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Phylogeography of *Ramalina menziesii*, a widely distributed lichen-forming fungus in western North America

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Abstract

The complex topography and climate history of western North America offer a setting where lineage formation, accumulation and migration have led to elevated inter- and intraspecific biodiversity in many taxa. Here, we study *Ramalina menziesii*, an epiphytic lichenized fungus with a range encompassing major ecosystems from Baja California to Alaska to explore the predictions of two hypotheses: (i) that the widespread distribution of *R. menziesii* is due to a single migration episode from a single lineage and (ii) that the widespread distribution is due to the formation and persistence of multiple lineages structured throughout the species' range. To obtain evidence for these predictions, we first construct a phylogenetic tree and identify multiple lineages structured throughout the species' range – some ancient ones that are localized and other more recent lineages that are widely distributed. Second, we use an isolation with migration model to show that sets of ecoregion populations diverged from each other at different times, demonstrating the importance of historical and current barriers to gene flow. Third, we estimated migration rates among ecoregions and find that Baja California populations are relatively isolated, that inland California ecoregion populations do not send out emigrants and that migration out of California coastal and Pacific Northwest populations into inland California ecoregions is high. Such intraspecific geographical patterns of population persistence and dispersal both contribute to the wide range of this genetically diverse lichen fungus and provide insight into the evolutionary processes that enhance species diversity of the California Floristic Province.

Keywords: Ascomycota, California Floristic Province, coalescent, diversification, long-distance dispersal, symbiosis

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Introduction

Understanding the phylogeography of genetic lineages is crucial to the study of populations because they provide useful insights about the evolutionary processes that lead to population subdivision and species formation (Avise 2000). Western North America provides numerous examples of how complex topography and climatic variation have shaped the diversification of lineages within and across species (Calsbeek *et al.* 2003; Carstens *et al.* 2005; Rissler *et al.* 2006). This large region has broad latitudinal temperature and precipitation gradients, as well as regional longitudinal variation, which separates coastal regions with marine climate-moderating influences in the west from the highly seasonal climates in the interior. The northern region of western North America, dubbed the Vancouverian

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Floristic Province (Raven & Axelrod 1978; Thorne 1993), was largely colonized after deglaciation and is characterized by low diversity (Heusser & Igarashi 1994; Soltis *et al.* 1997; Nielson *et al.* 2001; Carstens *et al.* 2005; Gugger *et al.* 2010). In the north, one might expect the evolutionary histories of species to be dominated by large-scale extinction/expansion cycles created by glaciation with corresponding patterns of migration during warming and cooling periods. The source of biodiversity within genera or within a species may be refugia in local unglaciated patches (Printzen *et al.* 2003; Carstens *et al.* 2005; Godbout *et al.* 2008) or recolonization from southern unglaciated regions (Taberlet *et al.* 1998; Hewitt 2000, 2004; Petit *et al.* 2003).

Conversely, in the unglaciated regions south of the Cordilleran ice sheet regions near the California/Oregon border south to northern Baja California known as the California Floristic Province, CFP (Stebbins & Major 1965; Raven & Axelrod 1978), taxa may be expected to be less affected by climatic fluctuations but still structured by topographic barriers, such as the north–south Coast and Sierra Nevada Ranges, and the east–west Transverse Ranges in the south. This topography, which shapes migration patterns before and after genetic bottlenecks created by climate fluctuations, likely contributes to the tremendous biodiversity and high degree of endemism in the CFP (Calsbeek *et al.* 2003), including more than 5500 native plant species, approximately 40% of which are endemic (Lancaster and Kay 2013). The CFP is recognized as one of the world's hotspots of biodiversity (Mittermeier *et al.* 2005). In a recent phylogenetic analysis of 16 angiosperm clades of California plant species, Lancaster and Kay (2013) conclude that the high biodiversity of the CFP primarily results from low extinction rates due to favourable climate rather than elevated speciation or immigration rates, although they also point out that the complex topography has shaped lineage diversification. If these processes are shaping species diversity, then they should affect intraspecific diversity where we can also observe the impact on lineage formation and migration patterns.

We investigated these theoretical expectations in the context of the phylogeography of the widely distributed lichen fungus *Ramalina menziesii* Taylor (Lecanorales, Ramalinaceae), which spans the temperate and boreal zones of the Vancouverian Floristic Province (Raven & Axelrod 1978; Thorne 1993) to the Mediterranean zones of the CFP and the subtropical zone of the Vizcaino desert in Baja California (Rundel 1974). This species intersects six ecoregions (*sensu*, Omernik 2004), each separated by topographic barriers and climate zones. Along its range, this species experiences several phorophyte (shrub or tree species) shifts across different ecoregions. Numerous studies focus on the phylogeography of species in the northern part of western North America (e.g. Soltis *et al.* 1997; Brunsfeld *et al.* 2001; Printzen *et al.* 2003; Carstens *et al.* 2005) and species in the CFP (e.g. Calsbeek *et al.* 2003; Lapointe & Rissler 2005; Burns & Barhoum 2006; Grivet *et al.* 2006; Rissler *et al.* 2006; Chatzimanolis & Caterino 2007). However, a study of a species that spans multiple regions provides a unique opportunity to assess the impact of geographical barriers, climate and ecoregion on the formation and spread of lineages across coastal western North America.

Ramalina menziesii provides a unique opportunity for studying the contributions of dispersal and population persistence because, as a cryptogam (organisms such as algae, mosses, fungi (lichenized or not) and ferns), it disperses via microscopic spores rather than seeds, which might facilitate movement across topographic boundaries. Many cryptogams are distributed across continents, intersecting multiple ecoregions (Brodo *et al.* 2001; Otte *et al.* 2005; Medina *et al.* 2011; Werth 2011). One hypothesis is that spore dispersal should enable these organisms to disperse over long distances and colonize new habitats (Fontaneto 2011). Alternatively, the wide species range could include a patchwork of local lineages that evolved and persisted in separate regions for long periods of time and dispersed when favourable climatic conditions allowed their survival in other regions. As a poikilohydric organism, the lichen requires moisture in the form of frequent fog in coastal sites. Inland sites are characterized by winter rain (Matthes-Sears *et al.* 1986), morning dew or proximity to water bodies (S. Werth, V. L. Sork, personal observation). These environmental requirements constrain potential locations to sites characterized by fog, through either advection fog caused by the movement of subtropical high-pressure centres resulting in transport and upwelling of cold currents adjacent to coastal Baja California or California, or fog in inland areas that is created by the assembly of cold air moving downwards from mountain regions and rising warm air masses in valleys. Thus, dry climate can be as much of a barrier to this species as mountain ranges. Taken together, these barriers may have isolated populations but not jeopardized local survival.

This study will explore the predictions of two key hypotheses: (i) that the widespread distribution of *R. menziesii* is due to a single migration episode from a single lineage and (ii) that the widespread distribution is due to the formation and persistence of multiple lineages structured throughout the species' range. To obtain evidence for these predictions, we have three specific objectives based on four low copy nuclear genes. First, we will construct a phylogenetic tree to identify unique clades and determine whether there is a

single widely distributed lineage, or multiple lineages structured throughout the species' range. We will also assess the extent to which four-locus genotypes are restricted to single ecoregions or distributed across ecoregions. Second, we will test whether ecoregion populations diverged from each other at different times. Understanding the timing of divergence between ecoregions will provide support for the importance of historical and current barriers to gene flow. Third, we will estimate migration rates among ecoregions. If the widespread distribution of the species and spatial patterns of genetic diversity are shaped by extensive gene flow, then we should find evidence of this gene flow among ecoregions. Our data provide a unique case study of how lineage formation and migration have shaped geographical patterns of genetic diversity in a species that is capable of both extensive dispersal and population persistence, processes that ultimately led to the evolution of biodiversity hotspots in western North America.

Methods

Study species

Ramalina menziesii is a haploid lichen-forming ascomycete, which forms a mutualistic symbiosis with a ubiquitous lichen photobiont, the green algae *Trebouxia decolorans* Ahmadjian (Werth & Sork 2008, 2010) and a species closely related to *T. jamesii* (Hildreth & Ahmadjian) Gärtner (Werth and Sork, in review). *Ramalina menziesii* is a predominantly sexual fungus (Werth & Sork 2008). In the fruiting bodies of the lichen, fungal spores form and then disperse, germinate and become lichenized if they encounter a compatible photobiont (Scheidegger & Werth 2009; Werth & Sork 2010). *Ramalina menziesii* is obligately associated with woody plant species (phorophytes) with a tendency towards certain species within local environments.

Ramalina menziesii is a common epiphyte in coastal western North America, ranging from Baja California in Mexico to southeastern Alaska (Rundel 1974), as indicated by our sampling localities (Fig. 1). Along its range, the species experiences several phorophyte shifts.

Study area and sampling design

The study area covers the entire range of *R. menziesii* from Baja California to Alaska (Fig. 1; Supporting Information Table S1A). We sampled 110 localities and collected one thallus of *R. menziesii* from 3 to 5 trees per locality, resulting in 509 samples. Multiple samples ($n = 72$) were collected from the UC Santa Barbara Sedgwick Reserve in southern California in a previous study (Werth & Sork 2008), but for the present analyses, we included only five randomly selected samples.

The localities were distributed among six ecoregions, which are defined by climate, physical landscape features and vegetation: (i) the Baja California inland (BI) region classified as subtropical desert; (ii) the Baja California coastal (BC) region that was characterized by coastal Mediterranean chaparral vegetation; (iii) the California coastal (CC) region consisting of Mediterranean coastal woodlands and chaparral sites situated west of the Coast Ranges, north of Baja California; (iv) the southern California inland region (CS) that included Mediterranean oak savannas in the foothills on the inland side of the Coast Ranges, the Central Valley and the southern foothills of the Sierra Nevada; (v) the northern California inland region (CN) that included northern oak woodlands, oak savannah and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco (Pinaceae)), forests on the inland side of the northern Coast Ranges, the northern part of the Central Valley and the northern foothills of the Sierra Nevada; and (vi) the Pacific Northwest (PN) region, a region that was partially glaciated during the last glacial period and consists of interior temperate broad-leaved forests and coastal temperate coniferous forests. To simplify analyses, we pooled haplotypes from the coastal ecosystems of the Pacific Northwest and the inland region of Oregon into one ecoregion (once preliminary analysis revealed that haplotypes within these two regions showed no genetic differentiation and extensive haplotype intermixing). In addition, we split the inland California ecoregions using climatic criteria when preliminary analyses revealed separate lineages in each region that hampered our ability to estimate divergence and migration among subpopulations with the combined data.

Laboratory procedures

We sequenced four unlinked, low copy nuclear genes: β-tubulin (*bet*), elongation factor 1-α (*efa*), glyceraldehyde 3-phosphate dehydrogenase (*gpd*) and an unidentified locus similar to glycine dehydrogenase (e-score 5×10–117, *uid*, Werth & Sork 2008). These markers were available through other fungal genetic studies (*uid*, Carbone & Kohn 1999; *gpd*, *efa*, Johannesson *et al.* 2000; *bet*, Myllys *et al.* 2002). DNA extraction, PCR and sequencing conditions followed the methods given in Werth & Sork (2008). A few samples from desert sites of the Baja California Peninsula did not amplify with the primer set used for β-tubulin by Werth & Sork (2008). To sequence these samples, two new forward primers were developed, BT3-SW1.F (sequence 5′-GCG YAT GAA CGT CTA CTT CA-3′) and BT3-SW2.F (sequence 5′-TGA ACG TCT ACT TCA ATG AG-3′,

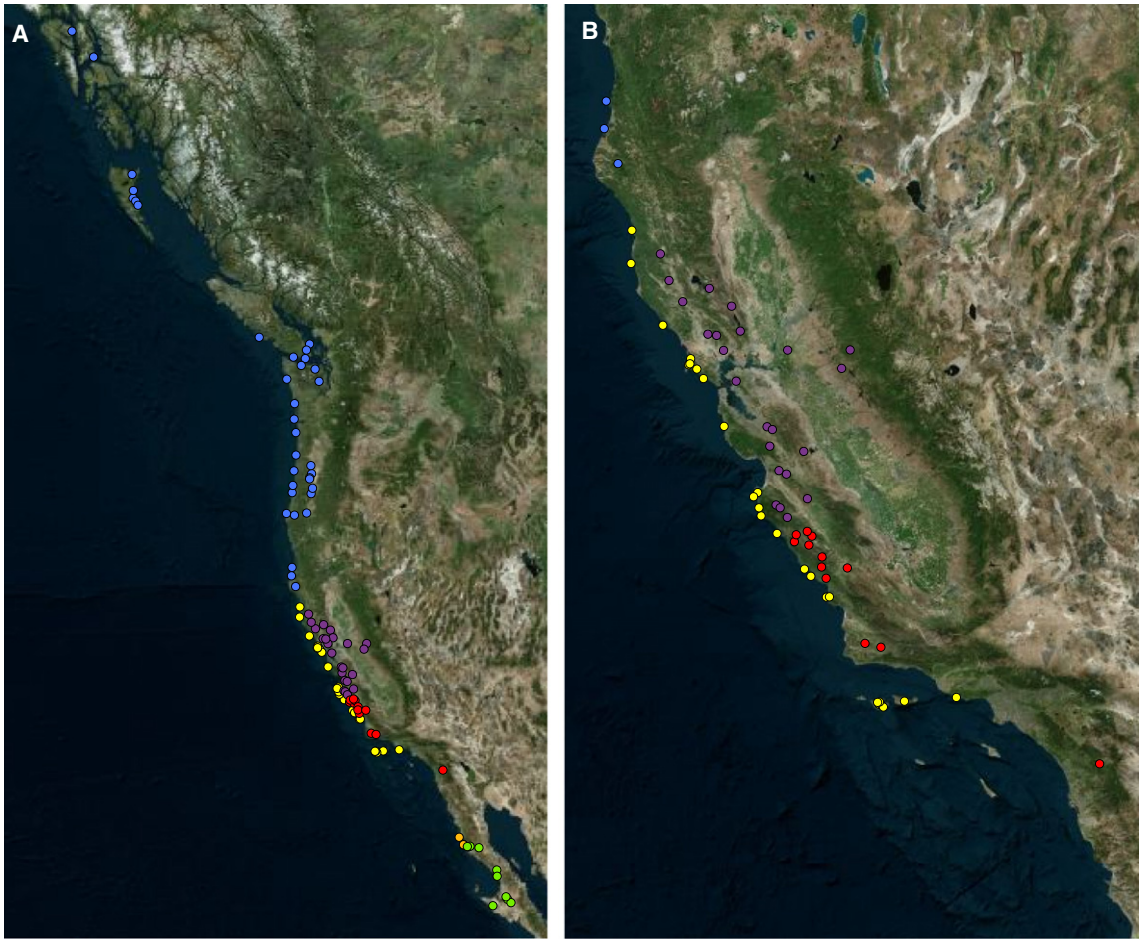

Fig. 1 Map of collection sites of *Ramalina menziesii* in western North America. Collection sites are colour-coded by ecoregion, as defined by habitat type and topographic barriers. Map source: Google Earth. BI = Baja California inland fog desert (green); BC = Baja California coastal chaparral (orange); CS = California southern inland ecoregion (red); CN = California northern inland ecoregion (purple); CC = California coast (yellow); PN = Pacific Northwest (blue). Map source: Google Earth.

amplicon 1 bp longer than when using Bt3-LM forward primer by Myllys *et al.* 2002). Together with the reverse primer Bt10-LM (Myllys *et al.* 2002), these primers produced a single product at annealing temperatures between 50 and 53 °C.

DNA sequence analysis

DNA sequences were edited and aligned with MEGA version 4.0 (Tamura *et al.* 2007). The sequences of the haploid fungus did not require phasing. Haplotype maps and GenBank accessions are given in Supporting Information Table S2. As an outgroup taxon, we used the closely related *R. sonorensis* collected in site BA11 (Table S2).

We used the Perl script IMgc to generate the longest nonrecombining block of DNA sequence for each locus while retaining the maximum sequence length obtainable without dropping individuals (Woerner *et al.* 2007). This removal was necessary for our coalescent analyses that assumed no recombination. For all analyses, we use the largest nonrecombining block of sequence.

Background information on genetic diversity and structure

As background information on subpopulations associated with each ecoregion, we calculated measures of genetic diversity and tested for selective neutrality and population size changes for the following measures using DnaSP version 4.50 (Rozas *et al.* 2003): number of polymorphic sites (s), nucleotide diversity (π), number of haplotypes (H), haplotype diversity (Hd), Tajima's (1989a,b), and Fu's F_S (1997). For each locus, significance of F_S and D was assessed for the six ecoregions in DnaSP with 1000 coalescent simulations; P-values were adjusted for multiple testing by controlling the false discovery rate (Benjamini & Hochberg 1995).

The distribution of genetic variation within and between populations of *R. menziesii* across the six ecoregions was assessed using analysis of molecular variance (AMOVA) in GENALEX version 6 (Peakall & Smouse 2006). We excluded four populations that contained only one sample.

Using the longest nonrecombining block of DNA sequence for each locus, we conducted Bayesian analysis of population structure using BAPS version 6.0 (Corander *et al.* 2003, 2004) with a codon model for the sequence data in mixture analysis to define groups of individuals. Several runs were performed with varying upper number of clusters, up to K = 40. For the partition of the data with the highest log likelihood (K = 8), the partitioning of the data was plotted in GENGIS version 2.2.0 (Parks *et al.* 2013). Admixed individuals were assigned to a given cluster if their probability of belonging to a group was ≥0.8.

Genetic lineages

To identify genetic lineages of *R. menziesii*, we estimated Bayesian Markov Chain Monte Carlo (MCMC) trees as implemented in the software BEAST. To reduce the run time, we resampled all ecoregions to 50 individuals prior to analyses. We used a coalescent prior assuming constant population size. Based on results from MRMODELTEST (Nylander 2004), we used the general time reversible (GTR) substitution model (Tavaré 1986) for the genes *bet* and *efa* and the HKY substitution model (Hasegawa *et al.* 1985) for *gpd* and *uid*. Three runs of 20 M generations sampling trees every 1000 generations with a burn-in of 10% were combined into a single tree, which was generated using unlinked tree topologies among loci. A maximum clade credibility tree was computed based on 54 000 trees with TREEANNOTATOR version 1.7.5 (Rambaut & Drummond 2007). Effective sample sizes >100 for all parameters, calculated with TRACER version 1.4.1 (Rambaut & Drummond 2007), indicated that runs had converged. *R. sonorensis* was the outgroup for the tree, which was drawn in FIGTREE version 1.2 (Rambout 2008, available from http://tree.bio.ed.ac.uk/software/figtree/).

To describe the haplotype networks of each locus, we created median-joining haplotype networks using NETWORK version 4.51 (Bandelt *et al.* 1999) and visually assessed haplotype distribution among ecoregions.

Divergence among ecoregions and patterns of migration

To assess divergence times and migration among ecoregions, we applied the isolation with migration model (Nielsen & Wakeley 2001) as implemented in IMa2 (Hey 2010). We chose this widely used program for estimating divergence times and levels of gene flow between recently diverged populations because it provides robust estimates of molecular demographic parameters as long as nonrecombined sequences are used (Strasburg & Rieseberg 2010). Individuals with missing loci (~10%) were excluded from the analyses, resulting in 462 individuals with data for all loci. Due to the lack of convergence of runs comparing more than two populations at a time, we tested pairwise comparisons of an ecoregion of interest versus the remaining (pooled) ecoregions (using the example of Strasburg & Rieseberg 2010). To estimate divergence time while minimizing parameters, we ran the model assuming no migration with three pairwise comparisons, recognizing that divergence times may be underestimated (Leaché *et al.* 2014). The Early Divergence model contrasted the pooled ecoregions of Baja California (BI, BC) with all remaining ecoregions (CC, CS, CN, PN). The Mid-divergence model contrasted the pooled inland ecoregions of California (CS, CN) with the pooled coastal ecoregions of California and the Pacific Northwest (CC, PN). The Late Divergence model contrasted the pooled unglaciated ecoregions of California (CC, CS and CN) with a largely glaciated region, the Pacific Northwest. After a burn-in phase of at least 2.9×10^6 (range 2.9×10^6 to 8.4×10^6) with priors obtained from preliminary runs ($m = 10$, $q = 10$, $t = 20$), a total of 300 000 trees were generated to sample the posterior distribution in multiple shorter (30 000 trees) runs, which were combined in an *L*-mode run to estimate parameters. Divergence time was converted into chronological time units based on substitution rates of nuclear genes reported for lichen-forming fungi in the family Parmeliaceae: β-tubulin (0.83×10^{-9} substitutions per site per year) (Leavitt *et al.* 2012b), RPB2 (1.391×10^{-9}) (Leavitt *et al.* 2012b) and MCM7 (1.649×10^{-9}) (Leavitt *et al.* 2012a). To provide estimates for low, medium and high mutation rates, respectively, we multiplied these rates with the number of sites (*bet*, $n = 810$, *gpd*, $n = 428$, *efa* $n = 109$, *uid*, $n = 354$) to calculate a mutation rate per locus, and then calculated the geometric mean across these rates. Thus, the scaled mutation rate estimates μ were 2.82×10^{-7} (low mutation rate), 4.73×10^{-7} (medium) and 5.61×10^{-7} (high). Divergence time was converted to chronological time units using the methods described by Hey (2007, 2010). We used 5 years as a generation time for *R. menziesii*, given that it is likely to be in the range of 2–10 years. If generation time is longer than 5 years, the population size estimates may be smaller, but this parameter was used only to examine relative order of magnitude. To estimate bidirectional gene flow among ecoregions, we used the same set of three pairwise comparisons and the same model parameters in IMa2, but now included migration in the models. We

then inferred migration rate (M), scaled population size (θ) and divergence time with three pairwise comparisons.

Results

Background information on genetic diversity and structure

Our alignments included 810 bp from *bet*, 499 bp from *efa*, 586 bp from *gpd* and 789 bp from *uid*, for a total of 2684 bp (Table S2A–D). Before removing recombined sections of the loci, the raw data included 79 haplotypes in *efa*, 72 in *uid*, 31 in *gpd* and 22 in *bet* (Table S2A–D), yielding a total of 264 four-locus genotypes. However, for our analysis, we considered only the largest nonrecombining block of sequence for each locus, which resulted in 16 haplotypes in *bet*, 21 in *uid* and *gpd* and 17 in *efa* and a total of 98 four-locus genotypes. Each ecoregion had unique haplotypes and all ecoregions shared haplotypes except the two Baja California regions (Fig. S1).

Overall, the diversity measures based on the largest nonrecombining block of DNA sequence indicate that CC and CS ecoregions have the highest values of nucleotide and haplotype diversity across ecoregions, with PN also high (see Table S3). Sample sizes across ecoregions varied from 18 to 125 individuals. For the ecoregion BI, the significantly negative Tajima's *D* at one locus and the significantly negative F_S at another locus indicate a possibility that these regions have experienced demographic expansion or purifying selection at those loci. However, none of the other ecoregions had unusual values of Tajima's *D* or F_S (Table S3) so we take these comparisons lightly.

The AMOVA revealed substantial structure among ecoregions ($\Phi_{RT} = 0.270$), among sites within ecoregions ($\Phi_{SR} = 0.252$) and among sites across the entire range ($\Phi_{ST} = 0.454$) (Table 1A). The BAPS mixture analysis revealed eight genetic clusters (Table 1B) and all but eight individuals (<2%) were 100% assigned to one specific cluster. Some ecoregions had only one unique cluster (BI, BC), while the other ecoregions were comprised of individuals derived from multiple genetic clusters.

Table 1 Analysis of genetic structure of *Ramalina menziesii* sampled across six ecoregions, based on four fungal low copy nuclear loci

Source	d.f.	SS	MS	Var.	%
A. Summary of hierarchical AMOVA results					
Among ecoregions	5	171.638	34.328	0.459	27
Among sites within ecoregion	97	224.775	2.317	0.312	18
Within sites	357	331.231	0.928	0.928	55
Total	459	727.643		1.699	100

Φ-statistics	
Among regions	$\Phi_{RT} = 0.270$***
Among sites within regions	$\Phi_{SR} = 0.252$***
Among sites relative to the total	$\Phi_{ST} = 0.454$***

Ecoregions	BI	BC	CS	CN	CC	PN
B. Summary of genetic clusters based on assignments via BAPS						
Cluster 1	0	0	3	20	0	0
Cluster 2	0	0	0	0	23	48
Cluster 3	0	0	27	69	29	19
Cluster 4	0	0	1	6	37	46
Cluster 5	0	0	22	3	2	0
Cluster 6	0	0	5	4	19	19
Cluster 7	41	0	0	0	0	0
Cluster 8	0	18	0	0	0	0

***, $P < 0.001$.

Genetic lineages

The Bayesian coalescent tree (Fig. 2A) based on DNA sequence data illustrates multiple unique clades suggestive of multiple diversification events. The tree suggests the most basal split within *R. menziesii* separates a clade now found only in the southern California inland ecoregion from all other individuals. The next branching within *R. menziesii* separates the remaining US and Canadian individuals from the Baja California individuals. Within the Baja California clade, there are two well-supported clades that align with the Baja California inland and Baja California coastal chaparral ecoregions. In addition, the tree includes a shallow set of haplotypes that were extremely widespread from California to Alaska (Fig. 2). Nested within one of these groups of haplotypes is a well-supported subclade endemic to the northern CA ecoregion, which is geographically restricted (Fig. 2).

The median-joining haplotype networks illustrate a distinct clustering of unique haplotypes for ecoregions BI, BC and CS (Fig. 3A–D). Across all loci, BI samples were represented as a unique clade. Also, samples from BC and BI locations each exhibited separate clades for three of the loci. Notably, haplotypes found in CC and PN were widespread and the networks exhibited a star-like topology for these geographical regions (e.g. Fig. 3C, uid).

Divergence times

Results from IMa2 yielded estimates of divergence times that indicate that populations within some of the ecoregions diverged at distinct times over a long time interval. The analyses yielded a consistent rank order in

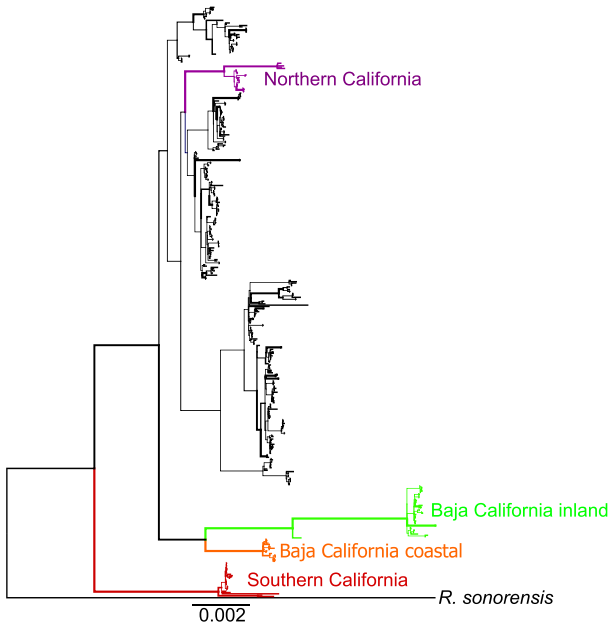

Fig. 2 Maximum clade credibility coalescent tree of *Ramalina menziesii*, based on data of fungal β-tubulin, elongation factor 1-α, glyceraldehyde 3-phosphate dehydrogenase and an unidentified locus closely resembling glycine dehydrogenase. The line weight is proportional to the posterior probability of nodes.

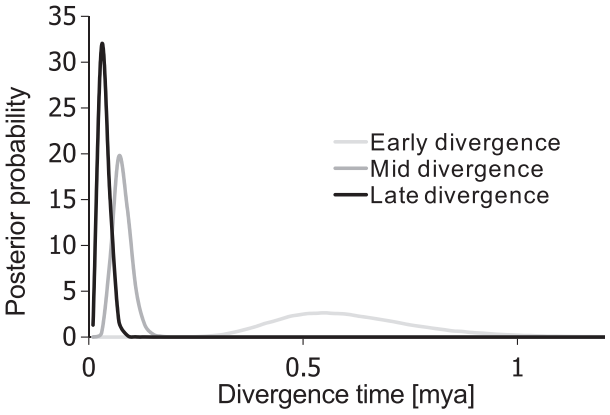

Fig. 4 Probability of divergence time estimates in the lichen-forming fungus *Ramalina menziesii* as quantified from IMa2 models, calculated assuming no migration. Time was converted into chronological time units by division with the 'medium' mutation rate, 4.731×10^{-7}. Early Divergence represents Baja California versus all other ecoregions (BI & BC versus CS, CC, CN & PN). Mid-divergence represents California inland ecoregions versus coastal ecoregions (CS & CN versus CC & PN). Late Divergence represents California unglaciated ecoregions versus glaciated ecoregion (CS, CC & CN versus PN).

divergence among ecoregions (Fig. 4): the two Baja California ecoregions diverged a long time ago from the rest of the ecoregion populations (Early Divergence); the divergence between the inland California ecoregions and the coastal and PN ecoregions was more recent (Mid-divergence); and the PN ecoregion was the youngest divergence (Late Divergence). With the assumption of medium mutation rate that gives a conservative estimate of divergence, our findings for Early Divergence indicated that the ecoregions BC and BI had remained isolated from ecoregions further north on the order of 1.2 Million years ago (Ma) (Fig. 4, Table S4). Our comparison of the pooled inland California regions versus the pooled California coast and Pacific Northwest regions results conservatively in an estimated divergence time of 148 000 years ago (Table S4). The most recent divergence times were estimated for the Pacific Northwest and the pooled California ecoregions

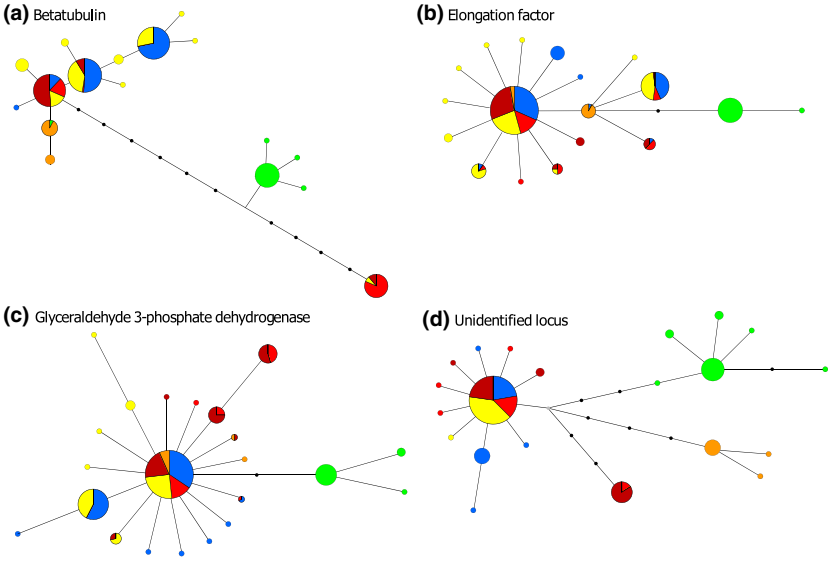

Fig. 3 Median-joining haplotype networks are presented separately for four fungal nuclear low copy genes. The size of pie charts (haplotypes) is proportional to the number of samples containing this haplotype. Dots indicate the location of median vectors.

(Late Divergence, Table S4) and yield minimum mean estimates of divergence time of at least 63 000 years ago. The posterior distributions on the divergence times are diffuse, making it difficult to precisely estimate divergence times but the peaks illustrate the rank order across three pairwise comparisons (Fig. 4).

Migration among ecoregions

Our IMa2 analyses with migration yielded wide 95% highest posterior density (HPD) intervals (Table 2); however, the values and trends we report here were found consistently in repeated runs and provide useful comparative pairwise estimates of migration. For most of the pairwise comparisons, we observe very little gene flow. Under the Early Divergence model, migration between the pooled Baja California ecoregions and the remaining ecoregions seems to be negligible (Table 2). Mean effective number of immigrants from Baja California to the rest of the range is close to zero and the lower 5% overlaps with zero, while migration to Baja California is very small and greater than zero. Under the Mid-divergence model, mean effective number of immigrants between the inland California ecoregions and the pooled California coast and Pacific Northwest ecoregions was directed towards CS and CN from the regions with the more recently evolved haplotypes (Table 2) and negligible towards CC and PN. In the Mid-divergence model, we observe the highest values of pairwise population gene flow and estimate a mean value of 9.73 effective migrants directed from the PN into the pooled ecoregions CS, CC and CN (Mid-divergence, Table 2), and very little migration northwards.

Discussion

The phylogeographical analysis of the widespread lichen *R. menziesii* provides evidence that neither hypothesis is entirely correct. Our phylogenetic and historical population analyses document multiple lineages with apparent long-term persistence and migration on the geographical distribution of biodiversity in western North America. Unlike the patterns found for other cryptogam species (e.g. Medina *et al.* 2011), our findings do not support the hypothesis that the widespread distribution of this lichen is due to a single, but extensive, migration episode. Instead, the AMOVA indicates a significant genetic structure of *R. menziesii* both across ecoregions and within regions, and the IMa analysis suggests that gene flow is restricted during the older historical periods. Moreover, our phylogenetic analysis reveals that this species has formed several unique clades, and several are found uniquely within specific ecoregions. These geographically restricted clades suggest the importance of lineage formation at different times and their long-term persistence as contributing factors to

Table 2 Estimates of population sizes, bidirectional migration rates and bidirectional effective number of migrants for pairwise population comparisons under three divergence models. The analysis was based on isolation with migration models (IMa2). Given are mean values for all parameters, 5% lower bound and 95% upper bound of the estimated highest posterior density interval. Estimates of population sizes for populations 0 and 1 and ancestral population (θ_0, θ_1 and θ_A, respectively) are listed for each divergence model. Migration rates are scaled by medium mutation rate estimate (see text), where $m_{0>1}$ is the rate at which population 0 received genes from population 1 forward in time and $m_{1>0}$ is the rate at which population 1 received genes from population 0. Population migration rates, $2Ne_0\ m_{0>1}$ and $2Ne_1\ m_{1>0}$, are scaled by effective population sizes, where $2Ne_0\ m_{0>1}$ is the effective number of migrants into population 0 from population 1 and $2Ne_1\ m_{1>0}$ is the effective number of migrants into population 1 from population 0

	θ_0	θ_1	θ_A	$m_{0>1}$	$m_{1>0}$	$2Ne_0\ m_{0>1}$	$2Ne_1\ m_{1>0}$
Early Divergence (Baja California versus all other ecoregions): 0 = BI & BC; 1 = CS, CC, CN & PN							
Mean	201 877	438 633	345 622	0.0002767	0.0000237	0.28	0.05
Lower 5%	113 093	320 255	0	0.0000686	0	0.04	0
Upper 95%	167 526	601 403	1 981 776†	0.0006079†	0.0000686†	1.02	0.21
Mid-divergence (Inland versus coastal ecoregions): 0 = CS & CN; 1 = CC & PN							
Mean	174 396	286 433	261 066	0.001838	0.0001585	1.6	0.23
Lower 5%	91 954	178 624	136 346	0.0004707	0	0.22	0
Upper 95%	150 615	434 405	2 112 837†	0.0040280†	0.0015160†	6.07	3.29
Late Divergence (California unglaciated ecoregions versus glaciated ecoregion): 0 = CS, CC & CN; 1 = PN							
Mean	480 911	98 296	239 927	0.004047	0.001019	9.73	0.5
Lower 5%	265 294	43 335	142 688	0.0000213	0.0000733	0.03	0.02
Upper 95%	458 187	201 877	2 112 837*,†	0.0000047†	0.0039520†	21.66	3.99

*Highest posterior density interval had a rough surface or was multimodal, which makes the value of the high point doubtful.
†Highest posterior density interval did not reach low values close to the upper or lower prior limit.

the widespread distribution of this lichen. Thus, our findings provide a similar pattern for diversity that reported by Lancaster and Kay (2013) who analysed 16 clades of Californian angiosperm plant species and concluded that low extinction rates (i.e. long-term persistence of populations) have contributed to the high biodiversity of the CFP.

Despite evidence that some clades are found mostly or entirely in one ecoregion, we also find evidence that suggests extensive, recent gene flow. The coastal ecoregions in California and Pacific Northwest have both numerous and widely distributed shared haplotypes, making it seem that a coastal migration zone habitat, with its fog belts and moist conditions, may have promoted long-distance dispersal. In addition, six of the eight population clusters are found in all of the California and Pacific Northwest ecoregions. Such extensive long-distance dispersal has been found in several other cryptogam species (Fernández-Mendoza *et al.* 2011; Geml 2011; Medina *et al.* 2011; Domaschke *et al.* 2012). Our findings also indicate that the widespread distribution of this particular cryptogam species is not only due to the persistence of ancient lineages within some of the separate ecoregions with restricted gene flow in the CFP, but is also enhanced by immigration of recently formed haplotypes that increases local biodiversity. Below, we discuss the details of the complex evolutionary history of *R. menziesii* and the insight they provide for the biodiversity of western North America.

Genetic lineages

The coalescent tree of *R. menziesii* suggests the presence of multiple ancient lineages, four of six that align separately with four ecoregions: BI, BC, CS and CN. Those clades, which are all found in unglaciated regions, may have developed under long-term isolation and climate stability with limited migration between ecoregions, conditions under which genetic drift (e.g. Printzen *et al.* 2003) may have led to the formation of isolated clades. A unique clade found in the fog desert ecoregion of Baja California (BI) is the oldest; our phylogeny indicates that the coastal Baja California clade diverged from that group. Our tree also indicates an old clade in southern California and a later split in northern California that is nested within a part of the tree with haplotypes that are ubiquitously distributed. We are cautious in interpreting their origins without additional and more informative loci. Nonetheless, the persistence of these clades suggests low extinction rates in this region due to stable climate and a lack of recent glaciation. Thus, the presence of several well-supported independent clades provides evidence that a key component of the diversity of *R. menziesii* in the unglaciated part of the species range is the evolution of independent lineages that have not yet dispersed elsewhere.

Along the coast and in the northern glaciated area of the distribution range, we do not find unique clades. *Ramalina menziesii* is capable of tolerating freezing temperatures that would have allowed the formation of separate lineages at high latitudes. For example, it occurs in proximity to major glaciers in coastal locations 100 km south of Glacier Bay National Park in Alaska (N58.4278, W134.785; K. Dillmann, personal communication). Moreover, some evidence points to northern refugia on Vancouver Island and the Queen Charlotte Islands for plant populations (Heusser 1989; Heusser & Igarashi 1994; Soltis *et al.* 1997) and lichen-forming fungi (Printzen *et al.* 2003). In this study, we find unique haplotypes in the specimens of *R. menziesii* sampled on but we do not find deep lineages indicative of long-term persistence such as those in the southern region. Thus, even Pacific Northwestern populations harbouring unique haplotypes such as those on the Queen Charlotte Islands may have colonized from more southern sources during the Pleistocene and may have actually survived in relictual populations.

In assessing the entire tree of *R. menziesii*, evidence strongly points to a southern origin of the species in southern California or Baja California. These regions are isolated from each other today with major climatic barriers between the fog desert, Baja California chaparral and inland California. The fog requirement of this species could result in isolation of regions when climate changes on land or switches in the patterns of ocean upwelling could have split formerly connected ancient populations or created pockets of suitable habitat in different regions that were colonized by long-distance dispersal of the fungal ascospores.

Divergence time

Our coalescent analysis of the pairwise divergence of pooled ecoregions indicates that several of the ecoregion populations diverged at different times: the oldest divergence took place between Baja California and the remainder of the populations, the divergence between the inland California ecoregions and the coastal California and Pacific Northwest ecoregions was more recent, and the divergence between the three California regions and the Pacific Northwest is most recent. The fact that our population structure analysis found intermixed clusters of coastal California and Pacific Northwest raises questions about when those two ecoregions diverged from each other. Nonetheless, the overall order of divergence of the older splits suggests that Pleistocene glacia-

tion has played a role in the evolutionary dynamics of the species and that the coastal ranges of California were at one point in time barriers for the expansion of the species that were overcome in more recent times.

The timescales of the divergences have large error bars, but the patterns strongly suggest divergence among most of the ecoregions is old and that *R. menziesii* has survived multiple glacial periods both in southern California and in Baja California. The split between Baja California and the four northern ecoregions (Early Divergence) is probably about 1–2 Ma, which corresponds to the Early Pleistocene. The earliest estimate we obtain is ~500 000 years ago and the oldest is about 3.5 Ma (Table S4), which is the broad time range shown in Fig. 4. The split between *R. menziesii* populations in the California inland and the California coastal ecoregions (Mid-divergence: CS and CN vs. CC and PN) is much more recent. This divergence took place about 125 000–248 000 years ago, which would have been during the relatively cool Late Pleistocene and possibly during a glacial period. However, again, the estimates indicate that this split could have taken place any time between 50 000 and 950 000 years ago, a period that proceeds the last Glacial Maximum cold period. During glacial periods, tree populations contracted substantially (e.g. Adam *et al.* 1981; Heusser 1995), which could have led to a corresponding contraction in lichen populations in California and the Pacific Northwest. Because tree populations did not begin to re-establish until after the glaciers retreated, we assume that the lichen population could not expand until their phorophyte species were present.

The more recent divergence between Pacific Northwest ecoregions and the pooled California ecoregions (Late Divergence) was estimated to be about 54 000 to 106 000 years ago (or older, given these estimates are conservative), which is a glacial period when lichen populations are likely to contract or even go extinct if no phorophyte populations would be present. The time estimates suggest that the lichen populations could have survived the last glaciation in the Pacific Northwest, which is theoretically possible given the cold tolerance of *R. menziesii*. However, given our separate finding that individuals in the CC and PN ecoregions clustered together genetically, we suggest that additional research would be needed to clarify this most recent divergence.

Patterns of migration among ecoregions

Overall, our analysis of bidirectional migration implies negligible migration in the Early Divergence model, a small amount of migration inland in the Mid-divergence model and, in the Late Divergence model, high migration from the Pacific Northwest south and some migration from the Pacific Northwest and coastal California ecoregions inland. This southern migration of the more northern haplotypes of *R. menziesii* into coastal and interior southern California has added to the genetic diversity of those regions. In contrast, the northernmost part of the range of *R. menziesii* located in previously glaciated areas does not show evidence of recent immigration from the older ecoregions. Moreover, the Baja California Peninsula has neither received recent immigrants, nor sent out migrants towards the north, suggesting its isolation from the ecoregions further north.

Migration from north to south seems counterintuitive because our phylogenetic tree indicates the ancient lineages are in the south, which should have historically sent out immigrants to northern regions. Perhaps a much earlier period of gene flow included movement of original lineages north followed by new lineage formation. However, occasional colonizing events are not the same as ongoing migration. Our species is showing a different pattern than that of other lichens in the region. Walser *et al.* (2005) investigated migration among populations of the old forest lichen *Lobaria pulmonaria* (L.) Hoffm. in British Columbia and found that populations on Vancouver Island were isolated from populations further inland, while inland populations exhibited substantial genetic exchange with one another and with one coastal population. In the Pacific Northwest lichen *Cavernularia hultenii* Degel. (Printzen *et al.* 2003), population expansion was detected from northern refugia into formerly glaciated areas, but no gene flow into southern areas, although the study included sites in Alaska, British Columbia, Washington, Oregon and California.

Our study is somewhat unusual in that the movement of recent migration out of the north into California and from coastal regions into inland regions enhances the biodiversity of this species in the CFP. One possible explanation is that wind direction during the last glacial period seems to have been primarily easterly off the Pacific Ocean (Bush & Philander 1999), which would have promoted dispersal of spores from west to east and inhibited gene flow from inland California to the coast. This hypothesis is supported upon greater scrutiny of the distribution of genetic clusters identified through BAPS (Fig. 5), where the clusters in California and Pacific Northwest separate out inland and coastal groupings, which are in agreement with morphological differences between coastal and inland populations (Boucher & Nash 1990). An additional factor for spore dispersal is that the fog conditions along the coast may have promoted survival and dispersal of spores along the coast, a suggestion supported by the

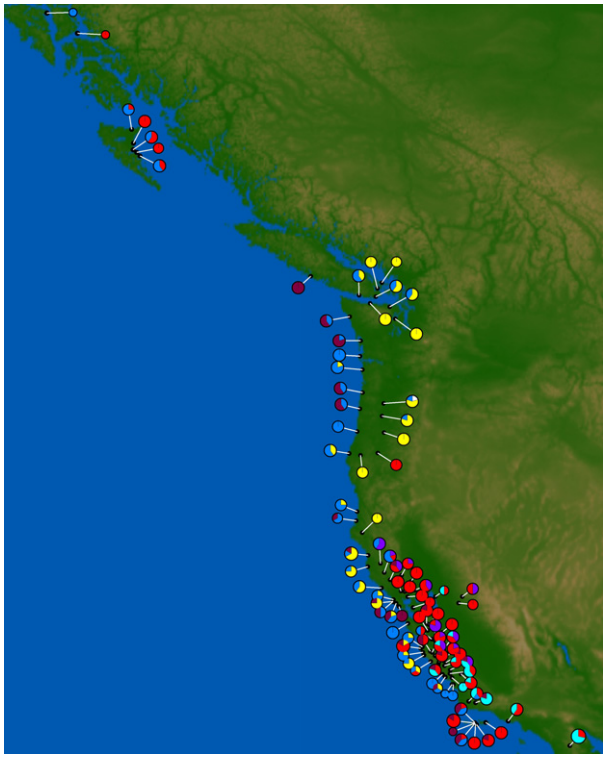

Fig. 5 Distribution of individuals associated with six population clusters of *Ramalina menziesii* identified by BAPS population structure analysis (see Table 1B). Two other clusters not shown are unique to the Baja California inland (BI) and Baja California coastal (BC) ecoregions, respectively.

distribution of population clusters along the coast from California to Pacific Northwest.

Implications for western North American biogeography

This study identifies two mechanisms that would make southern California a genetic diversity hotspot for *R. menziesii*. The inland southern California ecoregion contains an ancient lineage that has persisted for a long time, but not dispersed elsewhere, and numerous haplotypes that evolved more recently and immigrated into the region from elsewhere. In valley oak (*Quercus lobata* Née), an important phorophyte of *R. menziesii*, greater haplotype diversity is found in the inland foothills of the Coast Ranges relative to that on the western foothills of the Sierra Nevada (Grivet *et al.* 2006, 2008; Sork *et al.* 2010; Gugger *et al.* 2013), which further suggests that the western part of inland California may be a region of long-term persistence of populations for many species. The intraspecific dynamics we report here are consistent with Lancaster and Kay (2013) who concluded that population persistence in 16 plant angiosperm taxa is a critical factor in the high biodiversity of the CFP. However, in contrast to their conclusion that migration is less important, our study indicates that migration from other ecoregions has enhanced genetic diversity of this lichen in the CFP. Our study also differed by analysing a species that extends into Baja California, including the northernmost part of the Vizcaino Desert Ecoregion (Gonzáles-Abraham *et al.* 2008), adjoining the CFP. The fact that *R. menziesii* includes ancient lineages in Baja California illustrates the potential influence of this region on the evolutionary history of other temperate and Mediterranean taxa in western North America. Thus, for *R. menziesii*, by studying a species that spans western North America, we find evidence that California is a centre of biodiversity both because of the processes that take place within the region and because of its relationship to the surrounding ecoregions.

In conclusion, *R. menziesii* provides a valuable example of how the broad range of this cryptogam species has been shaped by both lineage formation and persistence, as well as long-distance dispersal along the coast and directed inland. Moreover, this study illustrates how latitudinal and longitudinal climate gradients and complex topography in western North America have helped shape and retain ancient diversity. This spatial and temporal heterogeneity allowed multiple demographic processes of population expansion, contraction and long-distance migration to promote high diversity of the CFP.

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V.L.S. and S.W. contributed to study design, sampling, data analyses and writing. S.W. performed the molecular analyses.

Data accessibility

Included in supporting information.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Haplotype sharing among ecoregions, calculated based on the largest nonrecombining block of DNA sequence in *Ramalina menziesii* and four loci: *bet*, *efa*, *gpd*, and *uid*.

Table S1 Samples of *Ramalina menziesii* collected throughout the species' range including: Locality identification code (ID), host species, habitat, state in the USA, Canada, or Mexico, ecoregion, latitude, longitude, elevation in metres, number of samples sequenced (*N*), and collectors.

Tables S2 Haplotype maps for the four genes in *Ramalina menziesii* indicating position, site number, consensus sequence (consensus), the type of polymorphism (t, transition; v, transversion; −, insertion or deletion), sample ID, and GenBank accession (Accession).

Table S3 Summary of diversity statistics and tests of demographic history by ecoregion and loci for four low copy nuclear genes with recombinants removed.

Table S4 Inference of demographic parameters for estimated population sizes and divergence times based on three pairwise IMa2 population models from assuming zero migration.

Table S5 Haplotypes of *R. menziessii* sampled throughout the species range.