (Nash, 2008). For example, two lichen species survived exposure to space conditions on an earth-orbiting satellite, and back on Earth they approached normal photosynthetic rates within 1 d after hydration (Sancho et al., 2007). The presence of lichens in extreme environments in the Arctic and Antarctic,

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and in desert regions (Rundel, 1978; Castello and Nimis, 1997; Domaschke et al., 2012; Pérez-Ortega et al., 2012) provides additional evidence of their unusual level of tolerance. Yet, this greatly enhanced ability to tolerate environmental stress and extreme conditions is not present separately in each partner of the mutualism (de Vera et al., 2008). Thus, the mutualists seem to synergistically create a symbiotic phenotype that has environmental tolerances greater than the fungal species and photobiont separately.

One mechanism for creating such an adaptable phenotype is association with specific photobiont strains. Many lichen fungi disperse through microscopic ascospores and then "relichenize" with a local photobiont partner, which is likely to be adapted to those local ecological conditions (Piercey-Normore and DePriest, 2001; Yahr et al., 2006; Werth, 2011). For example, ecologically similar but taxonomically unrelated lichen fungi often share closely related photobiont strains (Lücking et al., 2009; Peksa and Škaloud, 2011). The photobionts associating with individual lichen fungi exhibit large-scale geographic trends related to ecology and biogeography (Yahr et al., 2006; Wornik and Grube, 2010; Fernández-Mendoza et al., 2011; Widmer et al., 2012). While some mycobionts are highly selective for photobiont strains (Yahr et al., 2004; Fedrowitz et al., 2011, 2012; Dal Grande et al., 2012; Werth and Scheidegger, 2012), other lichen fungi associate with a wide range of taxa (Blaha et al., 2006; Nyati et al., 2013) or with divergent photobiont species or strains in different parts of their ranges (Altermann, 2009; Nelsen and Gargas, 2009). Fungal selectivity for photobionts is often low in extreme environments such as deserts, alpine, arctic, or antarctic habitats (Wirtz et al., 2003; Muggia et al., 2008; Pérez-Ortega et al., 2012). Collectively, these studies suggest that the successful establishment of the lichen is dependent on the lineage or genetic strain of the photobiont and the extent to which that photobiont taxon is locally specialized. Thus, knowledge of the ecology of the photobiont will enhance our understanding of how some lichens can evolve such broad tolerances.

To understand the extent to which a photobiont can become ecologically specialized, we assessed whether the evolutionary history of the *Trebouxia* photobionts of the epiphytic lichen *R. menziesii* is shaped by ecological specialization on phorophyte species after accounting for other factors such as geographic location. Like the fungal symbiont (Sork and Werth, 2014), the green-algal photobiont is likely to show genetic differentiation across multiple ecogeographic regions (ecoregions) in western North America, defined by predominant habitat type and topographic boundaries, which intersect with major climate zones of subtropical, Mediterranean, and temperate climate. However, epiphytic lichens are rarely thought to be specialized on a phorophyte because most lichens occur on multiple plant species (Barkman, 1958). Nevertheless, we predict ecological specialization of *Trebouxia* photobionts on phorophyte species. *Ramalina menziesii* tends to be found on several woody plant species within ecoregions, and the predominant phorophyte also differs among ecoregions. In epiphytic lichens, the association with phorophyte species differing in important properties, such as deciduous/evergreen, bark pH, bark chemistry, or bark roughness, could lead to differential survival among photobiont strains. In a local scale study of *R. menziesii*, photobionts sampled from different phorophyte species were genetically differentiated (Werth and Sork, 2010), while the fungal genotypes in those same individuals were not differentiated by phorophyte (Werth and Sork, 2008). Given that the photobionts were within dispersal range of each other, this finding raises the possibility that the genetic differentiation was due to local selection pressures on the algal genotypes.

Previously we studied the phylogeography of the widespread lichen fungus *R. menziesii* in coastal areas of western North America using DNA sequences of four nuclear low-copy genes and found genetic differentiation among ecoregions, but no significant divergence associated with the phorophyte species (Sork and Werth, 2014). Here we address two central hypotheses concerning the photobiont of the lichen *R. menziesii*: (1) that *Trebouxia* photobionts should show strong genetic differentiation across major ecoregions due to (a) restricted gene flow over topographic and climate barriers between ecoregions and (b) selection by local environmental conditions that amplifies genetic differences and (2) that *Trebouxia* photobionts are ecologically specialized on different phorophyte species within ecoregions, leading to genetic differentiation of photobionts sampled from different phorophyte species. Specifically, we have three goals. Our first aim is to identify the lineages of *Trebouxia* associating with *R. menziesii* throughout its range using a phylogenetic approach. Second, we quantify the hierarchical genetic structure associated with ecoregion and phorophyte species within ecoregion to find evidence for the second hypothesis. Third, we conduct separate multivariate ordination analyses to test for associations with local phorophyte species and climate environments. Our findings will highlight patterns of genetic diversity and differentiation of an understudied, yet ecologically significant, organism that plays a major role in all ecosystems. Despite the advantages of codispersal of symbionts when the vegetative propagules carry along photobiont cells embedded in fungal hyphae, and the developing propagules do not need to obtain a new photobiont partner (Dal Grande et al., 2012; Werth and Scheidegger, 2012), *R. menziesii* mainly disperses via fungal ascospores, which must then find a photobiont. This study assesses whether this strategy results in a unique selective advantage of locally adapted photobiont partners that could account for the wide distributions of many lichen species.

MATERIALS AND METHODS

***Taxonomy*—**Species names of fungi and algae followed Index Fungorum (http://www.indexfungorum.org, accessed 21 August 2013) and algaebase (http://www.algaebase.org/, accessed 21 August 2013), respectively. Names with taxonomic authorities of the *Trebouxia* algae and lichen fungi included in this study are given in Table 1. Plant names follow Roberts (1989) for the flora of Baja California and the Index of California Plant Names (http://ucjeps.berkeley.edu/about_ICPN.html, accessed 21 August 2013).

***Study species*—**The epiphytic lichen *Ramalina menziesii* is distributed from the Baja California Peninsula in Mexico north to southeast Alaska (Rundel, 1974; Brodo et al., 2001). The lichen disperses mainly sexually on a local scale (Werth and Sork, 2008). The photobiont has been previously determined as *Trebouxia decolorans* Ahmadjan based on samples from southern California (Werth and Sork, 2008, 2010; Werth, 2012). *Trebouxia decolorans* is a common photobiont in lichens and has previously been found in species such as *Xanthoria parietina* from Europe (Beck and Mayr, 2012; Nyati et al., 2013; Nyati et al., 2014) and in *X. hasseana* from western North America (Werth, 2012). Phylogeographic analyses of the mycobiont *R. menziesii* indicated that this species survived the Pleistocene glaciations in multiple refugia located in California and on the Baja California peninsula, as evidenced by the presence of several ancient fungal clades (Sork and Werth, 2014). California exhibited the highest genetic diversity as a result of the persistence of ancient fungal clades and recent migration from northern sites. Sites on the Baja California peninsula

TABLE 1. *Trebouxia* reference strains and sequences of known identity. The table gives photobiont species, strain number, the lichen it was isolated from, and GenBank accessions of ITS and *rbcL* loci.

Species	Strain	Isolated from	ITS	*rbcL*
T. arboricola de Puymaly	SAG-219-Ia	Free-living?	Z68705	AM158960
T. arboricola de Puymaly	P-3-I	*Xanthoria turbinata* Vain.	AJ969509	AJ969669
T. arboricola de Puymaly	P-7-Ia	*Xanthoria parietina* (L.) Beltr.	AJ969512	AJ969646
T. arboricola de Puymaly	P-83-Ia	*Xanthoria ectaneoides* (Nyl.) Zahlbr.	AJ969611	
T. arboricola de Puymaly	P-53-Ia	*Xanthoria ligulata* (Körb.) P. James	AJ969528	AJ969670
T. arboricola de Puymaly	P-360-IIa	*Xanthoria* sp.	AJ969609	AJ969665
T. decolorans Ahmadjian	P-97-Ia	*Xanthoria ectaneoides* (Nyl.) Zahlbr.	AJ969539	AM158965
T. decolorans Ahmadjian	P-120a-IIIb	*Xanthoria parietina* (L.) Beltr.	AJ969545	AM158966
T. decolorans Ahmadjian	P-121-IIcd	*Xanthoria parietina* (L.) Beltr.	AJ969550	AM158967
T. decolorans Ahmadjian	P-319-Ig	*Xanthoria parietina* (L.) Beltr.	AM159503	AM159504
T. decolorans Ahmadjian	P-320-IIb	*Xanthoria parietina* (L.) Beltr.	AJ969600	AM158964
T. decolorans Ahmadjian	P-320-IIf	*Xanthoria parietina* (L.) Beltr.	AJ969603	AM158963
T. decolorans Ahmadjian	none	*Ramalina leptocarpha* Tuck.	EU717935	
T. decolorans Ahmadjian	Rsp-BA11	*Ramalina* sp.	KF556651	
T. decolorans Ahmadjian	RspSH401	*Ramalina* sp.	KF556652	
T. flava Archibald	UTEX 181/IB 346	*Physconia pulverulenta* (Schreb.) Poelt	AF242467	AJ969637
T. gelatinosa Ahmadjian ex Archibald	P-57-Ia	*Xanthoria* sp.	AJ969532	AJ969642
T. gelatinosa Ahmadjian ex Archibald	P-270-Ia	*Teloschistes chrysophthalmus* (L.) Norman ex Tuck.	AJ969579	AJ969640
T. impressa Ahmadjian	OR2-Rroe	*Ramalina roesleri* (Hochst. ex Schaer.) Nyl.	KF556650	
T. jamesii (Hildreth & Ahmadjian) Gärtner	UTEX 2233/IB 336	*Schaereria tenebrosa* (Flot.) Hertel & Poelt	FJ626733	AJ969663
T. jamesii (Hildreth & Ahmadjian) Gärtner	OR2-Rfar	*Ramalina farinacea* (L.) Ach.	KF556649	
T. potteri Ahmadjian ex Gärtner	P-330-Ib	*Xanthoria ulophyllodes* Räsänen	AJ969605	AJ969636
T. potteri Ahmadjian ex Gärtner	UTEX 900/IB 332	*Lecanora rubina* (Vill.) Ach.	AF242469	AJ969635
T. showmanii (Hildreth & Ahmadjian) Gärtner	UTEX 2234/IB 337	*Lecanora hageni* (Ach.) Ach.	AF242470	AJ969661

remained isolated for a long time from sites further north and represented the oldest divergence of the fungus *R. menziesii*.

***Study area*—**Our study area included coastal western North America, from the Baja California Peninsula to southeastern Alaska, covering the entire range of the species in North America. Sites were grouped into six regions according to geographic location, vegetation type, and the location of natural boundaries (Sork and Werth, 2014). Within the Baja California Peninsula, samples were collected in inland (BI) and coastal sites (BC), which were geographically separated. The BI region consists of fog desert sites (Vizcaíno Desert) and the BC region of coastal chaparral. Sites on the California coast were classified as the California coastal region (CC) and include various types of coastal woodlands, e.g., those dominated by coastal live oak (*Quercus agrifolia* Née). Also found in this region were sites with various pine species and other oak species (*Q. tomentella* Engelm., *Q. pacifica* Nixon & C. H. Mull.), tan oak [*Lithocarpus densiflorus* (Hook. & Arn.) Rehder], and California laurel [*Umbellularia californica* (Hook. & Arn.) Nutt.], as well as riparian habitats with red alder (*Alnus rubra* Bong.) and arroyo willow (*Salix lasiolepis* Benth.). The southern (CS) and northern (CN) California inland regions contain oak savanna sites with valley oak (*Q. lobata* Née), blue oak (*Q. douglasii* Hook. & Arn.), and coastal live oak. These sites are geographically separated from ecoregion CC by the Coastal Ranges. All sites characterized by coastal temperate forests were grouped into the Pacific Northwest region (PN), which included sites ranging from Oregon to British Columbia. Habitats in this region comprise temperate mixed coniferous forest with Sitka spruce [*Picea sitchensis* (Bong.) Carrière] and western hemlock [*Tsuga heterophylla* (Raf.) Sarg.], and some broad-leaved forests with Garry oak (*Q. garryana* Hook.), saskatoon [*Amelanchier alnifolia* (Nutt.) Nutt. ex M. Roem.], Oregon ash (*Fraxinus latifolia* Benth.), and bigleaf maple *(Acer macrophyllum* Pursh). Northern coastal coniferous forests of Humboldt County, northern California, were included within PN because they represent a temperate floristic element different from the remaining sites in California. All collecting sites are listed in Appendix S1 (see Supplemental Data with the online version of this article).

***Sampling design*—**To infer the broad-scale patterns of genetic variability of the photobionts, we used samples from numerous sites (N = 108) across the entire range of *Ramalina menziesii*, with three to five lichen thalli per site collected for our phylogeographic study (Sork and Werth, 2014). Within a given site, we sampled individuals from different phorophyte trees and at a distance of at least 10 m to decrease the likelihood of repeatedly sampling the same clonal multilocus genotype of the lichen, though clonality was low in a previous study (Werth and Sork, 2008). For comparative purposes, our analyses included prior data from a site in southern California (Werth and Sork, 2008), thus including 72 samples that had been sequenced previously. The total sample size for *R. menziesii* employed in our study was 575.

***Molecular analysis*—**We chose to analyze DNA sequences of the algal nuclear ribosomal internal transcribed spacer (ITS) region and the chloroplast ribulose-1,5-bisphosphate carboxylase oxygenase (*rbcL*) gene (also termed RuBisCO), since these loci have successfully been used to determine the identity of *Trebouxia* and *Asterochloris* species (Werth and Sork, 2010; Werth, 2012; Nyati et al., 2014). DNA extraction, PCR, and DNA sequencing of the algal ITS region has been previously described in Werth and Sork (2010). For the *rbcL* locus, we used primers rbcLfwd (forward), and rbcLrev (reverse) (Nyati et al., 2014), for PCR, with rbcLfwd for DNA sequencing, otherwise following the protocol of Werth and Sork (2010).

Data analysis*—Identity of photobionts*—**DNA sequence alignments were performed with CLUSTAL W (Thompson et al., 1994) implemented in Mega version 5.10 (Tamura et al., 2007). To investigate the phylogenetic relationships among ITS and *rbcL* haplotypes and identify the photobiont species, we computed median-joining networks (Bandelt et al., 1999), including sequences of photobiont strains of known identity. The analysis was performed in Network version 4.6.1.1 (available from www.fluxus-engineering.com). The ITS data set included 72 sequences from one study site in southern California, which were included in the haplotype networks (GenBank accessions FJ705175–FJ705206).

To assess whether the ITS and *rbcL* loci could be combined in a joint phylogenetic analysis, we used the congruence among distance matrices (CADM) test (Legendre and Lapointe, 2004; Campbell et al., 2011) based on Kimura 2-parameter distance matrices (Kimura, 1980). We performed the test using the function 'CADM.global' implemented in the R library ape (Paradis et al., 2004). A Kendall's concordance statistic W value of 0 signifies complete incongruence of loci, while a value of 1 indicates complete concordance. The null hypothesis of complete incongruence among DNA distance matrices was tested with 999 permutations in R.

To obtain additional evidence about the phylogenetic relationships among haplotypes, we constructed Bayesian phylogenetic trees using the program MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). We used the program MrModeltest2 to identify the type of substitution model to be applied to

the data (Nylander, 2004). We constructed Bayesian phylogenetic trees in MrBayes using 10^6 iterations in four parallel chains, sampling every 100th step, thus recording 10 000 trees for each locus individually and for the combined data. Bayesian trees were annotated with TreeAnnotator version 1.6.0, which is part of the BEAST package (Drummond and Rambaut, 2007; Drummond et al., 2012), discarding 10% of the run as burn-in. Convergence of runs was assessed by inspecting traces of the parameters over the course of the run in Tracer version 1.5 (Rambaut and Drummond, 2007) and by effective sample size (Drummond and Rambaut, 2007). To evaluate the support of individual nodes on the phylogenetic trees, we used posterior probabilities from the Bayesian analyses.

Genetic diversity of photobiont pool —Haplotypes were mapped and data were transformed to different sequence formats using the program SNAP Map (Aylor et al., 2006). We calculated the number of polymorphic sites *s*, the number of haplotypes *H*, haplotype diversity Hd, and nucleotide diversity π for each ecoregion in the program DNAsp version 5.10.01 (Rozas et al., 2003).

Genetic structure and ecological specialization of photobionts—We inferred the regional genetic structure of the *Trebouxia* population associated with *R. menziesii* by calculating hierarchical analysis of molecular variance (AMOVA, Excoffier et al., 1992) in Arlequin version 3.5.1.3 (Excoffier et al., 2005). For this analysis, we grouped geographically proximate localities into larger subregions with 10–20 individuals each. The analysis was performed for six ecoregions (BI, BC, CC, CS, CN, PN), nesting subregions within ecoregion. To test the effect of phorophyte within each ecoregion, we did a second AMOVA in which we nested phorophyte within subregion, leaving out individuals with a single occurrence on a phorophyte species, and leaving out subregions that contained only a single phorophyte. We did not perform this analysis for ecoregions BC and BI because they contained too few individuals to provide an informative test of the associations of *Trebouxia* strains with phorophyte species.

To assess the potential for ecological specialization in the photobiont strains of *Ramalina menziesii*, we used redundancy analysis (RDA) and partial redundancy analysis (Ter Braak, 1986; Legendre and Legendre, 1998). The partial RDA models allow us to examine the effect of phorophyte on the genetic composition of sites after removing the effect of climate. As genetic data, we used a presence/absence of 121 ITS and *rbcL* haplotypes in 110 sampling localities. A set of variables relating to phorophyte (e.g., P.1) was used, representing principal components of the presence/absence of a phorophyte species in each of the 110 localities. For the principal component matrices of phorophytes (P.1, P.2, P.3), the cutoff-levels for eigenvalues were set to 1.0, 1.5, and 2.0, respectively. Our redundancy analysis (RDA) also included a set of climate variables. Global climatic data were downloaded from WorldClim (Hijmans et al., 2005) at 2.5 arc-min resolution and imported into the program DIVA-GIS version 7.5 (Hijmans et al., 2004), and 19 bioclimatic variables (BIOCLIM, Busby, 1991) were exported for our sampling sites. We used these 19 bioclimatic variables from each sampling locality to compute principal components, retaining axes at a cutoff-value of 1.0, 1.5, and 2.0, respectively (C.1, C.2, C.3). Partial RDA was used to test which variable sets contributed substantially in explaining haplotype distribution in the *Trebouxia* photobionts of *R. menziesii* (Borcard et al., 1992; Økland and Eilertsen, 1994; Økland, 2003). Specifically, RDA and partial RDA were performed using the R library 'vegan' and function 'rda' (Oksanen et al., 2010). Variance partitioning was performed using functions 'varpart' and 'rda' (library vegan). Significance of a given fraction of variance was assessed with a permutation test using 1000 permutations (function 'anova.cca' in R). The test statistic ('pseudo-*F*') was the ratio of constrained and unconstrained variance, each divided by their respective ranks (Oksanen et al., 2010). In RDAs, the matrices themselves were permuted to assess statistical significance. In partial RDAs, residuals from partial RDA were permuted for significance testing. For each RDA and each partial RDA, we did a single global test rather than multiple tests of the various RDA axes or terms.

RESULTS

***Identity of photobionts*—**We tested the null hypothesis of incongruence among loci using the CADM test (Campbell et al., 2011). A high Kendall's concordance statistic *W* demonstrated the congruence of the two loci ($W = 0.87$, $p = 0.001$). Thus, the results from the congruence among distance matrices (CADM) test indicated that ITS and *rbcL* could be combined in a single phylogenetic analysis.

The majority of photobionts associating with *Ramalina menziesii* across its range belong to lineages closely related to the widespread green algal species *Trebouxia decolorans* (Figs. 1, 3C). At least one additional algal species was present in our data, a species that is closely related to *Trebouxia jamesii* (hereafter referred to as "*T. jamesii*"). This species was most common in CC and BC and had a few occurrences in BI and PN. In the single-gene phylogenies, the split between *T. decolorans* and *T. arboricola* received no support, but in the combined phylogeny, the split between *T. arboricola* and *T. decolorans* was supported by a posterior probability of 95% and that of *T. jamesii*–related lineages from the aforementioned clades by a posterior probability of 100%. While our data do not contain "typical" *T. arboricola* haplotypes, some of the ITS haplotypes showed an affinity to *T. arboricola* in the median-joining network. However, this pattern was not found in *rbcL* and or in the Bayesian analysis of either locus, and thus we conclude that our samples did not include *T. arboricola* sensu stricto, i.e., specimens closely related to the cultured strain SAG 219-Ia.

ITS haplotypes of the *Trebouxia decolorans* clade showed clear affinities to ecoregions. For example, one group of closely related ITS haplotypes was restricted to BI, another was predominant in inland regions of California (CS, CN), and a third predominated in the Pacific Northwest. Haplotypes of the *rbcL* region (cpDNA) showed a similar geographic distribution, but no clear distinction between California interior sites and the Pacific Northwest. Haplotype maps with GenBank accessions are given in online Appendices S2 (*rbcL*) and S3 (ITS).

***Genetic diversity of photobiont pool*—**Our study is based on photobiont ITS sequences from 572 specimens of *Ramalina menziesii* (this study: 500) and photobiont *rbcL* sequences from 305 specimens, totaling an alignment length of 1239 bp (ITS: 671/540 bp, *rbcL*: 594/594 bp; numbers refer to the number of sites including/excluding gaps and missing data). ITS and *rbcL* differed considerably in variability. There were 130 haplotypes defined by 313 variable sites in ITS (removing gaps and missing data: 102 haplotypes, 182 variable sites). The overall haplotype diversity of ITS was 0.93, and nucleotide diversity was 0.03163, excluding gaps and missing data. A total of 19 haplotypes was found in the *rbcL* locus, where 32 sites were variable; there were no gaps or missing data. The overall haplotype diversity in *rbcL* was 0.728 and nucleotide diversity amounted to 0.00480. In a combination of both loci, when gaps and missing data had been removed, 75 haplotypes were found in our samples and 11 additional haplotypes in *Trebouxia* reference strains.

The photobiont's ITS region exhibited a higher level of diversity than the photobiont locus *rbcL*, as reflected in nucleotide and haplotype diversity (Table 2, Figs. 1, 2), which might in part be explained by the lower sample size of *rbcL*. Variability might also be lower as this locus is a coding region that could be under selection. The locus *rbcL* is also likely to have a lower mutation rate than ITS as haplotype numbers are much lower when comparing sequences of the same samples (Werth and Sork, 2010; Nyati et al., 2014). Haplotype diversity was fairly evenly distributed across ecoregions (Table 2, Fig. 2). Nucleotide diversity was highest in the ecoregions BI, BC, and CC, which included multiple algal clades related to *Trebouxia decolorans* and *T. jamesii* (Fig. 1).

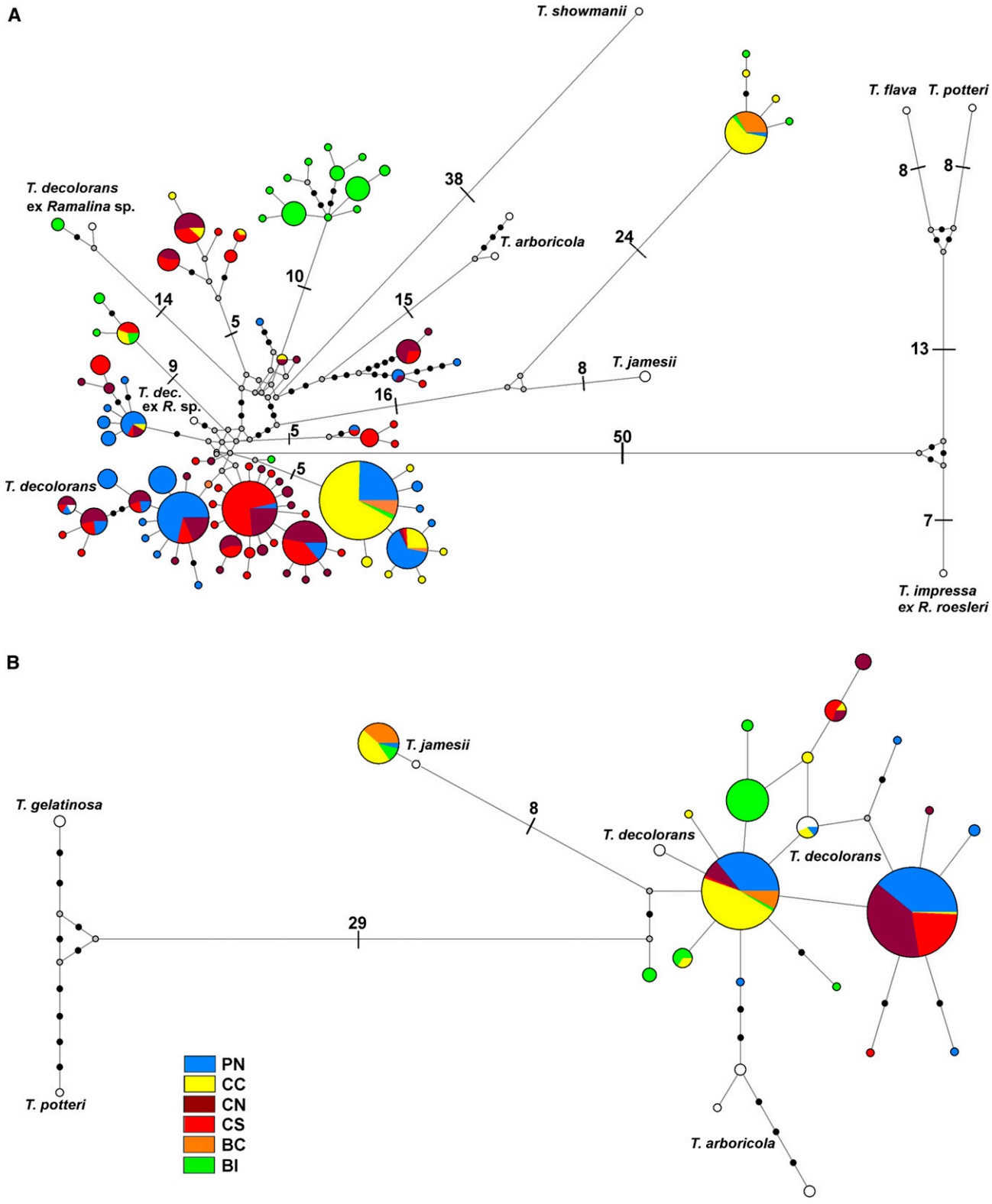


Fig. 1. Median-joining haplotype networks of the *Trebouxia* photobiont associated with *Ramalina menziesii* throughout the lichen's range. Known species of *Trebouxia* and photobionts from other species of *Ramalina* were included in the analysis. Missing data and gaps were excluded for the construction of networks. Missing (unsampled) haplotypes are shown as black dots, median vectors as gray circles. The remaining circles each represent haplotypes, color-coded by six ecoregions. White circles show individuals isolated from other fungal species than *R. menziesii*, or *Trebouxia* algae of known species identity. (A) Algal nuclear ITS region; data set includes 72 samples from site SW in southern California. (B) Algal *rbcL* region (cpDNA); data set without samples from SW.

TABLE 2. Diversity statistics and tests of demographic history for the *Trebouxia* algae associated with *Ramalina menziesii*. The table gives the number of sequences *N*, number of polymorphic sites *s*, nucleotide diversity π, the number of haplotypes *H*, haplotype diversity Hd, Fu's F_S, and Tajima's *D*. The locus abbreviations are ITS, internal transcribed spacer (nuclear ribosomal DNA), and *rbcL*, RuBisCO (chloroplast DNA). Mean values across loci are given in bold.

			Diversity statistics				Demographic statistics [a]	
Pop	Locus	*N*	*s*	π	*H*	Hd	F_S	*D*
BI	ITS	50	103	0.03486	23	0.910	1.50	−0.45
BI	*rbcL*	42	19	0.00471	7	0.546	1.26	−1.19
BI	**Mean**	**46**	**61**	**0.01979**	**15.0**	**0.728**	**1.38**	**−0.82**
BC	ITS	20	65	0.05128	6	0.789	15.74***	2.56**
BC	*rbcL*	17	11	0.00953	2	0.515	10.79***	2.75**
BC	**Mean**	**18.5**	**38**	**0.03041**	**4.0**	**0.652**	**13.27**	**2.66**
CS [b]	ITS	59	81	0.02223	22	0.930	0.62	−0.84
CS	*rbcL*	32	6	0.00148	4	0.286	0.14	−1.16
CS	**Mean**	**45.5**	**43.5**	**0.01186**	**13.0**	**0.608**	**0.38**	**-1.00**
CN	ITS	104	85	0.02268	35	0.941	−1.99+	−0.56
CN	*rbcL*	62	6	0.00173	5	0.389	0.15	−0.48
CN	**Mean**	**83.0**	**45.5**	**0.01221**	**20.0**	**0.665**	**-0.92**	**-0.52**
CC	ITS	123	103	0.03525	21	0.611	12.29***	0.20
CC	*rbcL*	64	17	0.00639	8	0.518	2.65	0.17
CC	**Mean**	**93.5**	**60**	**0.02082**	**14.5**	**0.565**	**7.47**	**0.185**
PN	ITS	150	124	0.01992	29	0.881	0.52	−1.61+
PN	*rbcL*	88	19	0.00158	8	0.563	−2.47+	−2.19**
PN	**Mean**	**119.0**	**71.5**	**0.01075**	**18.5**	**0.722**	**-0.98**	**−1.90**

[a] Tajima's *D* and Fu's F_s were based on the number of segregating sites; significance testing of F_S and *D* was performed assuming free recombination among sites for the specified locus. +, $p < 0.10$ (for Fu's F_S, $p < 0.05$); *, $p < 0.05$ (for Fu's F_S, $p < 0.02$); **, $p < 0.01$; ***, $p < 0.001$.

[b] In region CS, for all analyses, population SW was represented by five randomly drawn samples.

Genetic structure and ecological specialization of photobionts—Despite the different degrees of variability in the ITS and *rbcL* loci and that the two loci sample nuclear and chloroplast DNA, respectively, both exhibited rather consistent patterns of genetic structuring in analysis of molecular variance (Table 3). Populations of *Trebouxia* photobionts associating with *R. menziesii* exhibited substantial population structure by ecoregion (ITS: $F_{CT} = 0.227$***; *rbcL*: $F_{CT} = 0.221$*). This overall model also indicates significant genetic structure among subregions within ecoregions (ITS: $F_{SC} = 0.258$***; *rbcL*: $F_{SC} = 0.295$***).

The hierarchical AMOVA run separately for four ecoregions allowed us to assess the extent to which population substructure was shaped by the association with specific phorophyte species. In three of the ecoregions, there was a significant effect of phorophyte in at least one locus (Table 3). In ecoregions CC and CS, the *rbcL* locus was marginally significant ($0.05 < p < 0.10$) for phorophyte but, in both cases, genetic differentiation among phorophytes was rather high (F_{SC} = 0.312 and 0.208). Ecoregion CN showed an overall lower degree of population subdivision than the other regions with no significant differentiation among subregions. In this ecoregion, there was no significant differentiation according to phorophyte species.

To further identify evidence for ecological specialization, our redundancy analysis quantified the contribution of local climate and phorophyte species. Across three models when holding the effect of phorophyte species constant, we found that climate explained 11.3%, 18.9%, and 41.3% of the total variance explained (TVE), respectively, although in the third model this effect was not significant (Table 4). In contrast, phorophyte species explained 79.5%, 72.5%, and 55.55% of TVE, respectively, for the three models holding climate effects constant with both higher percentages explained and all three models being significant (Table 4). Thus, these multivariate models suggest stronger differentiation associated with phorophyte species than with local climate environment.

DISCUSSION

The photobionts of *Ramalina menziesii* exhibit extensive taxonomic diversity and intraspecific genetic variation throughout the range. Our findings provide evidence in support of our two central hypotheses. Like our previous phylogeographic analysis of fungal genes of *R. menziesii* sampled from Baja California through Alaska (Sork and Werth, 2014), we find significant genetic structure that supports our first hypothesis that *Trebouxia* photobionts should show strong genetic differentiation across major ecoregions. Unlike our previous study of the lichen-forming fungus, we find significant genetic structure across photobionts within ecoregion through AMOVA and significant genetic differentiation in the photobiont lineages associated with both phorophyte species and local climate using partial RDA analyses. These two results provide support for our second hypothesis that *Trebouxia* photobionts are ecologically specialized on different phorophyte species within ecoregions. Here, we discuss these patterns of genetic differentiation on range-wide and local scales in light of the opportunity they create for the ability of the lichenized fungus, *R. menziesii*, to have a widespread distribution.

Identity of photobionts—The main photobiont of *Ramalina menziesii* is *Trebouxia decolorans*. The split between *T. arboricola* and *T. decolorans* was well supported in the Bayesian analysis, and we found no evidence for "typical" *T. arboricola* (strains related to SAG219-2a) in *R. menziesii*. Nevertheless, it remains problematic to discriminate between the two photobiont species. Further studies with higher genetic resolution in

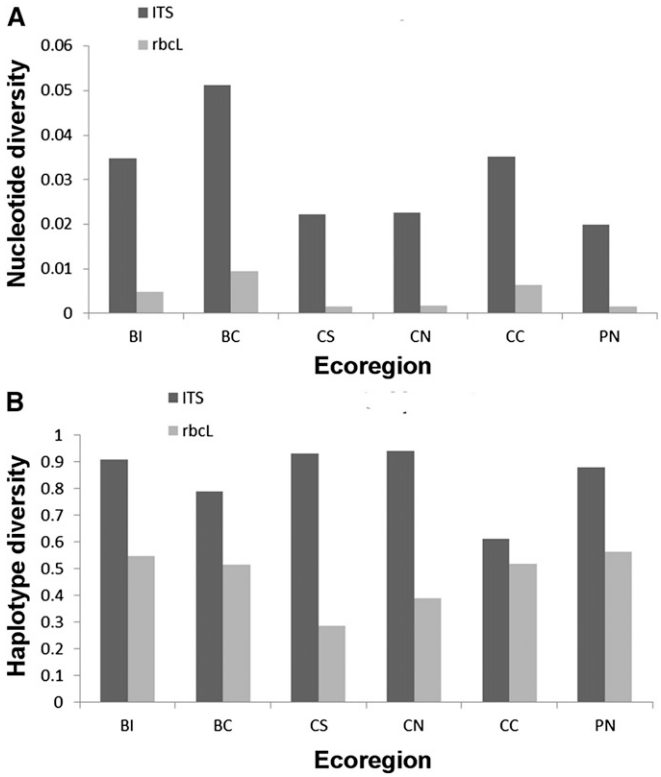


Fig. 2. Diversity statistics of *Trebouxia* photobionts associated with *Ramalina menziesii* in six ecoregions, representing the world distribution of the lichen. (A) Nucleotide diversity. (B) Haplotype diversity. Sites with gaps were excluded.

TABLE 3. Summary of AMOVA results for the *Trebouxia* photobionts of *Ramalina menziesii*, based on two loci and different hierarchical groupings of the data using pooled populations ("subregions"). For the overall model across all ecoregions, F_{CT} represents differentiation across ecoregions, F_{SC} represents population differentiation within ecoregions, and F_{ST} represents subpopulation differentiation across entire species range. For the separate models for ecoregions with sufficient samples sizes to examine genetic structure within subpopulations, F_{RT} represents differentiation across regional populations, F_{SR} represents populations on different phorophyte species within regional populations, and F_{ST} represents populations on different phorophyte species across ecoregion.

Locus	Statistics		
Across ecoregions	F_{CT}	F_{SC}	F_{ST}
ITS	0.277***	0.258***	0.464***
rbcL	0.221*	0.295***	0.451***
Separate models within ecoregions	F_{RT}	F_{SR}	F_{ST}
Ecoregion CC			
ITS	0.313**	0.226**	0.468***
rbcL	0.421*	0.312+	0.602***
Ecoregion CN			
ITS	0.043	0.029	0.071*
rbcL	0.064	0.009	0.072
Ecoregion CS			
ITS	–0.014	0.222***	0.211***
rbcL	0.030	0.208+	0.232*
Ecoregion PN			
ITS	0.259+	0.504***	0.633***
rbcL	0.114	0.329***	0.406***

Notes: +$P < 0.1$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

combination with morphology are required to better resolve the species boundaries. What is clear from our analysis is that the photobionts that we refer to as "*T. decolorans*" include multiple lineages, and some of these are as distantly related to the "*T. decolorans*" reference strain as to *T. arboricola*. Whether these lineages represent distinct species has to be determined in a future revision of the genus.

Previously, we identified *Trebouxia decolorans* to be the photobiont of *Ramalina menziesii* in an oak savanna landscape of southern California (Werth and Sork, 2008, 2010). Here, we confirmed this species as the predominant photobiont of *R. menziesii*, but our data also shows great variability of algal lineages (Figs. 1, 3). The green alga *T. decolorans* is a common photobiont in lichen fungi (Muggia et al., 2013) and has, for instance, been reported from *Xanthoria* lichens in Switzerland, France, and Germany (Beck and Mayr, 2012; Nyati et al., 2013, 2014). In an investigation of photobiont communities in California, *T. decolorans* was found in association with multiple fungal species: *Candelaria concolor* (Dicks.) Arnold, *Physcia adscendens* (Fr.) H. Olivier, *Ramalina farinacea* (L.) Ach., *R. menziesii, R. leptocarpha* Tuck., *Xanthoria hasseana* Räsänen, and *X. tenax* L. Lindblom (Werth, 2012). The same ITS haplotypes were shared among fungal species in the latter study, supporting the concept of groups of lichen-forming fungi sharing a common photobiont pool (Rikkinen, 2003). Photobiont-mediated fungal guilds have also been reported for other lichen-forming fungi (Dal Grande, 2007, 2011).

In 37 of our 575 *R. menziesii* samples (6.4%), we found photobionts belonging to another clade of *Trebouxia*, similar to a cultivated strain of *T. jamesii* (Figs. 1, 3). These photobionts occurred predominantly in coastal sites of Baja California (BC) and California (CC). The green alga *T. jamesii* sensu lato includes several distinct lineages, associates with a wide range of fungal taxa (Beck, 1999; Kroken and Taylor, 2000), and has a large geographic distribution. *Trebouxia jamesii* has been found to associate with the lichen fungus *Thamnolia vermicularis* (Sw.) Schaer. from Alaska, Costa Rica, and northern Norway (Nelsen and Gargas, 2009), with *Letharia vulpina* (L.) Hue from the inland western parts of the United States (Kroken and Taylor, 2000; Altermann, 2009), with parmelioid lichens of eastern Canada (Piercey-Normore, 2006, 2009), and with lecideoid lichens of Antarctica (Ruprecht et al., 2012). Even though the photobionts of *R. menziesii* related to *T. jamesii* likely occur in other parts of the range of *R. menziesii*, it is found in association with *R. menziesii* in the most southern part of the range of this lichen, and nowhere is it a common photobiont of *R. menziesii*.

Genetic diversity of photobiont pool— Across its range, the lichenized fungus *R. menziesii* associates with multiple clades belonging to two green algal species, forming a diverse and geographically structured photobiont pool. Haplotype diversity was more or less uniformly high across ecoregions, whereas nucleotide diversity was highest in BI, BC, and CC, as expected in these regions that included multiple photobiont species as well as several not closely related groups of haplotypes within species. The genetic diversity of the photobionts of *R. menziesii* is difficult to compare with other species because only three prior studies have performed a range-wide quantification of photobiont diversity for a single lichen-forming fungus. In the lichen *Cetraria aculeata*, trebouxioid photobiont diversity was highest in the temperate

TABLE 4. Results from partial redundancy analysis (RDA), partitioning variance in *Trebouxia* ITS and *rbcL* haplotypes (presence/absence) found in association with *Ramalina menziesii* onto thee variable sets of climate variables (C.1, C.2, C.3) and phorophyte (P.1, P.2, P.3). Axes resulting from principal component analysis were used as climatic and phorophyte variables. In the climate matrices, principal components were based on 19 bioclimatic variables in 110 sites. In the phorophyte matrices, principal components were based on presence/absence of 30 phorophyte species across 110 sites. The table gives the model and denotation of each term, sum of all canonical eigenvalues (EV), fraction of total variance (FTVE), the pseudo-*F* statistic and significance (*p*) from a permutation test using 1000 permutations, the number of phorophyte (Var.p) and climate (Var.c) variables in the model, and the eigenvalue cutoff (Cutoff) used to select PCA axes for the model. The symbol "|" stands for "conditioned on". Total variance was 0.817. For further details, refer to the Methods.

Model	Denotation	EV	FTVE	*F*	*p*	Var.p	Var.c	Cutoff
C.1 | P.1	Climate without phorophyte	0.03236	11.3	1.3	0.038	17	4	1.0
C.2 | P.2	Climate without phorophyte	0.03190	18.9	1.6	0.004	7	3	1.5
C.3 | P.3	Climate without phorophyte	0.01844	41.3	1.2	0.142	2	2	2.0
P.1 | C.1	Phorophyte without climate	0.22743	79.5	2.2	0.001	17	4	1.0
P.2 | C.2	Phorophyte without climate	0.12205	72.5	2.7	0.001	7	3	1.5
P.3 | C.3	Phorophyte without climate	0.02489	55.5	1.7	0.001	2	2	2.0

zone, as compared with polar and antarctic regions (Fernández-Mendoza et al., 2011; Domaschke et al., 2012). Photobiont haplotype numbers in *R. menziesii* (6–35 ITS haplotypes and 2–8 *rbcL* haplotypes per ecoregion) were comparable with those of *C. aculeata* (3–14 ITS haplotypes, 1–4 COX1 haplotypes, and 2–13 actin haplotypes per geographic region). In photobionts of *R. menziesii*, the highest diversity of clades (high nucleotide diversity) was found in southern localities on the Baja California Peninsula. These southern localities never experienced glaciations during the climatic fluctuations associated with Pleistocene cooling and warming cycles. Yahr et al. (2006) studied the photobionts associated with *Cladina subtenuis* (Abbayes) Hale & W.L. Culb. in the southeastern United States. Similar to what we found in *R. menziesii*, this lichen fungus associated with several closely related clades of green algae across its range. Other authors have looked at parts of wide distribution ranges. For instance, in European populations, *Lobaria pulmonaria* (L.) Hoffm. associates with multiple highly diverse gene pools of the green alga *Dictyochloropsis reticulata* (Tschermak-Woess) Tschermak-Woess. In *L. pulmonaria*, fungal and algal gene pools show similar spatial distributions (Widmer et al., 2012), implying the predominance of codispersal of the photobiont with the mycobiont (Dal Grande et al., 2012; Werth and Scheidegger, 2012). High haplotype diversity, comparable with that found in *R. menziesii*, has been reported for the photobionts of *Letharia vulpina*, a species complex found in Africa, Europe, and America; these taxa associate with several clades of *T. jamesii* in inland regions of western North America (Kroken and Taylor, 2000; Altermann, 2009). Thus, overall, the photobiont diversity we report here seems to be similar to that found in other lichens.

Photobiont genetic structure—In support of the first hypotheses, populations of lichen photobionts showed population subdivision at large spatial scales across ecoregions. This structure could have been shaped either by topographical boundaries that reduced gene flow among ecoregions or by selection due to climate and habitat differences between ecoregions. Such genetic structure has also been found in other studies. In the *Trebouxia* photobionts associating with *Thamnolia vermicularis* and *Cetraria aculeata*, two studies found population subdivision by ecogeographic regions (Nelsen and Gargas, 2009; Fernández-Mendoza et al., 2011), similar to what we found with respect to differentiation between ecoregions in the current study. The two studies compared populations of the lichens situated on different continents, and, not surprisingly, the genetic differentiation among ecogeographic regions was higher than what we found for *R. menziesii*. The photobiont of *Lobaria pulmonaria*, *Dictyochloropsis reticulata*, had regionally subdivided populations in Europe (Widmer et al., 2012), but there was also genetic differentiation between collecting sites on a local scale (Werth and Scheidegger, 2012).

Few prior studies have reported results concerning the genetic structure of *Trebouxia decolorans* associated with lichen fungi. Nyati and coworkers (2013, 2014) found little population structure in *T. decolorans* associating with *Xanthoria* spp. on a local scale, but genetic differentiation was substantial between geographically distant sites. Genetic differentiation was also low at a local scale in *T. decolorans* associating with *R. menziesii* (F_{ST} = 0.009–0.047, Werth and Sork, 2010).

Ecological specialization of photobionts—Our findings support our second central hypothesis that ecological specialization is shaping the genetic structure of the photobionts. First, we found substantial differentiation between the photobionts of lichens growing on different phorophyte species in AMOVA. While we cannot rule out that this genetic differentiation was not initially created by reduced gene flow once algal populations established on different phorophyte species, this tendency to be found on only a subset of available plants creates the opportunity for differential selection on algal populations found on different phorophyte species. If it was due to reduced gene flow alone, we would have found high genetic structure, but it would not be associated with the species of woody plant occupied by the algal strain. It remains to be seen whether this pattern will be confirmed for other lichen symbioses. Other studies report subtle (Muggia et al., 2008) or large (Nyati et al., 2013) genetic differences between photobionts of lichens growing in epiphytic and saxicolous habitats.

Second, we found a significant effect of phorophyte on variation in ITS and *rbcL* haplotypes of *Trebouxia* sp. in RDA multivariate analysis. Previous work on other lichens suggests that ecological specialization might well represent a general pattern in lichen photobionts (Yahr et al., 2006; Fernández-Mendoza et al., 2011). For example, evidence of ecological specialization has been reported from *Trebouxia* and *Asterochloris* photobionts (Yahr et al., 2006; Nelsen and Gargas, 2009; Peksa and Škaloud, 2011). *Asterochloris* photobionts associating with *Cladonia subtenuis* exhibited population subdivision according to habitat (Yahr et al., 2006). Other authors found evidence for ecological specialization in the *Asterochloris* photobionts of *Lepraria* and *Stereocaulon* lichens (Peksa and Škaloud, 2011). In the latter study, photobionts grouped according to the ecology of their mycobionts.

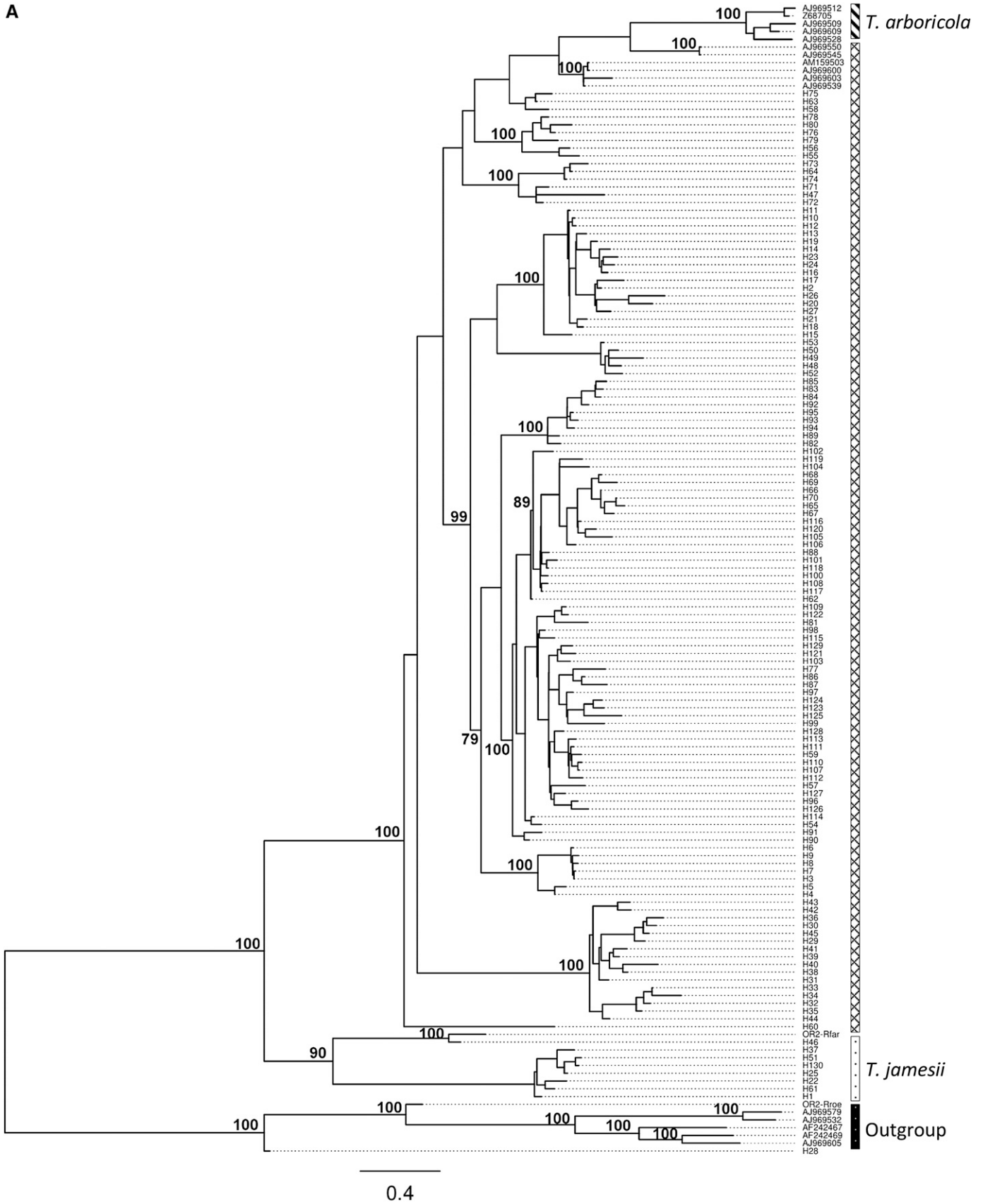


Fig. 3. Bayesian phylogenetic trees of *Trebouxia* photobionts of *Ramalina menziesii.* Support values of nodes represent posterior probabilities from the Bayesian analyses. (A) Nuclear ribosomal ITS region. (B) Chloroplast locus *rbcL.* (C) Combined tree of ITS and *rbcL.*

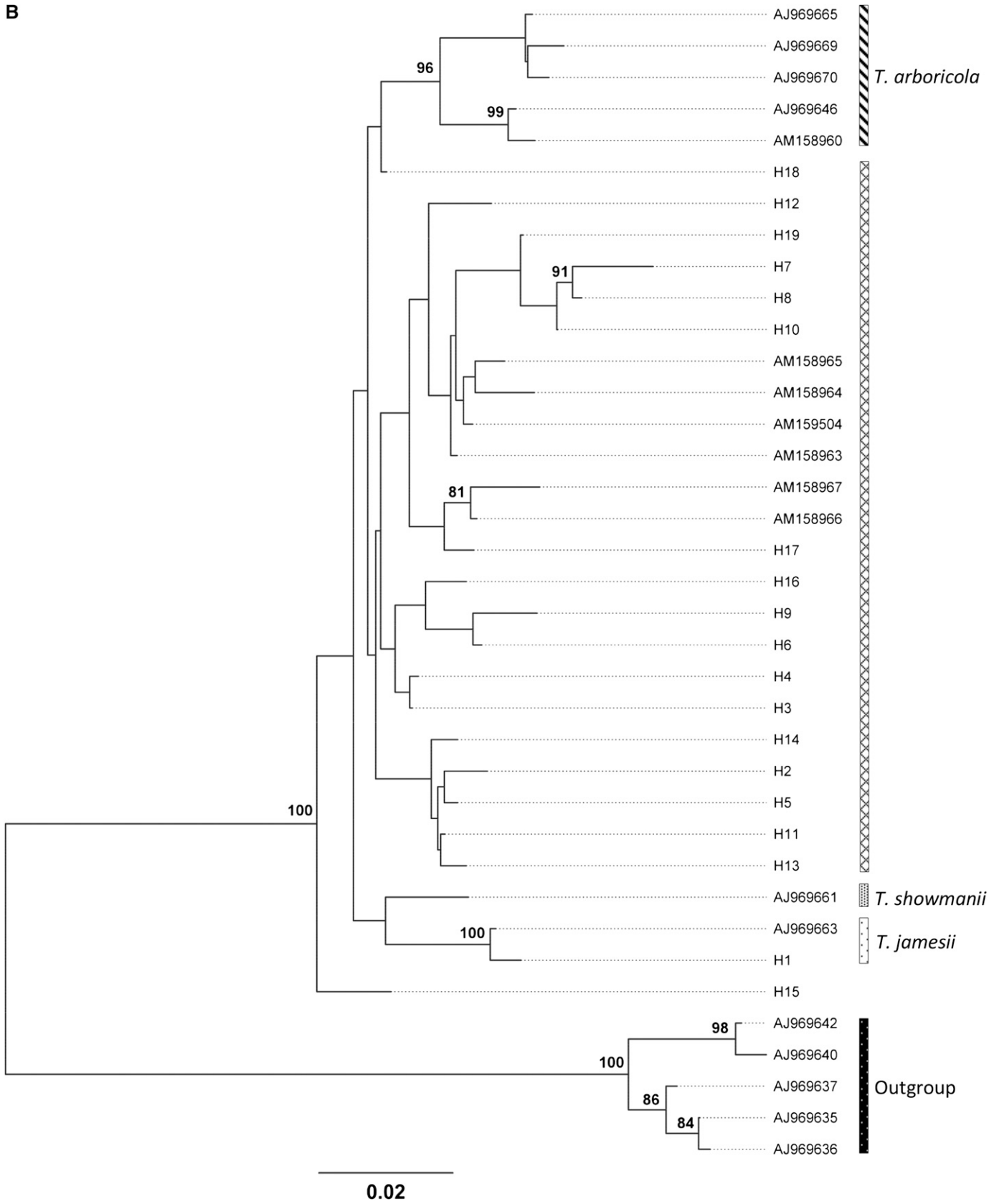
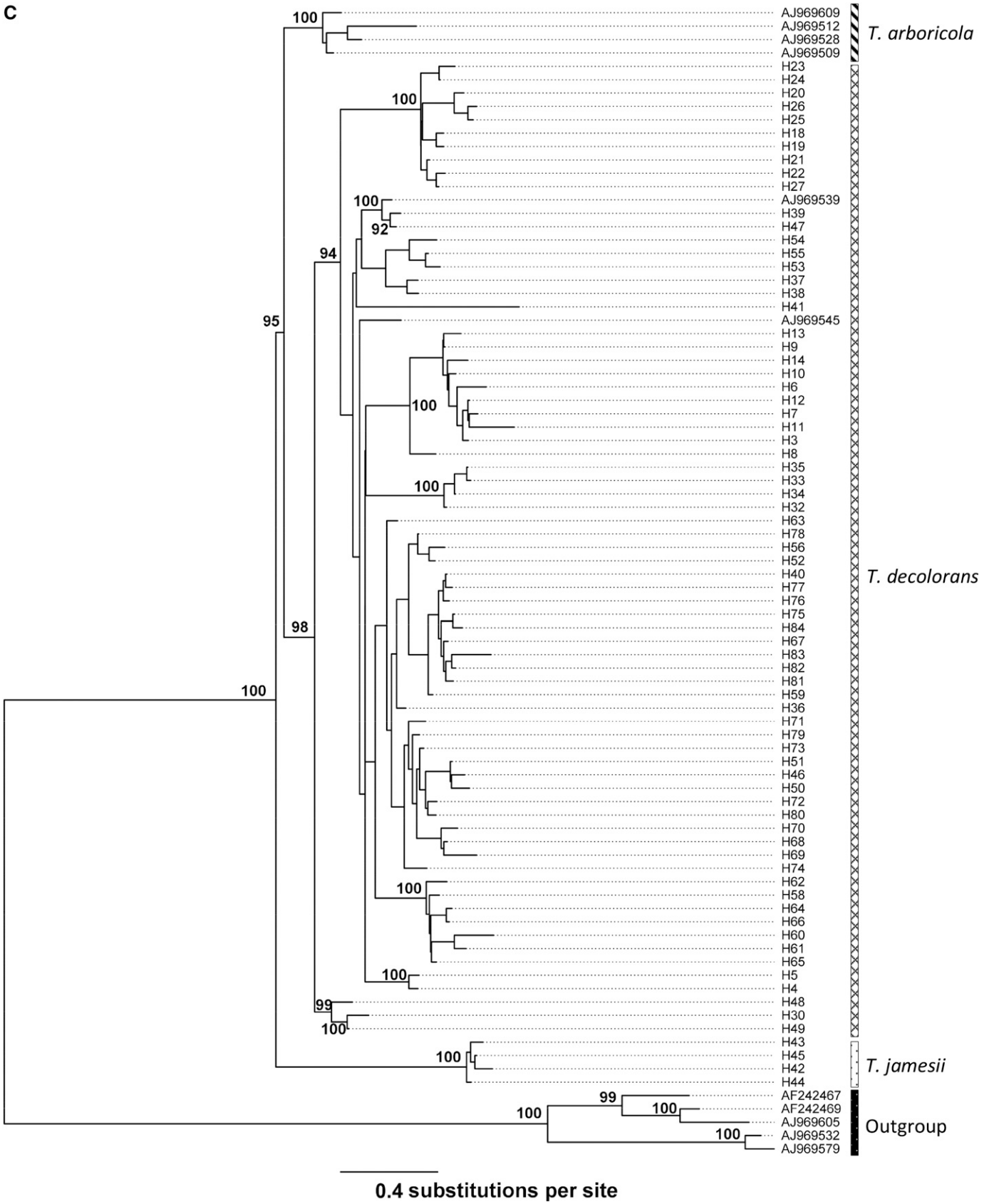


Fig. 3. Continued.



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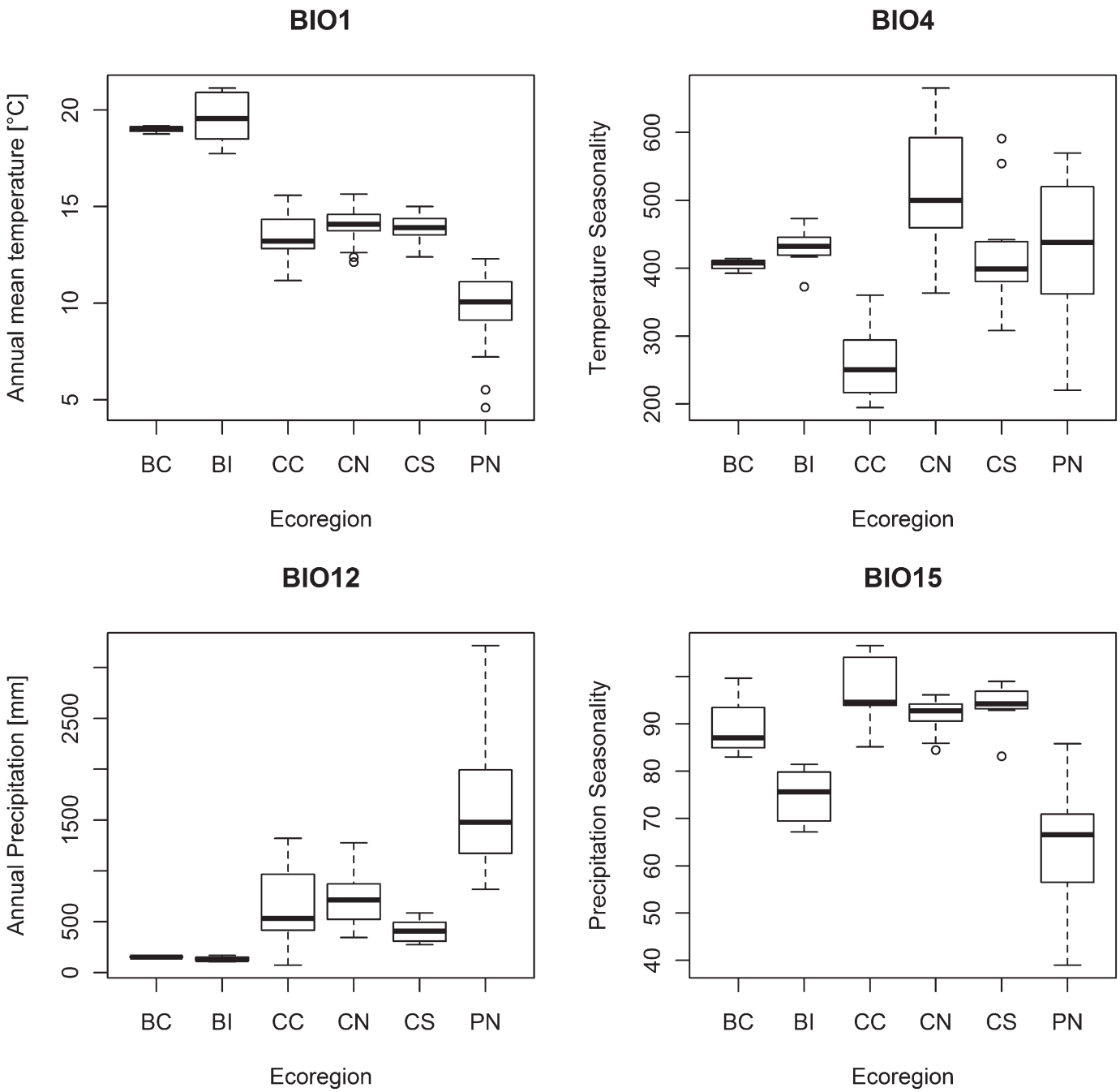

Fig. 4. Boxplots characterizing the macroclimate of ecoregions, showing four bioclimatic variables, mean annual temperature (BIO1), temperature seasonality (BIO4), annual precipitation (BIO12), and precipitation seasonality (BIO15). Climate data: Bioclim/Worldclim (Busby, 1991; Hijmans et al., 2005).

Third, we found evidence that multivariate genetic differentiation was also associated with local climate conditions, which differed markedly among ecoregions (Fig. 4). In our RDAs, we found that two of the three models had a significant portion of the variance explained by climate when holding phorophyte species constant. These findings indicate that genetic differentiation due to local climate is another source of local adaptation in the algal populations. Overall, the multivariate analyses showed a high proportion of total variance explained by both phorophyte species and climate, and both contribute to ecological specialization of the algal populations.

In conclusion, our data make a strong case for the ecological specialization of *Trebouxia* photobiont populations above and beyond the genetic differences due to ecoregion and local climate, and they suggest that the occurrence of *Trebouxia* haplotypes depends on macroclimatic factors and substrate ecology (phorophyte species). Fungal ascospores that germinate and incorporate locally adapted algal strains will develop a lichen individual that benefits from the algal specialization on local environmental conditions. This process may be an important factor in promoting the wide distribution of *R. menziesii* across multiple western North

American ecoregions and phorophyte species from Baja California to Alaska. Moreover, these findings illustrate a great advantage of independent symbiont dispersal leading to symbiotic associations with locally adapted photobiont strains. Future work might look for evidence of ecological specialization in other widespread lichen species that are dispersed by fungal ascospores rather than vegetative propagules.

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