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GEOGRAPHIC STRUCTURE IN A SYMBIOTIC MUTUALISM

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GEOGRAPHIC STRUCTURE IN A SYMBIOTIC MUTUALISM

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DISSERTATION ABSTRACT

An increasing number of studies analyze the geographic structure of species interactions, including symbioses, but few address mutualistic symbioses, systems where phylogenetic congruence can be expected when there is vertical inheritance of symbiotic partners. In this study, I examine fungal-algal partnership patterns and geographic structure of the fungus *Letharia vulpina* s. lat. (Ascomycota) and its partners, multiple clades of *Trebouxia jamesii* (Chlorophyta), across Western North America. *Letharia* lichens are unusual in that multiple gene genealogies delimiting taxa on both sides of the symbiosis are available from a prior study. This previous work provides a robust phylogenetic context for the study of a multi-species interaction network that occurs at a large geographic scale. The primary methodology employed is DNA sequencing of seven fungal loci and two algal loci. The study provides: 1) Formal description of a lichenized fungus, *Letharia lupina* sp. nov., that is distinct from *Letharia vulpina* s. str., based on morphology, geographic distribution, fungal DNA sequences, and algal partner. *L. lupina* and its algal partners form "Mountain Wolf" lichens. 2) A map of the geographic distribution of five clades of Mountain Wolf algae in Western North America. 3) A comparison of geographic structure of Mountain Wolf fungi with Mountain Wolf algae. 4) A comparison of the geographic structure of Mountain Wolf algae with co-distributed plant and animal taxa in Western North America.

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That this thesis is useful to the famous, powerful, sexy people of Edgewood Blue.

DISSERTATION INTRODUCTION

Phylogenetic studies show that symbiotic organisms demonstrate the entire range of concordance (and lack of concordance) in their evolutionary histories (Thompson 1994). These studies usually compare two suites of interacting species and ask whether there is evidence for cospeciation (Page 2003). Increasingly, studies have looked for parallels in the intraspecific population structures of interacting species (Althoff & Thompson 1999, Thompson 1999, Roy 2001, Jerome & Ford 2002, Anderson et al. 2004, Parker et al. 2004, Jones et al. 2006). While phylogeography is the study of how genetic variation within closely related organisms is structured geographically (Avise 2000), comparative phylogeography is the study of congruence among unrelated taxa in their geographic distribution and population structure (Riddle & Hafner 2004). Because comparative phylogeography has only begun to be used for symbiotic mutualisms, we know little about the geographic scale at which these interacting species have phylogenetically congruent histories.

Here I compare the phylogeographic structure and partnership patterns of the fungus *Letharia vulpina* 'lupina' and *Letharia vulpina* 'vulpina' (Ascomycota) and their algal partners, species of *Trebouxia* (Chlorophyta), across Western North America. Wolf (*Letharia)* lichens are unusual in that multiple gene genealogies delimiting species on both sides of the interaction are available (Kroken & Taylor 2000, 2001). This previous work provides a robust phylogenetic context for the study of a multi-species interaction network that occurs at a large geographic scale. My primary objectives are:

1. To determine the extent of congruence in the phylogeographic structures of symbiotic species that have vertical inheritance of symbiotic partners and that participate in interaction networks.
2. To compare the geographic structure of lichenized fungi and algae with that of plants and animals in Western North America.
3. To determine whether high partnership specificity is driven by geographic opportunity.

Chapter 1 provides a formal description of the lichenized fungus, *Letharia lupina* sp. nov., which combined with its algal partners forms "Mountain Wolf" lichens. In Chapter 2, the geographic structure Mountain Wolf algae is inferred and compared with the geographic structure of co-distributed plants and animals of Western North America. Chapter 3 provides an analysis of the geographic structure in Mountain Wolf fungi, compares geographic structure of the fungus with that of the alga, and describes geographic patterns in the symbiosis itself. Each chapter has its own introduction, but three topics merit an overture here. First, the significance of each chapter is summarized. Second, background on the taxonomic and phylogenetic context of the two main taxa of interest in this study, *Letharia lupina* and *Trebouxia jamesii*, is provided. Lastly, any geographic study involving an obligate symbiosis confronts the problem of what constitutes an individual, a species, and a population. Lichens pose additional complications in each of these three areas, and below I clarify my use of terminology.

Significance

The formal description of a new fungal species in Chapter 1 is important for its novel use of the fungal-algal symbiotic relationship as a taxonomic character. Sequence data from symbionts have been used in phylogeographic studies of parasite hosts (Criscione and Blouin 2007, Nieberding et al. 2004), and there is serious discussion of their utility in taxonomy (Jousselin et al. 2009, Nieberding and Olivieri 2007). It is the tradition in lichenology that at least one morphological or biochemical character is required in order to describe a lichen fungus species, regardless of the genetic discontinuities revealed by molecular work. The newly described species *Letharia lupina* is morphologically distinct from its congener *Letharia vulpina* only in allopatry: morphological characters are not entirely reliable to distinguish the species in sympatry. However, the two fungi, in addition to forming separate phylogenetic clades at multiple loci, pair with separate clades of algae (also recognized with molecular tools) in allopatry and in sympatry. The description of this new fungal species is supported by two independent sources of molecular data: the fungus and its obligate algal symbiont.

The second chapter expands the context of the dissertation to include the comparative phylogeography of plants and animals of Western North America. Microorganisms are strongly underrepresented in phylogeographic studies (Beheregaray 2008) and any post-Pleistocene history is depauperate without them. This is the first study to compare geographic structure of a terrestrial alga with that of widespread plants and animals of Western North America. As such, it broadens our view of how

diverse taxa move across shared landscapes, especially in response to post-Pleistocene climate change.

A priority in the study was to sample the algal partners across the entire geographic range of a single lichenized fungus. As a result, the study is the first description of the geographic structure of a lichenized alga from the perspective of a single fungal species. The study contributes to our understanding of cryptic geographic patterns in symbioses in general and in lichens in particular by revealing genetic diversity in the symbiotic relationships that was not evident in the morphology or genetic markers of the dominant partner. Symbiotic partners can drive geographic structure in what otherwise appear to be weedy, widespread inconsequential taxa (from a conservation standpoint), such as Mountain Wolf lichens.

The third chapter is a rare comparison of geographic structure on both sides of a symbiotic mutualism. We know almost nothing about geographic structure in these pivotal species interactions. Vertical inheritance of symbiotic partners should be an important factor shaping geographic structure in such species interactions because successful partnerships do not need to be formed anew at each generation. The fact that Mountain Wolf lichens have an obvious, unambiguous mechanism for vertical inheritance led me to expect that there would be "populations" of fungal-algal partnerships that had swept geographic areas. For example, I expected a signal of a few highly successful dual fungal-algal partnerships in British Columbia where lichens had colonized returning conifers after the retreat of the ice sheets. Instead, I found that every lichen thallus in the study is genetically unique. A combination of high

genetic diversity in each partner, recombination in the alga, recombination in the fungus, and partner switching, all likely contributed to a perfectly unique data set. Apparently, vertical inheritance of symbiotic partners does not shape geographic structure at the large spatial scales employed here. This underlies the importance of multiple spatial scales in the design of studies that concern mode of partner inheritance and geographic structure in symbioses.

Fungal Taxonomic and Phylogenetic Context

Lichenized fungi are nutritional specialists that rely on the photosynthetic products of algal and/or cyanobacterial photoautotrophs for their carbon, and they are a major phenomenon in the fungal kingdom (Lutzoni et al. 2001). Approximately one-fifth of all fungi form lichens, and within the phylum Ascomycota, nearly half of all species employ the lichen lifestyle (Hawksworth 1988). The question of whether lichenized fungi are parasites or mutualists pivots on what definition of "benefit" is used and what time scale is invoked. Over evolutionary time, the fungal-algal relationship appears to be a mutualism as the algae would otherwise not achieve the large population sizes nor enjoy the diverse ecological niches that they currently occupy (Purvis 2000).

It has been argued that symbiosis with autotrophic organisms is a synapomorphy (a shared and derived trait) among major groups of fungi (Tehler et al. 2003), but it appears that lichen symbioses have arisen multiple times among the fungi (Lutzoni et al. 2001). About 20 species of lichenized Basidiomycota are recognized and over 13,500 species of lichenized Ascomycota have been described (Hawksworth

1988). Within the Ascomycota, lichenization likely arose twice (Lutzoni et al. 2001). The vast majority of lichenized fungi fall within the Lecanoromycetes, a class composed almost entirely of lichenized fungi.

The focal fungi of this study fall within the genus *Letharia*. *Letharia* (Th. Fr.) Zahlbr. is in the Parmeliaceae, a large family of lichenized fungi nested within the class Lecanoromycetes. *Letharia* is a small genus, usually characterized by only two formally described (accepted) species (Brodo et al. 2001, Ryan 2001). *Letharia columbiana* has frequent conspicuous fruiting bodies and no soredia/isidia whereas *Letharia vulpina* has rare fruiting bodies and copious soredia/isidia. These two morphologically described species are easy to identify in the field. Previous authors have attempted to split the genus or formalize species subtaxa (Acharius 1810, Schade 1955), but these taxonomic changes did not find acceptance. It is too soon to tell if recent species descriptions, based at least partially on molecular work (McCune & Altermann 2009 and Chapter 1), will achieve wide acceptance.

Algal Taxonomic and Phylogenetic Context

Lichenized fungi partner with photoautotrophs in four phyla (sensu Margulis & Schwartz 1998): Chlorphyta (green algae), cyanobacteria, Phaeophyta (brown algae), and Xanthophyta (yellow-green algae). Despite this broad phylogenetic amplitude in symbiosis, it is estimated that over half of all lichenized fungi pair with a green alga belonging to the genus *Trebouxia* Puymaly (Ahmadjian 1982). The genus consists of 17-27 morphologically described species (Takeshita 2001) that are recognized by their distinct central and lobed chloroplast observed with a simple slide squash and light

microscopy. The genus is subdivided into groups based on chloroplast morphology (Gärtner 1985, Friedl 1989, Takeshita 2001), life cycle characters (Tschermak-Woess 1989 and Takeshita 2001), and pyrenoid ultrastructure (Friedl 1989). Both chloroplast morphology and life cycle characters are based on aposymbiotic algal cultures. DNA sequence data has yet to further inform a formal taxonomic revision of the genus, but useful phylogenetic trees based on the ITS locus are nonetheless found in the literature (Friedl & Rokita 1997, Helms et al. 2001, Friedl & Bhattacharya 2002, Friedl and Büdel 2008, Kroken & Taylor 2000, Piercey-Normore 2006).

Letharia fungi pair with a group of closely related algal clades within the morphologically described species *Trebouxia jamesii* Hildreth et Ahmadjian (Kroken and Taylor 2000). *T. jamesii* was originally described based on an algal culture from the North American lichenized fungus *Shaereria tenebrosa*. The characters in the key to the taxon include 1) presence of vegetative reproduction, 2) a continuous starch sheath, and 3) weak autotrophy on standard media (Hildreth and Ahmadjian 1981). In his revision of the genus, Takeshita (2001) verified the morphological description of this species based on life cycle characters, chloroplast shape, cell size, and cell wall thickness. *T. jamesii* occupies the taxonomic rank of "species" based on morphology, ultrastructure, and life cycle characters of cultures, but DNA sequences suggest that the morphospecies is made up of multiple reproductively isolated taxa. Members of *T. jamesii* have been compared by their DNA sequences based on two loci: ITS and two actin I introns (Kroken and Taylor 2000). Both loci show multiple clades within *T. jamesii*, and congruence between the two loci is evidence for the presence of multiple

phylogenetic species. Formal revision of the taxon has not been attempted, so I use the terms "algal lineage" or "algal clade" instead of algal species.

Lichen Individuals

Lichens often form discrete thalli that are morphologically uniform. Molecular work indicates that individual thalli of some lichens consist of only one fungal and one algal genotype (Kroken and Taylor 2001a, Walser et al. 2003), but multiple fungal or algal genotypes have also been found within individual thalli (DePriest 1993, Bhattacharya et al. 1996, Romeike et al. 2002, Piercey-Normore 2006, Robertson & Piercey-Normore 2007). The mechanism for the occurrence of multiple genotypes within a thallus is likely either a fusion event among soredia (dual fungal-algal dispersal propagules) (Schuster et al. 1985), or a fusion event among hyphae of different germinating ascospores (Ott 1987), or between separate thalli (Jahns 1987, Sanders & Lucking 2002). In addition, secondary fungi add biodiversity to the lichen thallus (Sun et al. 2002, Suryanarayanan et al. 2005, Fahselt 2008), but play an unknown role in lichen physiology and morphology. Apparently the DNA of these secondary fungi occurs in such low concentration that it usually does not register in whole lichen extract-based DNA amplifications (PCR) that are employed here. *Letharia* lichens have been found to have consistent respective fungal and algal genotypes throughout the thallus (Kroken and Taylor 2000, 2001a), so it is safe to assume that a single thallus "individual" consists of a single fungal-algal relationship. *Letharia* lichens have not been screened for secondary fungi.

Another problem in the identification of individuals is the fact that many lichens have mechanisms for dual fungal-algal propagation. Soredia, isidia, and thallus fragments are clones of the source thallus and can be considered as part of the same individual (clones of the same genet). The average dispersal distance for most dual fungal-algal propagules is not know, but Werth et al. (2006) were able to quantify the expected dispersal distance of *Lobaria pulmonaria* lichen soredia to be within 200 meters. Here, multiple sampling of clones was minimized by spacing collections at least 200 meters apart. This strategy was surprisingly successful with *Letharia* lichens: no two thalli in the study have identical combined fungal-algal genotypes (Chapter 3).

Lichen Species

Although lichens are stable dual organisms consisting of a fungus and an alga, the symbiosis has no taxonomic standing under the International Code of Botanical Nomenclature. Technically, there are no lichen species, only lichenized fungi and lichenized algae. Yet these stable fungal-algal partnerships deserve recognition. In practice, lichens are commonly named for the fungal component. This creates ambiguity in the literature as the word "lichen" has two meanings: it refers to the symbiotic dual-organism, and it refers to the fungus as a taxon to the exclusion of the alga (or cyanobacterium) (Goward 2008). For clarity, here the word "lichen" always refers to the fungus *and* its algal partner. Synonyms for "lichen" include "dual organism" and "fungal-algal partnership." When referring to the components of lichens, I employ "lichenized fungus", "lichenized alga," "partner," "symbiont," or "biont." There are also contexts, particularly in Chapter 3, when I want to refer to a

specific set of lichens that consists of a single fungal species (*Letharia lupina*) and any one of five algal lineages (see Chapter 2). Since there is no formal way to name this stable and morphologically consistent set of relationships, I employ the common name, Mountain Wolf lichen, coined by Trevor Goward (1999). Mountain Wolf lichens are a rankless, informal, but useful taxon that consist of the fungus *Letharia lupina* and its algal partners, and they are the primary protagonists of this study.

Lichen Populations

Lichenized fungi lend themselves to populations studies because it is possible to recognize many species with macroscopic morphological characters. Lichenized algae are hard to study because they are morphologically cryptic and many, including *Trebouxia jamesii,* pair with multiple fungal taxa (Tschermak-Woess 1988, Beck 1999, Kroken and Taylor 2000, Romeike et al. 2002). A true population study of lichenized algae would have to include representative individuals that pair with all the appropriate fungal partners at any one site (Beck 1999, Yahr et al. 2004). In Chapter 2, it is shown that the algal partners of *Letharia lupina* are not restricted to relationships with that fungus, but sampling is overwhelmingly of *Letharia lupina* lichens. As a result, the data are not representative of the full algal population, only of those algae that pair with *Letharia lupina*.

There are populations of lichenized fungi and populations of lichenized algae, but are there populations of lichens? That is, are there populations of stable fungal-algal partnerships? Lichen populations may exist to the extent that stable fungal-algal partnerships show spatial structure over the geographic distribution of the component

fungal and algal species. Because Mountain Wolf lichens are understood to reproduce and disperse primarily through asexual dual fungal-algal propagules (isidia/soredia), I expected to find geographic structure in the fungal-algal genetic type combinations, especially when narrowing the question to a single fungal species and a single algal lineage. In Chapter 3, I describe the geographic structure of Mountain Wolf lichen fungal-algal partnerships and find little evidence for partnership populations. Despite asexual reproduction and dispersal, the success of the Mountain Wolf lichen symbiosis appears to be based on modular relationship between symbiont genetic types, not on a perpetual marriage.

Gregory Bateson cautioned us that "the map is not the territory, and the name is not the thing named" (Korzybski 1941, Bateson 1972), and so it is with the language of lichens in particular and with scientific study in general. Suffering the perennial limitation of language, a dissertation is an attempt at forming the most useful possible map to a mysterious territory. I have made the above clarifications to best match my language to the territory of the lichen symbiosis, as I have come to know it.

CHAPTER 1

LETHARIA LUPINA (PARMELIACEAE): A NEW SPECIES BASED ON FUNGAL AND ALGAL MOLECULAR CHARACTERS

ABSTRACT

Western North America is the global centre of diversity for *Letharia*, a distinctive genus of lichenized fungi belonging to the Parmeliaceae, and characterized by a shrubby, fruticose habit and the presence of vulpinic acid. Previous molecular studies using multiple fungal nuclear loci revealed the existence of two distinct phylogenetic species within the morphologically defined species *Letharia vulpina* (L.) Hue. Because *L. vulpina* s. lat. is prominent in air pollution monitoring, it has become important to understand whether this widespread taxon is actually a single species or a complex of closely related species. Here we used DNA sequencing of the fungal ITS and two anonymous loci to genetically characterize the fungi. We also used the algal ITS and an actin I intron to genetically identify the algal symbionts. Our studies reveal that the two phylogenetic species within *L. vulpina* s. lat. not only consistently differ in fungal genetic characters, but also form mutually exclusive partnerships with separate algal clades within *Trebouxia jamesii* s. lat.. Accordingly, we describe the morphologically rarely sexual species *Letharia lupina* sp. nov. from western North America and Western Eurasia. *Letharia lupina* differs from *L. vulpina* s. str. in morphology, ecology and distribution, although none of these characters is consistently and unambiguously diagnostic for the fungal species. For rapid species identification, fungal ITS restriction digests and an algal actin I intron AFLP provide simple, reliable alternatives to DNA

sequencing. An epitype is selected for the lectotype of *Lichen vulpinus* to clarify the use of the epithet.

INTRODUCTION

Letharia (Th. Fr.) Zahlbr. is a small but highly distinctive genus of lichen fungi belonging to the Parmeliaceae. Member species are characterized by a coarse, densely branching, fruticose habit, internal cartilaginous strands, and the presence of cortical vulpinic acid (Stephenson and Rundel 1979; Ryan 2002). Two species have traditionally been recognized – the morphologically rarely sexual *L. vulpina* (L.) Hue with copious isidia/soredia, and the frequently fertile *L. columbiana* (Nutt.) J.W. Thomson which lacks obvious asexual reproductive structures. Previous work using multiple fungal nuclear loci has revealed the existence of several additional taxa, especially in *L. columbiana* s. lat. (Kroken & Taylor 2001; McCune & Altermann 2009), though also within *L. vulpina* s. lat., as discussed in the present paper.

Letharia vulpina s. lat. is a widespread species documented from western North America, Europe (Fennoscandia and the Alps), the Caucasus mountains, and the Atlas mountains of North Africa (Gams 1955; Deil 1984; Brodo et al. 2001). *Letharia vulpina* s. lat. is often abundant in the dry coniferous forests of western North America, though it tends to be sparse elsewhere. It is currently listed as rare and threatened in many parts of Europe, especially Fennoscandia (Tønsberg et al. 1996; Trass 1997; Vitikainen et al. 1997; Gärdenfors 2000).

A key characteristic of *Letharia* is the copious production of vulpinic acid - a brilliant yellow cortical pigment that has led to the widespread use of *L. vulpina* s. lat. as a source of colorful dye (Mead 1972; Turner 1979; Casselman 1996). Vulpinic acid is toxic, and in earlier times *L. vulpina* s. lat. was used in Northern Europe as an active ingredient in various preparations against foxes and wolves (Schneider 1904; Santesson 1939). Some native peoples of western North America also used this species medicinally, e.g., in the treatment of inflammation and running sores (Chestnut 1902). Its tolerance to atmospheric (Sigal and Nash 1983) has lately earned it a prominent role in air pollution monitoring in western North America (Fenn et al. 2007; Geiser & Neitlich 2007; Jovan & Carlberg 2007) since it can be tested in both polluted and unpolluted environments.

Goward (1999) was the first to suggest that *L. vulpina* might encompass two species distinguishable on morphological and ecological grounds, e.g., branching, color, density of isidia/soredia, and altitudinal distribution. He referred to the new species as *Letharia* "sp. 1" and gave it a common name: "Mountain Wolf" lichen. Through multi-locus DNA sequencing, Kroken and Taylor (2000, 2001a) confirmed that two fungal phylogenetic species are indeed present in North America, and that each pairs with distinct algal phylogenetic species nested within the morphologically-based taxon *Trebouxia jamesii* s. lat.. They nicknamed the fungal phylogenetic species *L. vulpina* 'vulpina' and *L. vulpina* 'lupina,' but did not go on to formally describe the latter taxon. They found no overlap in the geographic distribution of the two lichens. More recent molecular work has found only *L. vulpina* 'vulpina' in Europe and the Caucasus

(Høgberg et al. 2002; Arnerup et al. 2004), though a single tree trunk in Morocco has been found to support both phylogenetic species (Arnerup et al. 2004).

As part of a large-scale geographic study of *Letharia* fungal-algal partnerships by the first author, we used DNA sequencing to identify the fungal and algal lineages present in *L. vulpina* s. lat.. We had two primary objectives. First, we wanted to know whether Goward's putative morphological differences between the two lichen phenotypes are consistent throughout western North America. Second, we wished to learn to what extent the mutually exclusive fungal-algal partnership pattern observed by Kroken and Taylor is stable across different geographic regions.

MATERIALS AND METHODS

Three hundred and five specimens of *L. vulpina* s. lat. were collected from throughout the range of *Letharia* in western North America, with the addition of one specimen each from Turkey, Switzerland, and Sweden. In an effort to capture maximum genetic diversity in the fungal and algal components, we sampled across a wide variety of altitudes, substrates, and microenvironments. The North American specimens were collected from 105 sites (**Figure 1.1**), with one specimen ultimately used from each of 49 sites, and an average of five specimens from each of the remaining 56 sites. Fifteen fertile specimens were included in the study. All voucher specimens have been deposited in the University of California Berkeley herbarium. **Table 1.1** provides a summary of collection and other data for 27 representative specimens from throughout our study area.

Three fungal loci were amplified and sequenced: fungal ITS, and two anonymous loci, DO and 11. Two algal loci were used: algal ITS and an actin I intron, using the actin primers 3T and 4T. Specimen preparation, DNA extraction, primer selection, and PCR were carried out for all five loci in accordance with Kroken and Taylor (2001a). PCR products were cleaned either with a QiaQuick PCR purification kit (Qiagen Incorporated) or using the enzymes Exonuclease I and Shrimp Alkaline Phosphatase (USB Corporation). In both cases, we proceeded according to the manufacturer's instructions. All PCR amplifications yielded single bands with the exception of the actin I intron locus of the epitype specimen which yielded 2 bands. Preliminary sequencing showed that lighter of these two bands was the target sequence. The band was cut out of a 1% agarose gel and cleaned with a PrepEase Gel Extraction Kit (USB Corporation) according to the manufacturer's instructions.

PCR products were sequenced in opposite directions either using an ABI PRISM® 3100 Genetic Analyzer or an ABI 3730 xl DNA Analyzer. Sequences were aligned with Sequencher (Gene Codes Corp.) or ClustalW in MEGA4 (Tamura et al. 2007), and then corrected by eye. Maximum parsimony trees were computed in MEGA4. Gaps were treated as a fifth character. Trees were obtained using the close-neighbor-interchange algorithm with search level 3 in which the initial trees were generated with the random addition of sequences (10 replicates).

NCBI accession numbers for selected specimens and four loci are indicated in **Table 1.1**. Accession numbers for the entire dataset follow. Fungal ITS: FJ161369-

FJ161667. Fungal locus 11: FJ133287-FJ153585. Algal ITS: FJ170466-FJ170764. Algal actin intron I: FJ170821-FJ171119. Each accession number is linked to the complete locus alignment through the PopSet database at NCBI. In the case of fungal locus DO, there is no alignment available because there were only two haplotypes, one corresponding to all *L. lupina* (FJ041055) and the other to all *L. vulpina* (FJ041056).

Fungal species were also diagnosed using restriction digests on the fungal ITS locus, which proved to be an efficient, accurate and inexpensive alternative to DNA sequencing. The restriction enzyme Eco0109I (New England Biolabs) was used in 50 μL reactions at 37° C overnight on PCR products cleaned with QiaQuick (Qiagen Corporation). Bands were visualized on a 1.5% agarose gel using SYBR Gold stain (Molecular Probes, Inc). Digested *L. lupina* yields two fragments, 156 bp and 535 bp. *Letharia vulpina* yields three fragments, 156 bp, 246 bp, and 289 bp. The 156 base pair fragments are not always visible on the gel. Algal clade membership can be quickly and accurately diagnosed by the size of the PCR amplicon, inasmuch as the two actin intron clades differ in PCR product size by about 70 bp, Clade A having the longer fragment. The algal clade is readily observed by running an agarose gel with the actin intron PCR product.

RESULTS

Three hundred and eight specimens of putative *L. vulpina* s. lat. were sequenced at three fungal loci and two algal loci, yielding a total of 1,540 sequences. For five specimens the fungal sequences were consistent with *L. columbiana*, and presumably

represent immature *L. columbiana* collected as *L. vulpina* s. lat.; this material has been excluded from further consideration. Also excluded were three additional sterile specimens that could not be placed within *L. vulpina, L. lupina* or *L. columbiana* s. lat. despite sequencing at three additional loci (18s, locus 2 and locus 13, see Kroken & Taylor 2001). The latter three specimens share a unique IGS haplotype and may be members of a new cryptic taxon, or they may be hybrids. Our final data set therefore consisted of 300 specimens.

The three fungal loci yielded eight fixed polymorphisms (**Table 1.2**), thus making it possible to distinguish unequivocally between the two fungal species. Of our 299 specimens, 261 yielded sequence patterns consistent for *L. vulpina* s. str.*,* whereas the remainder were consistent for *L lupina*. Of the 15 fertile specimens examined, 12 proved to be *L. lupina*.

The maximum parsimony bootstrap consensus tree for the algal ITS locus shows strong support for two algal clades, labeled A and B in **Figure 1.2**. The average genetic distance between the two algal clades is 51 base pairs. The previous analysis by Kroken and Taylor (2000) inferred multiple phylogenetic species of algae within Clade B, and one phylogenetic species within Clade A. The ITS tree differs from that published by Kroken and Taylor (2000) in that there are two distinct subclades of algae nested within Clade A.

Each of the two fungal species consorts with only one of the two algal clades (**Figure 1.2**). This analysis is based on the reduced data set presented in **Table 1.1**, and

confirms that the two fungal species consistently draw on separate algal partners: *L. vulpina* s. str. always pairs with algal ITS Clade A and *L. lupina* always pairs with algal ITS Clade B. The complete data set of 299 specimens yields the same pattern.

The algal actin intron sequence data is entirely congruent with the algal ITS tree. The actin intron DNA sequences divide into two distinct groups that parallel algal ITS Clades A and B, and are accordingly referred to by us as actin groups "A" and "B" (**Figure 1.3**). The two groups differ at 50% of the actin I intron sites, making phylogenetic tree construction unreliable (Kumar & Filipski 2007). This deep sequence difference strongly supports the genetic independence of the two algal lineages. The strict fungal-algal partnership pattern found with the algal ITS data holds true: *L. vulpina* s. str. always pairs with algal actin group A and *L. lupina* always pairs with algal actin intron group B.

Letharia lupina Altermann, Goward *sp. nov.*

Lethariae vulpinae *similis sed sequentia moleculari loco DO et loco 11 regionis ITS differt. Alga photobiontica genetice cum algis* Lethariae vulpinae *non congruit.* Similar to *Letharia vulpina*, but with distinct DNA sequences at ITS, locus DO and locus 11. Associates with an algal partner genetically distinct from the algal consort of *L. vulpina.*

TYPE. U.S.A. OREGON: Umatilla County, Umatilla National Forest, Lincton Mountain Road, 45°47'40"N, 118°9'41"W, 1354 meters, on wood, 29 June 2006, *Altermann 226,* (UCB holotype; UCSC, US, UPS, isotypes).

Description. Thallus shrubby-fruticose, highly variable, brilliant lemon yellow (typical material) to chartreuse green, except usually brown to black at tips and often whitish toward base or brownish at extreme base, varying from loosely subpendent (typical) to tightly tufted, at maturity 5-20 cm long and 4-8 cm wide, copiously branched, the branches terete to more often angular-ridged in cross-section, coarse, 1-3 mm wide in basal portions, mostly irregularly branching except more or less isotomic-dichotomous toward tips, lined on ridges with pseudocyphellae and with sparse (typical) or dense globular to weakly cylindrical isidia 0.1-0.3 mm long, these sometimes more or less replaced by non-corticate or weakly corticate "soredia."

Apothecia. Rare, appearing late in development, usually only on large thalli, 0.75-1.5 (-5) mm across, solitary, sessile; disc: pale brown to dark brown, dull to shiny, deeply concave when young, becoming less so with age; thalline margin: strongly raised and inflexed when young, few to no fibrils, 1-3 (-5) mm long, longer fibrils sometimes branching, underside of margin strongly foveolate and always sorediate; epihymenium: ca. 13 μm, brown; hymenium: 45-58 μm, hyaline; ascospores: ellipsoid, 5-7 × 3-4 μm, hyaline.

Pycnidia: Rare, laminal, immersed, usually with a dark brown to black rim; ostioles: ca. 75 μm diameter. Conidiophores ± type V (Vobis 1980). Conidia 7-9 μm × 1 μm, straight.

Chemistry. Vulpinic acid and atranorin in the cortex, with norstictic acid in the hymenium of the apothecia (Kroken & Taylor 2001).

Etymology. *Lupinus* is based on the traditional common name "Wolf Lichen" (Latin "lupus" = wolf), which alludes to the former use of this toxic lichen by Northern Europeans, who mixed it with fat and applied it to carcasses as bait for wolves (Santesson 1939).

Distribution. *Letharia lupina* is widely distributed in western North America (**Figure 1.1**), where it is much more frequently encountered than *L. vulpina*. Of 299 specimens examined by us, 260 (87%) proved to be *L. lupina* while only 33 (13%) belonged to *L. vulpina* s. str. *Letharia lupina* is also more widespread, alone occurring east of the continental divide into Alberta, Saskatchewan, Montana, Wyoming, Southwestern South Dakota, and Nevada. Similarly, *L. vulpina* is essentially restricted to lower elevations (<1600 m even in southern localities), while *L. lupina* extends upwards to treeline, with a total recorded elevational range between 190 m and 3370 m.

Though data are sparse, *L. vulpina* s. str. in the Old World appears to be relatively much more frequent and widespread. Recent molecular studies have confirmed its presence in Italy, Sweden, Switzerland, Turkey, Morocco, and the Caucasus (Høgberg et al. 2002; Arnerup et al. 2004). By contrast, *L. lupina* is known to

date only from mountainous areas of Morocco (Arnerup et al. 2004) and Switzerland (**Table 1.1**). The identity of the algal partner in the single Swiss specimen has been checked by us, and can now be confirmed as belonging to Clade B, as in North America.

Substrates. *Letharia lupina* is commonly found on old fence posts as well as on the decorticated trunks and branches of a wide variety of conifers, especially *Pinus*. It has also been documented from conifer bark, including that of *Abies, Calocedrus, Picea, Pinus,* and *Pseudotsuga*. On hardwood trees it most commonly colonizes wood, as in the case of *Arbutus*, *Arctostaphylos*, *Populus*, and *Salix*, though we have also seen it on the corticated branches of *Betula* and *Quercus*. Granitic outcrops also occasionally support this species.

Letharia vulpina (L.) Hue

Nouv. Arch. Mus., sér. 4, 1:57 (1899). --*Lichen vulpinus* L., *Species plantarum* 2: 1155 (1753); type without location, C. Linnaeus (LINN 1273.298 – upper left specimen, lectotype designated by Jørgensen et al. 1994:367); --*Evernia vulpina* Ach. *Lich Univ.* 443 (1810); SWEDEN. DALARMA, Idre par., Grundagssätern, V om vägen, 23 km N Idre, 62°03'N, 12°44'E, 685 m, 30 Sep 2008, *Hermansson 16600* (UPS L-173246 epitype).

The Linnean type specimen (LINN 1273.298) for *Lichen vulpinus* is a lectotype without a designated locality (Jørgensen et al. 1994). Though the lectotype specimen is morphologically congruent with typical material of *L. vulpina* – at least as we know it in western North America – we cannot exclude the possibility that it actually represents *L. lupina*. Accordingly, we hereby designate an epitype with DNA sequencing for the fungal internal transcribed spacer region, anonymous fungal locus DO, anonymous fungal locus 11, algal internal transcribed spacer region, and an algal actin I intron (NCBI Accessions: GQ398408-GQ398412).

Notes. Based on differences in their respective North American distributions as summarized in **Figure 1.1**, *L. lupina* clearly has a comparatively broad ecological amplitude in inland western North America, while *L. vulpina* appears to be specially adapted to summer-warm localities subject either to nighttime dew or to frequent fog. The northern Idaho population of *L. vulpina* appears to be an outlier, a pattern observed in a variety of organisms associated with mesic forest ecosystems (Brunsfield et al. 2001; Carstens et al. 2005). In addition, the only Sierra Nevada sites found with *L. vulpina* fall within the "Oroville anomaly," an area of higher precipitation and an unusual mixture of lichens typical of two distinct lichenological geographic regions: the California north coast ranges and the Sierra Nevada (Jovan and McCune 2004).

DISCUSSION

The results of our study are consistent with the hypothesis that *L. lupina* warrants recognition as a species distinct from *L. vulpina*. Not only do the fungal

phylogenetic species differ from one another on molecular grounds, they maintain their separate identities in localities where the fungal species co-occur, and consistently consort with separate clades of *Trebouxia jamesii* s. lat.. Five of our collection sites throughout the latitudinal range of *L. vulpina* supported both fungal species within 200 m of one another, that is, within the expected approximate limits of ready dispersal for sorediate species (Werth et al. 2006): Spahats Falls, British Columbia; Lower Stein Valley, British Columbia; White Pass, Washington; Castle Crags State Park, California; and Ventana Wilderness, California. The Stein Valley locality, moreover, yielded two fertile specimens of each fungal species. Given that no unexpected algal pairings were detected at any location, we conclude that each fungus is truly specific to its respective algal clade. Thus, phylogenetic constraint, not ecological opportunity, seems to limit each fungal species' breadth in algal partnerships.

Currently, no distinction is made between *L. vulpina* and *L. lupina* lichens in air pollution monitoring studies, and this could confound interpretation of results. It would be valuable to test whether the two lichens differ in their pollution tolerance or in bioaccumulation of commonly tested pollutants. The existence of multiple sites where the two taxa grow sympatrically makes such a study feasible.

Although Goward (1999) originally posited the existence of *L. lupina* based largely on morphological grounds, our study now makes clear that the morphology of this species readily intergrades with that of *L. vulpina* (Kroken & Taylor 2001; Ryan 2002; Arnerup et al. 2004). Phenotypic overlap is especially evident where the two

species grow in sympatry; elsewhere these species are more or less morphologically distinct. The two species' similarity in sympatry may reflect matching morphological development under identical or at least widely overlapping ecoclimatic conditions. *L. lupina* differs from *L. vulpina* in at least three characters: first in color (lemon yellow versus greenish yellow, respectively); second in branching (long and sparsely ramified versus short, abundantly ramified); and third in isidia production (sparse versus rather copious) (Goward 1999). Also diagnostic for L. *lupina* is the presence of bright yellow cortical patches, which contrast both with the rather more greenish isidia and with the similarly greenish cortical tissues immediately surrounding the isidia. This gives the thallus a characteristic "two-tone" appearance not observed by us in *L. vulpina* and, for that matter, by no means always present in *L. lupina*.

DNA sequencing is increasingly being used to clarify phylogenetic relationships among lichenized fungi, especially above the rank of species (Lutzoni et al. 2001, 2004; Crespo et al. 2007). DNA sequencing is also being used to support morphological species recognition (Tibell and Beck 2001; Miadlakowska et al. 2002; Molina et al. 2004; Divakar et al. 2005a, 2005b; Seymour et al. 2007) as well as species distinguished primarily on secondary chemistry (LaGreca 1999; Bayerová et al. 2005; Slavíková-Bayerová & Orange 2006). A non-lichenizing species of Ascomycota, *Coccidioides posadasii*, has been described on the basis of DNA sequence characters, physiological characters, and without morphological characters (Fisher et al. 2002), but to date no lichenized species has been described lacking clear cut morphological characters.

There is legitimate concern in lichenology that molecular-based taxonomic revision at the rank of species is impractical and could have the undesirable effect of excluding key taxonomic end-users (Purvis et al. 2000). In their review of species concepts in lichens, Grube and Kroken (2000) offer an alternative to raising morphologically cryptic taxa to the rank of species by suggesting the subspecies rank and adding an "agg." (aggregate) appendix to the name. While the use of "agg." may be appropriate for the purposes of informal field identification, we believe that *L. lupina* provides a compelling case for formal description at the rank of species.

At least four arguments can be advanced in favor of recognizing *L. lupina* as a distinct species. First, adopting a subspecies rank for *L. lupina* would result in a polyphyletic *L. vulpina* agg.. *Letharia lupina* and *L. vulpina* s. str. are not closest relatives, as *L. vulpina* s. str. is genetically more similar to the *L. columbiana* 'rugosa' lineage, whereas *L. lupina* is more similar to the remaining three lineages of *L. columbiana* ('gracilis,' 'barbata,' and 'lucida') (Kroken & Taylor 2001). Second, the present study indicates multi-locus fixed polymorphisms on *both* sides of the symbiosis. The *L. lupina* lichens consist not only of different fungal phylogenetic species but of a different *symbiosis* than that of *L. vulpina* s. str.. This fundamental biological difference between the lichens holds up throughout their geographic ranges. Third, *L. lupina* and *L. vulpina* can be distinguished in the field in most situations. Only in localities of sympatry are morphological points of distinction inadequate for reliable identification. And fourth, relatively simple molecular techniques in the form of restriction digests and AFLPs

bring genetic identification within reach of taxonomic end-users with access to community college level molecular tools.

Finally, Grube and Kroken (2000) have suggested that three criteria should be met prior to the introduction of formal taxonomic changes based on molecular data: 1) the data set should incorporate a large sample size spanning all sub-taxa; 2) the resulting clades must achieve high support values through confidence values or statistical tests; and 3) a thorough review of the taxon's nomenclatural history must be undertaken. We submit that all of these criteria are satisfied in the present case involving *L. vulpina* and *L. lupina*.

Table 1.1 Select Specimen Location Information And Genetic Identity

NCBI accessions are in the following locus order: fungal ITS, locus 11, algal ITS, and actin I intron.

Study ID #	Location	Date	Collector	Fungal species	Algal clade	Fungal Accessions	Algal Accessions
CANADA: Alberta							
AB1c	West Jumpingpound, 30 miles west of Calgary	Jul-05	D. Glass	*L. lupina*	B	FJ161370 FJ153288	FJ170467 FJ170822
BL2a	Bow Lake, 90 km north of Banff	Oct-05	D. Glass	*L. lupina*	B	FJ161400 FJ153318	FJ170497 FJ170852
CANADA: British Columbia							
GM8	Greenstone Mountain, summit area	May-05	T. Goward	*L. lupina*	B	FJ161445 FJ153363	FJ170542 FJ170897
OH1	South of 100 Mile House along Hwy 97	Apr-05	S. Stevenson	*L. lupina*	B	FJ161516 FJ153434	FJ170613 FJ170968
PG1	Teapot Mountain, north of Prince George	Jun-05	S. Stevenson	*L. vulpina*	A	FJ161647 FJ153565	FJ170744 FJ171099
SWITZERLAND							
SW2	Canton Graubünden, Silvaplana	Sep-99	T. Goward	*L. lupina*	B	FJ161566 FJ153484	FJ170663 FJ171018
TURKEY							
TK1a	Ala Dag, 29 km South of Bolu	Jun-07	T. Spribille	*L. vulpina*	A	FJ161663 FJ153581	FJ170760 FJ171115
USA: CALIFORNIA							
AR1	Calaveritas Drive, Arnold, Calaveras County	Jan-04	B. Poulson	*L. lupina*	B	FJ161379 FJ153297	FJ170476 FJ170831
EP3a	Ebbetts Pass, Alpine County	Jul-04	S. Altermann	*L. lupina*	B	FJ161432 FJ153350	FJ170529 FJ170884
SD1a	Mt. Laguna, San Diego County	Oct-03	T.H. Nash	*L. lupina*	B	FJ161540 FJ153458	FJ170637 FJ170992
SJ7a	San Jacinto Wilderness Area, Riverside County	Jul-06	K. Knudsen	*L. lupina*	B	FJ161548 FJ153466	FJ170645 FJ171000
PU1a	Plumas N.F., Feather River Rd, Yuba County	Dec-03	C. Dillingham	*L. vulpina*	A	FJ161649 FJ153567	FJ170746 FJ171101
SD2	Palomar Mountain St. Park, San Diego County	Apr-05	K. Knudsen	*L. vulpina*	A	FJ161654 FJ153572	FJ170751 FJ171106
USA: IDAHO							
ID11	Giant White Pine State Park, Latah County	Apr-06	C. Bjork	*L. lupina*	B	FJ161458 FJ153376	FJ170555 FJ170910
ID6a	Coeur D'Alene Mountains, Shoshone County	Sep-05	C. Bjork	*L. lupina*	B	FJ161463 FJ153381	FJ170560 FJ170915
ID7a	Kaniksu National Forest, Boundary County	Jun-05	C. Bjork	*L. vulpina*	A	FJ161640 FJ153558	FJ170737 FJ171092
ID9a	Near Orofino Creek, Clearwater County	Jun-05	C. Bjork	*L. vulpina*	A	FJ161641 FJ153559	FJ170738 FJ171093

Study ID #	Location	Date	Collector	Fungal species	Algal clade	Fungal Accessions	Algal Accessions
USA: MONTANA							
BR5a	Bitterroot N.F., French Basin, Ravalli County	May-05	A. Pipp	*L. lupina*	B	FJ161403 FJ153321	FJ170500 FJ170855
MT3a	Lincoln State Forest, Lewis and Clark County	May-05	A. Pipp	*L. lupina*	B	FJ161509 FJ153427	FJ170606 FJ170961
USA: OREGON							
WL1	Wallowa-Whitman N.F., Wallowa County	Jun-06	S. Altermann	*L. lupina*	B	FJ161614 FJ153532	FJ170711 FJ171066
RS1	I5 rest stop, Douglas County	Mar-05	S. Altermann	*L. vulpina*	A	FJ161653 FJ153571	FJ170750 FJ171105
WN5	Highway 126 Willamette N.F., Linn County	Mar-05	S. Altermann	*L. vulpina*	A	FJ161665 FJ153583	FJ170762 FJ171117
USA: WASHINGTON							
WA6a	Del Rio Road, Douglas County	May-05	C. Bjork	*L. lupina*	B	FJ161605 FJ153523	FJ170702 FJ171057
WA7a	Badger Mountain, near ski area, Douglas County	Aug-05	C. Bjork	*L. lupina*	B	FJ161606 FJ153524	FJ170703 FJ171058
WP8	Snoqualmie National Forest, Yakima County	Jun-06	S. Altermann	*L. vulpina*	A	FJ161667 FJ153585	FJ170764 FJ171119
USA: WYOMING							
BH9	Bighorn N.F., S. Tongue River, Sheridan County	Jun-05	S. Bell	*L. lupina*	B	FJ161397 FJ153315	FJ170494 FJ170849
IK2	Black Hills National Forest, Crook County	Jul-06	M. Zimmerman	*L. lupina*	B	FJ161465 FJ153383	FJ170562 FJ170917

Table 1.2 Eight Fixed Polymorphisms Across Three Loci

ITS and locus DO position numbers are based on NCBI PopSet alignments that are linked to the accessions associated with this paper. A cyotsine at ITS position 388 yields an Eco0109I restriction site.

Locus	**ITS**			**11**		**DO**		
Position	69	388	499	29	198	113	122	156
L. vulpina	C	C	C	C	C	A	T	T
L. lupina	T	T	T	T	T	G	C	C

Figure 4.1 Geographic Distribution of *L. vulpina* and *L. lupina*

Map is based on 316 specimens sampled across 105 sites. Map combines data from this study and Kroken and Taylor 2001. Base map is from MapPad 2.0 (Keltner 1996).

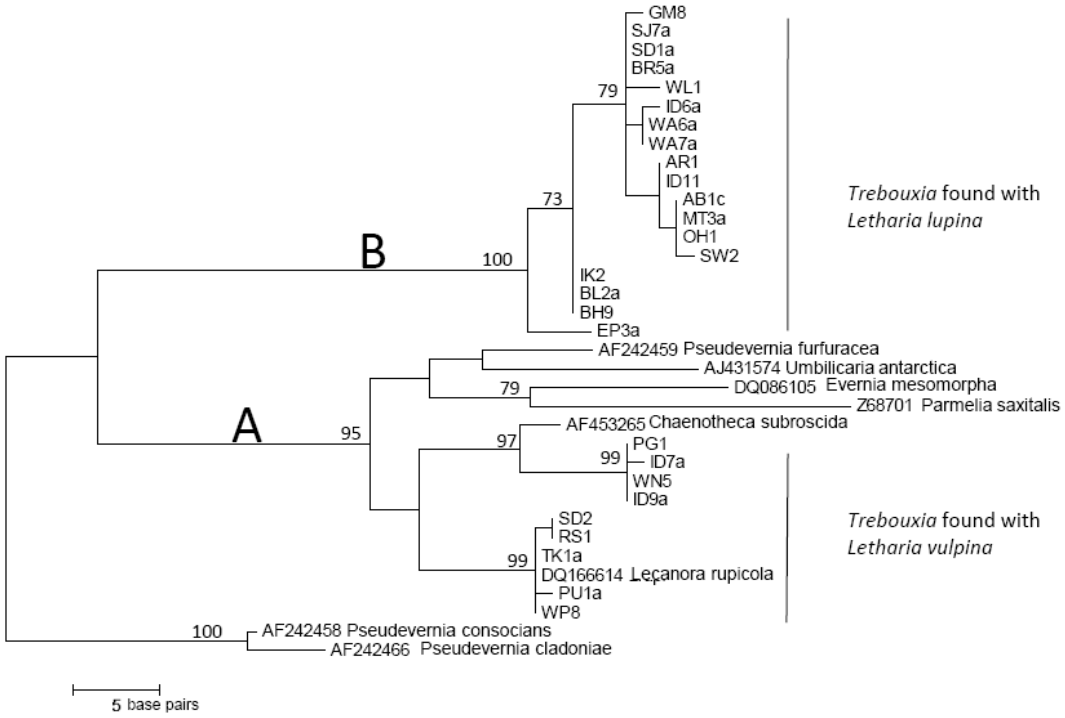


Figure 1.5 *T. jamesii* ITS Maximum Parsimony Consensus Tree

Tree is based on 1000 replicates. Three and four-character codes are individual sample identification codes (see **Table 1.1**). Eight-character codes are NCBI accession numbers. Numbers at nodes represent bootstrap support for the clade. Only bootstrap values over 70% are shown. The tree is rooted by the algae that partner with *Pseudevernia consocians* and *Pseudevernia cladoniae*. There are 746 positions in the alignment, 88 of which are parsimony informative.

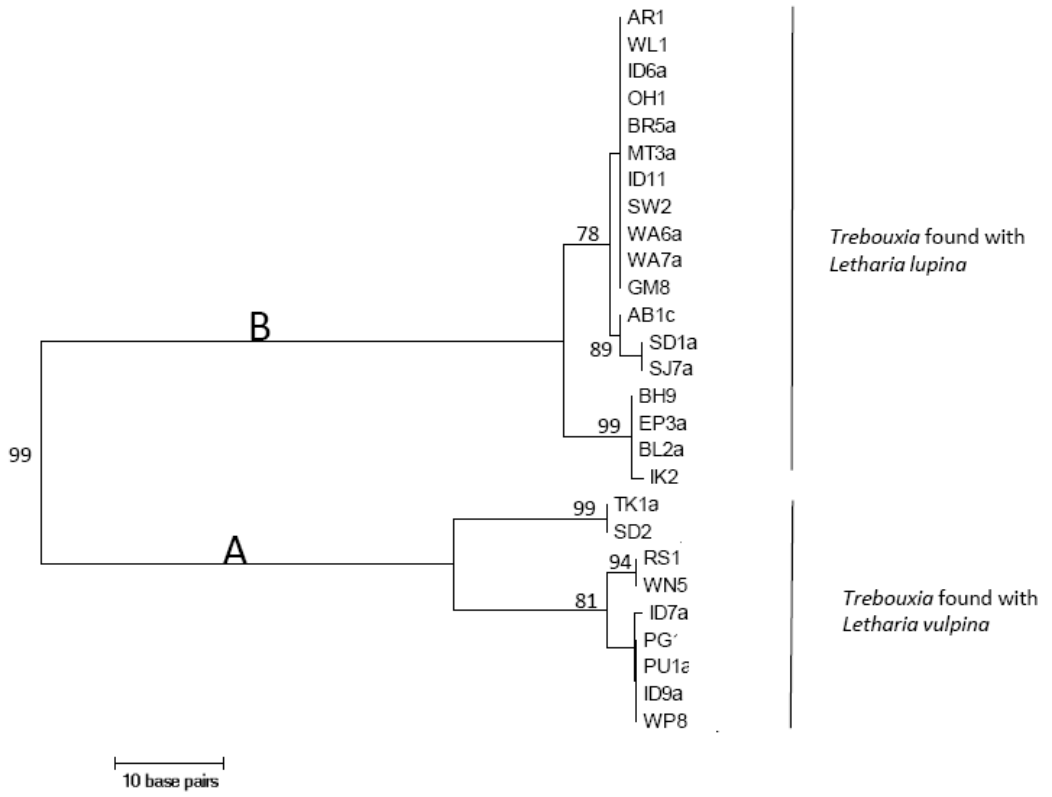


Figure 1.6. ***T. jamesii*** **Actin Intron Maximum Parsimony Consensus Tree**

Tree is based on 1000 replicates. Numbers at nodes represent the clade's bootstrap support. Only bootstrap values of 70% are shown. There are 280 positions in the alignments, 122 of which are parsimony informative. Three and four-character codes are individual sample identification codes (see **Table 1.1**). The tree is midpoint rooted.

CHAPTER 2

GEOGRAPHIC STRUCTURE OF MOUNTAIN WOLF LICHEN ALGAE OF WESTERN NORTH AMERICA

ABSTRACT

This study establishes that terrestrial green algae are important drivers of genetic structure in widespread lichen fungal-algal symbioses and that green algae share some of the same phylogeographic patterns found in Western North American plants and animals. *Trebouxia* Puymaly algae are the most common photosynthetic partners found in lichens, but the geographic structure of these widespread green algae is little studied. DNA sequencing of the ITS locus and an actin I intron demonstrates that *T. jamesii* (Hildreth & Ahmadjian) Gärtner present in widespread and abundant Mountain Wolf lichens (fungal partner: *Letharia lupina*) have a well developed geographic structure in Western North America. Five genetic lineages exhibit distinct geographic distributions. Pairwise population Φst within each of the two most widespread lineages suggest gene flow at a large geographic scale. Southern California is the center of algal lineage diversity for these algae. One of the five lineages also partners with another widespread lichen fungus, *Hypogymnia imshaugii* Krog, indicating a lack of specificity for *L. lupina*. Parallels in algal geographic structure with that of co-distributed plants and animals are discussed for Southern California, the Rocky Mountains, the Great Basin and the California-Oregon border region.

INTRODUCTION

The movement of populations across the landscape is important in understanding current distributions of taxa and in predicting future distribution shifts that result from climate change. Pleistocene glaciations have affected the genetic structure of numerous plants and animals of Western North America (Conroy and Cook 2000, Abrogast et al. 2001, Knowles 2001, Jerome and Ford 2002, Dobes et al. 2004, Brunsfeld and Sullivan 2005, DeChaine and Martin 2005, Kuchta and Tan 2005, Song 2006). Species and species complexes currently distributed over large portions of this region are valuable for inferring post-Pleistocene colonization patterns shared with other taxa. The broadest possible sampling of life-forms within a region enables a distinction between general historical processes that influence all taxa from processes specific to individual taxa (Thompson and Calsbeek 2005). In this study, DNA sequences are used (1) to determine the genetic identity of widespread symbiotic algae, (2) to map their geographic distributions, (3) to investigate the geographic structure of their genetic diversity, (4) to examine their fungal specificity, and (5) to compare their geographic structure with that of other taxa of Western North America.

The Cordilleran ice sheet reached northern Washington, Idaho and Montana at the last glacial maximum approximately 18,000 years ago (Waitt and Thorson 1983). Most organisms found today in regions previously covered by ice sheets had to have dispersed there from regions south of the ice or from northern glacial refugia (Pielou 1991). Environments (south of the ice sheets) suitable for the taxa displaced by the extreme climate changes were discontinuous because the Cascade Mountains, Rocky

Mountains and Sierra Nevada Mountains were heavily glaciated, and the surrounding areas would have experienced dramatic restructuring of habitats (Hewitt 1999). The roughly longitudinal orientation of the western cordillera has tended to act as a barrier to east-west dispersal, giving rise to corresponding east-west genetic discontinuities in many taxa (Swenson and Howard 2005, Jaramillo-Correa et al. 2009). Included among these are phylogenetic splits in western conifers such as *Pinus contorta*, *Pinus ponderosa* and *Pseudotsuga menziesii,* all of which provide important substrates for Wolf lichens (Godbaut et al. 2008, Latta and Mitton 1999, Li and Adams 1989). If Mountain Wolf lichens share a common history with their substrate trees, then Mountain Wolf lichen algae might be expected to exhibit a similar east-west phylogenetic split.

Within Western North America, the Pacific Northwest is the only region that has already accumulated several comparative phylogeographic studies (Soltis et al. 1997, Brunsfeld et al. 2001, Carstens et al. 2005). For taxa with wide latitudinal distributions, these studies often show decreased genetic diversity of northern populations and a strong zone of genetic discontinuity near the California-Oregon border sometimes referred to as the "Soltis Line" (Brown et al. 1997, Brunsfeld et al. 2007, Forister et al. 2004, Soltis et al. 2007). Two hypotheses are invoked to explain the latter pattern. The Leading Edge Hypothesis asserts that post-Pleistocene colonists moving into recently deglaciated regions experienced exponential growth in the low competition "leading edge" environment (Hewitt 1996). Additionally, diverse genetic types from the southern refugial populations that arrived later may have been at a disadvantage as the early genetic types had already occupied most of the available ecological niches. The

resulting genetic structure, based on a founder effect, is one of decreasing genetic diversity as one travels north, especially into the vast areas formerly covered by Pleistocene ice sheets. The Soltis Line has also been explained by the "North-South Recolonization" Hypothesis, a process in which bottlenecked populations emerge from northern and southern refugia after the Pleistocene forming a line of contact between alternative genetic types in the region of the California-Oregon border (Soltis et al. 1997).

Whether the Leading Edge Hypothesis, the North-South Recolonization Hypothesis, or more complex hypotheses (Rich et al. 2008) are relevant to lichenized algae is a question best approached in terms of the genetic structure of the algae as well as the likely location of glacial refugia. There is little evidence that nunataks and other northern refugia would have supported the conifers used as substrates by Mountain Wolf lichens (but see Anderson 2006, Brubaker et al. 2005). A recent study based on microfossils and molecular work for *Pinus contorta* (Lodgepole Pine), a substrate species for Mountain Wolf lichens, infers at least four southern Pleistocene glacial refugia for this tree: 1) North Dakota and Montana 2) the Eastern Cascade Mountains 3) the Western Cascade Mountains, and 4) coastal British Columbia or Alaska (Godbout et al. 2008). Today, *Pinus contorta* is a substrate for Mountain Wolf lichens in Idaho, British Columbia, Alberta and Saskatchewan, and it is safe to assume that it would have served as a substrate for these lichens during the Pleistocene as well. The tree may have grown right up to the edge of the Cordilleran ice sheet (Heusser 1969), facilitating colonization of new land as the ice retreated. Through association with Lodgepole

Pine, Mountain Wolf lichen may have been an early colonizer of formerly glaciated regions, becoming established from multiple southern Pleistocene refugia.

Farther south, biogeographic patterns across the California Floristic Province are influenced by the complex geologic history, sharp climatic gradients, and shifting climate due to changes in the California Current during the Pleistocene (Calsbeek et al. 2003, Herbert et al. 2001). Prominent phylogeographic discontinuities affecting numerous animal taxa occur across the Central Valley and across the Transverse Ranges. These discontinuities have been attributed to the unique geological and climatological history of California (Calsbeek et al. 2003). Most of the studies that contribute to our current understanding of phylogeographic patterns within the California Floristic Province concern taxa, such as ornate shrews, California thrashers, foxtail pines, and flightless beetles, that are not nearly as widespread (or as continuous in distribution) as Mountain Wolf lichens (e.g. Moldonado et al. 2001, Sgariglia & Burns 2003, Eckert et al. 2008, Caterino & Chatzimanolis 2009). However, widespread taxa including an owl, a butterfly and a moth, show some of the same phylogeographic breaks as the more narrowly distributed species (Barrowclough et al. 1999, Forister et al. 2004, Rich et al. 2008).

Lichens

Lichens are symbiotic systems that typically include a green algal photosynthetic partner (Ahmadjian 1967) in addition to the ascomycete fungus. Lichens can have large geographic ranges. Some taxa span entire hemispheres, while others occur both in arctic and antarctic regions (Galloway 2008). Molecular techniques have demonstrated

that some geographically widespread lichenized fungal taxa are actually made up of multiple phylogenetically independent species (Kroken and Taylor 2001a). Even these more narrowly defined fungal species can have vast geographic ranges (Kroken and Taylor 2001a, Murtagh et al. 2002, Wirtz et al. 2008).

Letharia lupina Altermann, Goward & Kroken is an obligately lichenized ascomycete commonly found throughout much of Western North America. Typically its lichens form large, shrubby tufts which, owing to their brilliant yellow to chartreuse color, stand out on the bark and wood of a variety of conifers in dry woodlands from northern Mexico north to British Columbia and from the Cascade Mountains east to the Black Hills of North Dakota (Chapter 1). The fungus rarely forms sexual fruiting bodies and is understood to disperse primarily through dual fungal-algal propagules, usually isidia and sometimes soredia (Schade 1954). *Letharia lupina* lichens (earlier included within *Letharia vulpina* L., see Chapter 1 for points of distinction between the two species) have been shown to have a pronounced tolerance for air pollution (Sigal and Nash 1983), making them a highly useful lichen in air quality monitoring studies (Fenn et al. 2007, Geiser & Neitlich 2007, Jovan & Carlberg 2007).

Letharia still holds the distinction of being the only multi-species lichen-forming fungal genus that has received detailed phylogenetic attention on both sides of the symbiosis. Kroken and Taylor used multiple gene genealogies to infer six fungal species and seven algal species within *Letharia* lichens (Kroken and Taylor 2000, 2001a, McCune and Altermann 2009). Basing their observations on 16 thalli collected from California north to British Columbia, Kroken and Taylor were able to confirm that at

least three *Trebouxia* lineages partner with *L. lupina* fungi. This level of diversity makes Mountain Wolf lichens suitable candidates for more detailed studies of the geographic structure of lichenized green algae.

Taxonomic concepts within the lichenized algae have traditionally been based on cell morphology and anatomy with special emphasis placed on the ultrastructure of the pyrenoids (Tschermak-Woess 1988, Takeshita 2001, Friedl and Budel 2008). Recent molecular work has shown that this approach often fails to resolve many phylogenetically independent algal lineages (Kroken and Taylor 2000), but the degree to which lichenized algae are morphologically cryptic is unknown and understudied. Although lichenized algae are increasingly being subjected to molecular investigation, multiple loci have only been used in two studies (Kroken and Taylor 2000, Piercey-Normore 2004). Neither of these resulted in a revision of algal taxonomy.

Mountain Wolf algae may also form lichens with fungi unrelated to *L. lupina*. The Forked Tube lichen (Brodo et al. 2001) is another geographically widespread lichen of Western North America, and it is commonly found growing in association with Mountain Wolf lichens. The fungal component of Forked Tube lichens, *Hypogymnia imshaugii,* has abundant fungal sexual reproductive structures (apothecia), but it produces no obvious dual fungal-algal asexual reproductive/dispersal propagules such as soredia or isidia. It is not clear how the germinating ascospores of such fungi obtain their algal partners in order to form lichens. There is evidence that *Trebouxia* algae grow free-living on the substrates occupied by lichenized fungi (Bubrick et al. 1984, Sanders 2005), but *Trebouxia* are usually regarded as obligate symbionts

(Ahmadjian 1988, 2002). Alternatively, lichenized fungi that lack dual fungal-algal dispersal mechanisms may "commandeer" the algae dispersed by sorediate or isidiate lichens of the same community. The often considerable abundance of these two lichens with contrasting reproductive modes on the same tree trunks begs the question whether *H. imshaugii* fungi make use of the same algae as *L. lupina*. Low specificity for fungal species complicates the study of algal geographic structure because multiple lichen taxa have to be analyzed simultaneously. On the other hand, finding algae that pair with both *L. lupina* and *H. imshaugii* would support the hypothesis that strictly sexual lichenized fungi rely on locally abundant isidiate lichens, such as Mountain Wolf lichens, for their symbiotic partners.

MATERIALS AND METHODS

Study System

Although apparently rare in the old world (Chapter 1, Arnerup et al. 2004), Mountain Wolf lichens are abundant and geographically widespread in Western North America. Wherever there are dry coniferous forests or there is dead wood high in the canopy of rain forests (McCune et al. 2000), Mountain Wolf lichens are often present, especially above 1000 meters in elevation. Obvious breaks in the lichen's distribution include California's Central Valley, the Columbia Plateau, and the Great Basin, although in the vast Great Basin, *L. lupina* lichens are occasionally found in sparse subalpine coniferous forests. In Southern California, *L. lupina* lichens have a patchy distribution in "sky islands" of evergreen forests above 1400 meters in elevation.

Sampling

Two hundred fifty-nine specimens from a phylogenetic study differentiating *Letharia vulpina* L. and *L. lupina* were used in this study (Chapter 1). Specimens were collected from 80 sites (**Table 2.1**, **Figure 2.1**) spread across most of the North American range of this lichen. Unsampled areas include the Rocky Mountains of Colorado, Southwestern Yukon, Alaska, Baja California, and Northern Mexico, all which have sparse populations of wolf lichens. Baja California and Northern Mexico are undersampled for lichens in general, and while wolf lichens have been reported on a few occasions from this region (e.g. Ryan 2001), I consider Southern California to be at or near the southern extreme of the Mountain Wolf lichen population distribution. One to three specimens were collected from 46 sites and 4-9 specimens (average 5.6 specimens per site) were collected from the remaining 34 sites. Within sites, 95% of collections were spaced at least 100 meters from other specimens in order to avoid collecting vegetative clones. This distance was chosen based on Werth et al. (2006), who in a study of *Lobaria pulmonaria* found that a majority of soredia disperse less than about 100 m from the source thallus. The soredia of *L. pulmonaria* are similar in size and shape to the vegetative propagules of Mountain Wolf lichens.

Mountain Wolf lichens were collected from fence posts (12 specimens), from granite (2 specimens), and from 30 species of plant, including 20 species of conifer. The three most common conifer substrates were *Pseudotsuga menziesii*, *Pinus ponderosa*, and *Pinus contorta*, comprising 43% of the substrates identified to species.

In addition, eleven specimens of the Forked Tube lichen (fungus = *Hypogymnia imshaugii*) were included in this study to characterize the algal partner. Seven specimens were collected from Stanislaus National Forest (Site 42; **Table 2.1**) with an additional specimen from Palomar Mountain, Cleveland National Forest (Site 47) and another from site Boise National Forest (Site 54). Two additional specimens were collected from sites not listed in **Table 2.1**: one from Gazos Creek Watershed (San Mateo County, California, N37° 11.72' W122° 16.13'), and one from Mount Hood National Forest (Hood River County, Oregon, N45° 10.66' W121° 41.75'). The specimens were compared against authentic material, and have been deposited in the University of California Berkeley herbarium.

Molecular methods

Sample material of *Letharia* was determined to species using molecular techniques. The fungal partner was verified by sequencing three loci (the internal transcribed spacer region and two anonymous loci, DO and locus 11), and the algae were characterized using the algal ITS locus and an actin I intron. For detailed molecular methods, see Chapter 1). I also attempted to include the second actin I intron used by Kroken and Taylor (2000), but PCR amplification success was wildly inconsistent.

Phylogenetic analysis

New algal sequence data from 258 Mountain Wolf lichen thalli and 11 Forked Tube lichens were combined with the data from 14 Mountain Wolf lichens and 22

collections of *Letharia columbiana* sensu lato lichens previously published by Kroken and Taylor (2000). Sequences were aligned with Sequencher (Gene Codes Corp.) and then corrected by eye. Collapse 1.2 (Posada 1998) was used to identify identical algal sequences, and duplicate sequences were removed for the phylogenetic analysis. To determine the best-fit model for sequence evolution, MrModeltest version 2.3 (Nylander 2004) was employed, and for the Bayesian phylogenetic analysis MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003) was used. The settings were: random starting trees for 1,000,000 generations sampled every 100 generations for the ITS locus and 500,000 generations sampled every 100 generations for the actin I intron.

The ITS tree was rooted based on a search of the NCBI database for closely related algae. For the actin locus, an arbitrary outgroup was chosen because the alignment with potential outgroups, including the algae that pair *L. vulpina*, was highly ambiguous. For all subsequent analyses, only the *L. lupina* lichen algal sequence data generated in this study were used.

The two algal loci were tested for congruence with the Partition Homogeneity Test (PHT or Incongruence Length Difference Test, Farris et al. 1994) using PAUP 4.0b10 (Swofford 2002). The settings were: random stepwise addition, maximum parsimony heuristic search, 1000 replicates, TBR, and maximum trees = 500. The null hypothesis of locus congruence is rejected when $P < 0.001$ (Darlu and Lecointre 2002). This test was carried out both with combined lineages and within lineages. The PHT was applied to the combined lineage data set as an indication of congruence between maximum parsimony trees for the two loci. The SH test (Shimodaira and Hasegawa

1999) was used to compare the topologies of the ITS and the actin Bayesian trees, also in PAUP. Branches with less than 90% posterior probabilities were collapsed to run this test. To avoid incongruence within lineages due to recombination, the datasets were pruned to contain only two individuals per algal lineage. Haplotype networks were calculated in TCS version 1.21 (Clement et al. 2000) using only the haplotypes from North America.

Population Delineation

The nearly continuous geographic distribution of Mountain Wolf lichens in Western North America complicates delineation of discrete populations, thereby creating a potential obstacle to the recognition of geographic structure. In addition, there is a trade-off between sampling many individuals per site and sampling many sites throughout the geographic range of a species. Because broad geographic sampling was favored in this study, there are generally too few samples per site to be legitimately representative of a geographic "population," even if the lichens had been distributed in discrete geographic clusters. To infer broad trends in spatial genetic variation, data from 64 sites were divided up into 12 groups based on geographic proximity, physiographic barriers, and on trends in haplotype frequencies (**Figure 2.1**). Due to geographic isolation, the fifteen remaining sites representing 18 individual thalli were excluded from the spatial analysis. Excluded sites are located in Nevada (4 sites), British Columbia (2 sites), Saskatchewan (1 site), Utah (1 site), Idaho (4 sites), Washington (2 sites) and California (1 site).

Spatial analysis of molecular variance (SAMOVA: Dupanloup et al. 2002) yields clusters of sampling groups according to the a priori assigned number of populations (K) based on a simulated annealing procedure. The analysis takes into account both genetic and geographic distances to identify genetically and geographically cohesive populations. SAMOVA analyses were carried out for each locus separately.

SAMOVA may be useful in defining the number of populations within a large data set by comparing F_{ct} values, but it is not specifically designed for this purpose (Dupanloup et al. 2002). In tests of this application of SAMOVA, Dupanloup et al. found that the largest F_{ct} values corresponded with the correct number of populations, but the F_{ct} values were not significantly different from each other. In running additional simulations, they found high variation in assigning the correct number of populations, with the correct number of populations depending on differentiation between populations, isolation by distance within populations, and the number of loci used. The SAMOVA approach is used here not to find a correct *number* of populations, but to infer spatially and genetically cohesive clusters of collection site groups that will be treated as populations for the remaining analyses. K=2 to K=9 populations with 100 simulated annealing processes were tested for each algal locus. Collection site groups were pooled into populations based on clusters with high F_{ct} values, clusters found under multiple values of K, and congruence between group clusters at each locus.

Population structure

Overall and pairwise Φst were used with partitions by population to describe genetic and geographic differentiation using Arlequin 3.1 (Excoffier et al. 2005). Φst incorporates both frequency and genetic distance information into the analysis. Both combined lineage and individual lineage analyses were carried out. Sample sizes were large enough to carry out individual lineage analyses within algal lineage 1 and algal lineage 3. For algal lineage 1, the Eastern Rocky Mountains and Transverse Range populations were excluded from the analysis due to small sample size. For algal lineage 3, only the British Columbia, Western Rocky Mountains and Eastern Rocky Mountains populations had large enough sample sizes for analysis. Arlequin was also used to calculate standard indices of genetic diversity.

"Isolation by distance" is a correlation between genetic differentiation and geographic distance (Wright 1943). Isolation by distance should be found between populations that share gene flow proportional with the geographic distance between them. Mean number of pairwise genetic distances were plotted against geographic distance between collection site groups to estimate isolation by distance within the most geographically widespread algal lineage.

RESULTS

Phylogenetic Analysis

When the data from this study are combined with the data from Kroken and Taylor (2000), there is evidence for seven algal lineages. All seven clades have greater than or equal to 0.95 support for at least one locus on the Bayesian trees (**Figure 2.2**

and **Figure 2.3**). The Bayesian phylogenetic analyses are congruent with those of Kroken and Taylor's previous analyses based on maximum parsimony and maximum likelihood (2000). For clarity, I use the same clade numbering system, but refer to the clades as "lineages" instead of species. Four points of variance with Kroken and Taylor's results can be noted. First, the topology of the Bayesian trees' deeper nodes for both the ITS and actin I intron trees differs from that of Kroken and Taylor, but this is resolved when branches with less than 95% Bayesian posterior probabilities are demoted to polytomies. Second, in the Bayesian ITS analysis, lineage 3 and 5 are unresolved, and only the actin I locus supports their separation. Third, lineage 1 is neither supported nor contradicted by the actin I locus Bayesian analysis as the taxon forms a paraphyletic group with Alga 2, Alga 3, and Alga 9, and only the ITS locus Bayesian analysis supports the recognition of this clade. Fourth, a new, small, and geographically restricted clade is supported by the ITS Bayesian tree. I refer to this clade as lineage 9. Like lineages 1, 2, 3, and 5, lineage 9 is only resolved as a monophyletic group based on one of the two loci in the Bayesian analysis. In each case, the data from the uninformative locus do not conflict with tip clades in the more resolved locus.

Three individuals with unique ITS and unique actin haplotypes do not clearly group with any of the above lineages. The affected ITS-actin haplotype combinations are 129-503, 507-504, 108-203. The first two were found in Mountain Wolf lichen thalli collected in the Peninsular Ranges and the third was found in a *Letharia columbiana*

'barbata' thallus collected in Washington State (individual TC1-1, Kroken and Taylor 2000). These individuals may be algal lineage hybrids.

The Forked Tube lichen algae are polyphyletic. Four individuals from ITS algal clade A algae (Chapter 1) were found (haplotypes 701, 702, 801, and 807 in **Figure 2.2**). The algae from these four individuals are more closely related to algae otherwise found in *L. vulpina*, *Lecanora rupicola*, or *Chaenotheca subroscida* lichens and not in *L. lupina* lichens. One of these Forked Tube lichen specimens was collected from a site where the only *Letharia* lichens confirmed nearby are *L. vulpina* (Gazos Creek, California). One specimen was collected from a site in Idaho where *L. lupina* lichens were found nearby, but not *L. vulpina*. Two specimens were collected from sites where both *L. vulpina* and *L. lupina* lichens were present (Palomar Mountain, California, and Mt. Hood, Oregon).

The specimens of Forked Tube lichens from the Stanislaus National Forest (California) site all contain clade B algae (Chapter 1). A single individual with an ITS and actin haplotype combination identical to *L. lupina* lichen Alga 1 algae was found in Stanislaus National Forest, California. The algae from two other individuals from Stanislaus National Forest clearly fall within algal lineage 4. The remaining four individuals have actin haplotypes from a new clade that is closely related to Alga 1, Alga 2 and Alga 3. However, the ITS locus groups these individuals with the Alga 4 lineage. The ITS-actin haplotype combinations of these algae from these last four Forked Tube lichens are not congruent with the known Mountain Wolf lichen algal lineages, suggesting some phylogenetic independence.

Geographic distribution of algal lineages

Figure 2.4 summarizes the genetic distances and geographic distribution of the five algal lineages through haplotype networks and a map of algal lineage frequencies by collection site. The five algal lineages found to pair with *L. lupina* fungi differ markedly in their geographic distributions. Alga 1 and Alga 3 are both abundant and widespread, especially in the northern latitudes, but Alga 1 is rare east of the continental divide and Alga 3 is rare west of the Rocky Mountains, especially south of the Canadian border. The geographic distributions of Alga 1 and Alga 3 overlap mostly in the Rocky Mountains of Idaho and in South Central British Columbia. The other three algal lineages have smaller geographic distributions and are found mostly within greater Southern California area and the Sierra Nevada Mountains.

Population delineation

Under the modified SAMOVA analysis, both loci agree that the Alberta and Wyoming collection site groups are very similar despite geographic distance, the Northern and Southern Cascade Mountains groups are similar, and the Idaho and Montana groups are similar (**Table 2.2** and **Table 2.3**). In addition, the Alberta and Wyoming cluster received the high F_{ct} score in each analysis. The analysis indicates that the collection site groups can be divided up into nine genetically and geographically cohesive populations: Eastern Rocky Mountains (Alberta and Wyoming groups combined), British Columbia, Western Rocky Mountains (Idaho and Montana groups combined), Blue Mountains, Cascade Mountains (Northern and Southern

Cascade groups combined), Sierra Nevada Mountains, Central Coast, Transverse Ranges, and Peninsular Ranges (**Figure 2.5**).

Diversity indices

The Cascade population enjoys low diversity rankings in all indices at the actin locus but has an intermediate ranking at the ITS locus (**Table 2.4**). Like the Eastern Rocky Mountains, the Cascade population only has one algal lineage. When compared with other populations for Alga 1 only, the Cascade Mountains' relative diversity ranking climbs for both loci (**Table 2.5**). In other words, the Cascades are impoverished in algal lineages, they are not impoverished in Alga 1 haplotype diversity.

The Eastern Rocky Mountains rank low in all diversity measures at both loci in the combined lineage analysis (**Table 2.4**).

Within California, the Sierra Nevada and Central Coast populations have no private alleles at either locus whereas the Transverse (ITS unique haplotype fraction = 0.29) and Peninsular Range (actin intron unique haplotype fraction =0.27) populations have the highest fractions of private alleles as well as superlative relative genetic diversity rankings according to a variety of indices (**Table 2.4**).

The data set includes 89 different ITS – actin I intron combined haplotypes, 58% of which are private, occurring only once. That is, 58% of the haplotype combinations consist of a unique ITS or actin haplotype or they are a unique combination of ITS and actin haplotypes. Populations differ both in the frequency distribution of ITS-actin combined haplotype "clones" (data not shown) and in the frequency of unique haplotype combinations (**Table 2.6**). In contrast with the

Peninsular and Transverse Ranges, neither the Sierra Nevada Mountains nor the Coast Ranges have any private alleles at either locus (**Table 2.4**). This pattern of low haplotype endemism extends to the ITS – actin I intron haplotype combinations as well. The Sierra Nevada Mountains and Central Coast populations rank lowest among all populations in percent unique haplotype combinations (6% and 11%, respectively), whereas the neighboring Transverse Ranges rank the highest (40%) (**Table 2.6**).

Southern California has the largest number of Mountain Wolf algal lineages, but is it also the center of within-lineage diversity? Only Alga 1 has a wide enough geographic distribution and large enough sample sizes to make population comparisons. For Alga 1, the Peninsular Ranges have an asymmetrical pattern of genetic diversity in that they have the lowest relative diversity at the ITS locus but the highest relative diversity at the actin locus (**Table 2.5**). The center of actin I intron diversity is the Peninsular Ranges while the center of ITS diversity is far to the north in the Blue Mountains and the Cascade Mountains.

Population structure

According to the pairwise Φst combined lineage analysis, genetic differentiation among the nine populations varies from none to very great (**Table 2.7**). For example, the Eastern Rocky Mountain population has very great differentiation with all other populations at both loci. Although British Columbia has very great differentiation from the Eastern Rocky Mountains, it has moderate differentiation with the neighboring Cascade Mountains, and no significant population differentiation with the Western Rocky Mountains. In **Figure 2.6**, neighboring populations with no

statistically significant genetic differentiation at both loci are pooled together to form six regions, each of which has a unique frequency of algal lineages. Region I consists of the Eastern Rockies population from Alberta southeast to Wyoming and South Dakota. Region II pools British Columbia, Western Rocky and Blue Mountain populations. Region III consists of the Cascade Mountain populations. Region IV pools the Sierra Nevada and Central Coast populations, and Regions V and VI correspond with the Transverse and Peninsular Range populations, respectively. The differing frequencies of algal lineages in each region show strong overall geographic structure in Mountain Wolf lichen algal lineages. Regions I and III are homogeneous for algal lineage over a vast geographic scale, whereas regions V and VI are highly heterogeneous for algal lineages at a relatively small geographic scale. Regions V and VI of Southern California are hotspots for algal lineage diversity.

The data allow for analyses of intraspecific structure for the two most abundant and widespread algal lineages, Alga 1 and Alga 3, but these show little to modest internal structure. The geographic distribution of Alga 1 is sufficiently widespread to compare seven of the nine populations in which it occurs. **Table 2.8** shows the pairwise Φst within algal lineage 1 for both loci. At the actin I locus, most pairwise population comparisons exhibit no differentiation with the exception of all pairwise combinations with the Peninsular Ranges. The Peninsular Ranges are greatly differentiated from all other populations. In contrast, the ITS locus shows that the Peninsular Ranges have great differentiation only with distant British Columbia and the Western Rockies populations. Moderate differentiation is evident for seven out of the

twenty-one pairwise population comparisons. Such differences are illustrated by frequency diagrams for the four most common Alga 1 ITS haplotypes in the data set (**Figure 2.7**). The modest number of significant pairwise comparisons is congruent with the lack of isolation by distance exhibited at either locus (**Figure 2.8**).

The Cascade Mountains populations differ from neighboring populations by having only a single algal lineage (Alga 1) present, but the genetic differences between the populations cannot be attributed to the Cascade Mountains' impoverished lineage diversity alone. Restricting a pairwise Φst analysis to members of Alga 1, (**Table 2.8**), the actin locus presents no significant signal of differentiation with the three neighboring populations, but at the ITS locus, there is moderate genetic differentiation between the Cascade Mountains and its neighbors to the north and south. This differentiation is evident when comparing the frequency of the most common ITS haplotypes in each population (**Figure 2.7**). The Cascade Mountains are the only population where haplotype 103 is the most frequent. In British Columbia, the Blue Mountains and the Western Rocky Mountains populations share the same most common haplotype (101). In the Sierra Nevada Mountains, haplotype 102 dominates.

Alga 3 spans three populations but has small sample sizes in British Columbia and the Western Rocky Mountains. The Alga 3 Φst was not significant for either locus, showing no overall population structure, although pairwise population Φst indicate statistically significant but moderate (Φst = 0.088) differentiation between the Eastern and Western Rocky Mountains at the ITS locus. A pairwise Φst analysis of Alga 3 for each locus that was partitioned by state or province instead of population

revealed no significant pairwise differentiation between collections from Alberta , British Columbia, Wyoming, and Idaho (data not shown).

DISCUSSION

Algal lineage recognition and rank

Kroken and Taylor (2000) previously assigned six of the seven algal lineages (**Figure 2.2** and **Figure 2.3**) the rank of "species" without formal description. While acknowledging that these taxa are likely to be evolutionarily independent, I call them "lineages" because the practice of species recognition within lichenized algae still requires morphological characters (Friedl and Büdel 2008), and these lineages do not differ morphologically (Kroken and Taylor 2001a). Five lineages (Alga 1, Alga 2, Alga 3, Alga 5, and Alga 9) pair with *L. lupina* and form the core taxa of interest for this study. Alga 4 and Alga 6 have both been found with *L. columbiana* s.l. lichens, but neither lineage has been documented for *L. lupina* lichens. Although the five core lineages appear to be reproductively isolated (with the exception of three possible hybrid specimens), most have monophyletic support at only one of the two loci. Species recognition based primarily on molecular characters is more robust with multiple informative loci (Avise and Ball 1990, Baum and Shaw 1995, Taylor et al. 2000, Taylor 2001). Future sequencing of additional loci will help clarify the appropriate taxonomic rank of these morphologically cryptic taxa.

Phylogenetic scale of algal partnerships

The simultaneous partnering of algal lineages with *Letharia* and *H. imshaugii* fungi suggests that there may be guilds of lichenized fungi that share a common pool of green algae, an ecological pattern that has previously been described in the cyanolichens (Rikkinen et al. 2002, Summerfield et al. 2006, Lücking 2009). Guild relationships expand the concept of the lichen symbiosis beyond binary fungal-algal partnerships. The scale of the lichen symbiosis is enlarged to include networks of fungal and algal taxa that may have biologically important patterns of interdependency. For example, guilds would allow lichenized fungi lacking dual fungal-algal dispersal propagules (such as Forked Tube lichens) to rely on sorediate or isidiate lichens (such as Mountain Wolf lichens) for the dispersal of their algal partners (Beck 1999).

This sharing of algal taxa imposes an important caveat with regard to the Forked Tube lichen. The present study was originally designed to provide an indication of the geographic scale of fungal-algal partnerships between one fungal species (*L. lupina*) and all of its algal partners. It was not designed to infer geographic structure of the algae regardless of fungal partner, nor was it designed to estimate the *phylogenetic scale* of algal partnerships. Because some of the algal ITS-actin haplotype combinations are identical in Mountain Wolf and Forked Tube lichens, a more complete estimate of geographic structure of any Mountain Wolf algal lineages has to include algal partners of *H. imshaugii* and an unknown number of other lichens. Determining how many taxa to include requires community-wide sampling as has been carried out in reef building cnidarians and their photosynthetic dinoflagellate symbionts

(LaJeunesse et al. 2004). In lichens, community wide sampling of green algal partners has so far only been achieved at the geographic scale of a single rock (Beck 1999 but see Yahr et al. 2004).

Only one population study besides the present one has mapped the distribution of multiple algal lineages that pair with a single fungal species over a large geographic range. Yahr et al. (2006) documented the geographic distribution of four Asterochloris group *Trebouxia* ITS lineages (clades) that pair with the lichenized fungus *Cladonia subtenuis*, similar to the finding of five algal lineages in Mountain Wolf lichens. The *C. subtenuis* study included eleven sites in Florida north to New Jersey and west to the Ozark Mountains. The authors found significant geographic structure among the sites. In contrast with Mountain Wolf lichens, algal lineages of *Cladonia subtenuis* lichens were more diverse in the northern portion of the sampled range and homogenous in the southern extreme of the geographic range. These findings, when combined, illustrate that genetic diversity in lichens can be driven by the algal partner, the morphologically cryptic and lesser-known component (Nelsen and Gargas 2009). Future studies in lichen phylogeography should include the photosynthetic partner in the symbiosis.

Genetic continuities and discontinuities

The all-lineage, pairwise Φst provide support for the boundaries between the six designated algal regions (**Figure 2.6**). The within-lineage analyses further support the discontinuities between the Cascade and British Columbia populations (Alga 1), the Cascade and California populations (Alga 1), the Peninsular Ranges and all others (Alga 1), and the Eastern and Western Rockies populations (Alga 3). Within-lineage

differences between neighboring populations are small. With several ITS-actin clones spanning more than 5 states or provinces, little genetic differentiation between neighboring populations, and no pattern of isolation by distance, widespread algal lineages appear to have gene flow at a large geographic scale. On the other hand, moderate population structure between non-neighboring populations shows that widespread algae are not panmictic. Additional markers are required to better estimate the geographic scale of gene flow in lichenized algae.

The algae of British Columbia are less genetically diverse than those from the Cascade Mountains, the Western Rocky Mountains, and the Eastern Rocky Mountains, but the differences are small. It appears that lichenized algae had effective dispersal into British Columbia after the Pleistocene ice sheets melted, maintaining substantial genetic diversity. This runs contrary to the Leading Edge Hypothesis. Computer simulations show that a high rate of long distance dispersal events can lead to impoverished genetic diversity in the sink population (Bialozyt 2006) as expected in the Leading Edge Hypothesis, whereas slow colonization can preserve genetic diversity (Ibrahim et al. 1996), as we see seem to see in lichenized algae. The Leading Edge Hypothesis has been especially useful in explaining the genetic patterns in mesic plants of the Pacific Northwest (Soltis et al. 1997), but seems less applicable in the case of lichenized algae. Relatively rapid dispersal by weedy lichen propagules may account for the incongruence between the taxa. Possibly this incongruence can be accounted by the documented ability of lichens – and other cryptogams - to disperse more efficiently than most vascular plants (Jørgensen 1979, Munoz et al. 2004).

The genetic continuity between the British Columbian and Western Rocky Mountain populations (forming Region II, **Figure 2.6**) suggests that British Columbia was colonized by propagules from the Western Rocky Mountains, but the Cascade population is another likely source. The Cascade and British Columbian populations share at least two of the most common haplotypes at both loci in algal lineage 1 (the only algal lineage the two populations have in common), but the frequencies of these haplotypes differ, leading to a signal of differentiation in the Φst analysis. These data favor the Western Rocky Mountains population as the source of Alga 1 propagules for British Columbia but do not exclude the Cascade population. The data are not informative about the source of British Columbia's Alga 3 population because there was no significant population differentiation between the British Columbia populations and the two southern source populations, Western Rocky Mountains and the Eastern Rocky Mountains.

The relative rarity of Alga 1 in Alberta (as compared with British Columbia: **Figure 2.4c**) invites two possible explanations. The first explanation involves an inability to disperse, while the second invokes an inability to establish. If the Canadian Rocky Mountains form a barrier to west-east dispersal, then the algal lineages of Alberta must have arrived there from refugia south of the Pleistocene icesheet. The data, however, do not support this inference, inasmuch as contemporary albertan Alga 3 haplotypes are no more similar to their counterparts south of the ice than to those west of the Rocky Mountains. On the other hand, establishment in lichens appears to be a more important limit to colonization than dispersal (Werth et al. 2006). It seems

more likely that the relative rarity of Alga 1 in western Albert is based on an inability to establish and thrive under the predominantly summer wet climatic conditions east of the cordillera. Such differences in precipitation patterns could also explain the apparent absence of Alga 1 from Wyoming (Norris et al. 2006). Physiological differences between Alga 1 and Alga 3 could be corroborated with laboratory cultures. Reciprocal transplants could provide a useful comparison of how well the two algal lineages perform under different environmental conditions.

Southern California

The algae of Southern California (here defined as the Transverse and Peninsular Range populations) are remarkable for their within-site diversity. For example, in the Transverse Ranges, four different algal lineage were found within hundreds of meters of each other at each of two sites when sample sizes were only n=6 and n=7 per site. Southern California, especially the Transverse Ranges, is clearly the center of algal lineage diversity for Mountain Wolf lichens. This makes sense in the context of comparative phylogeography since these ranges are associated with genetic splits within and between a variety of animal taxa including birds, reptiles, mammals, amphibians and invertebrates (Alexander and Burns 2006, Calsbeek et al. 2003, Chatzimanolis and Caterino 2007, Forister et al. 2004, Rissler et al. 2006, and Vandergast et al. 2008 and citations within), inspiring authors to refer to Southern California an evolutionary "hotspot" (Calsbeek et al. 2003, Davis et al. 2008, Vandergast et al. 2008). Compared with animals, plants have received relatively little attention in this geographic region. In conifers, several large scale studies excluded the

populations of Southern California (Steinhoff 1983, Fazekas et al. 2008, Godbaut et al. 2008). In a coarse-scale study that did include Southern California populations, the conifer *Pinus flexis* shows no significant difference between Southern California and Sierra Nevada populations (Mitton et al. 2000). On the other hand, *Datisca glomerata* (Datiscaceae) shows a a phylogeographic discontinuity in Southern California, with higher diversity south of the Transverse Ranges (Liston et al. 1992), congruent with lichen algae. Additional phylogeographic studies of widespread plants should include sites in Southern California.

The Great Basin

The Great Basin currently forms a formidable barrier to direct gene flow between the Sierra Nevada and Rocky Mountains (Harper et al. 1978). As a result, the Sierra Nevada population is more genetically differentiated from the Western Rocky Mountains than from the Cascade Mountains populations. Interestingly, Kroken and Taylor found Alga 3 in two specimens of *Letharia columbiana* s.l. in the Sierra Nevada Mountains (Kroken and Taylor 2000), and Alga 3 was found in a Mountain Wolf specimen from the White Mountains at the western extreme of the Great Basin (**Figure 2.4c**). Alga 5, otherwise only in Southern California, was found in a Mountain Wolf lichen in the Desatoya Mountains of Central Nevada. One possible explanation for these seemingly anomalous disjunctions would be historic gene flow through the Great Basin.

Lichen algae are not thought to have any long distance dispersal independent of the fungal partner. Birds (Bailey and James 1979, McCune et al. 2000) and wind

(Munoz et al. 2004) can always be implicated in long distance dispersal of dual fungal-algal lichen propagules, but gene flow over more moderate geographic scales (Lättman et al. 2009) may have occurred via the Great Basin during the Pleistocene when Mountain Wolf lichen substrate conifers grew there at lower elevations (Hamrick et al. 1994, Thompson and Anderson 2000). Abundant conifers from the Sierra Nevada to the Rocky Mountains could have allowed Alga 3 to disperse south (or, less parsimoniously, north) and allowed Alga 5 to disperse north from Southern California. Although northwestern Nevada and southwestern Idaho represent the shortest dispersal route between the Sierra Nevada and Western Rocky Mountains, appropriate substrate conifers are very sparse there today (Critchfield and Allenbaugh 1969), and there is little macrofossil or palynological data for this region. However, packrat midden macrofossils in Southern Nevada provide evidence for abundant *Pinus longaeva* (Bristlecone Pine) during the Pleistocene. This dispersal corridor between the western and eastern extremes of the Great Basin during the Pleistocene has been referred to as the "axial route" (Wells 1983). Mountain Wolf lichens grow exclusively on the wood of *Pinus longaeva* at Great Basin National Park in eastern Nevada, and they are also found on *Pinus longaeva* in the White Mountains of California. These trees or other conifers may have served as a Pleistocene link between Sierra Nevada and Rocky Mountain populations of Mountain Wolf lichens.

Cascade Mountains

The genetic differentiation of the Cascade Mountain population from the Sierra Nevada Mountains population takes on a special significance because a number of

mesic plant species share a phylogeographic transition zone in this area (Soltis et al. 1997). Despite congruence in the location of a phylogeographic break across diverse taxa, the nature of the discontinuity is not congruent. In contrast with the mesic plant taxa, Alga 1 enjoys increased genetic diversity on the north side of the transition zone (**Table 2.5**). A congruent phylogeographic break with higher diversity to the north was found in the pollinating seed parasite *Greya politella* (Lepidoptera: Prodoxidae), which has a unique clade of haplotypes in Southern Oregon (Brown et al. 2007, Rich et al. 2008). Spotted owls (*Strix occidentalis*) also have a congruent shift between subspecies in this region (Barrowclough et al. 1999). Based on one locus, Mountain Wolf lichen algae share a modest phylogeographic break with other taxa near the Soltis line, but our data are not consistent with the inferred phylogeographic history of mesic plant taxa and several amphibians (Brown et al. 1997, Brunsfeld et al. 2007, Soltis et al. 2007). Mountain Wolf algal phylogeographic structure in this areas appears to be most congruent with two widespread winged animals: the pollinating seed parasite *Greya politella* and the spotted owl *Strix occidentalis*.

The Cascade Mountain population is a curiosity as it stands apart from all of the other populations. It is home to only one algal lineage, that lineage has significant genetic diversity, but the population is somewhat genetically isolated from all of its neighbors. A convenient explanation for these data is found in the hypothesis that the Willamette Valley of Oregon served as a glacial refugium for Alga 1, as has been proposed for the important substrate conifer *Pseudotsuga menziesii* (Tsukada 1982, Li and Adams 1989). Demographic expansion from such a local refugium could explain the

data from this anomalous population. Unfortunately, neither Fu's F for Tajima's D tests for demographic expansion could be used on this data set due to recombination within both algal loci.

Canadian and Rocky Mountains Populations

The overlapping distribution of Alga 1 and Alga 3 from Southern Idaho up to South-Central British Columbia coincides with other putative post-Pleistocene secondary contact zones for closely related pairs of species or subspecies, including several conifers and their associated tree squirrels (Abrogast et al. 2001, Norris et al. 2006, Steinhoff and Fins 1983, Swenson and Howard 2005, Woodland 1982). In *Pinus contorta* ssp. *latifolia*, Godbaut et al. (2008) inferred that both the Cascade Mountains and the Rocky Mountain ranges formed barriers to east-west gene flow during the Pleistocene. For *Pinus ponderosa* there is a sharp cline in genetic markers in west-central Montana that is understood to be a zone of secondary contact between subspecies separated during the Pleistocene (Latta and Mitton 1999). Sharp east-west genetic clines that suggest post-Pleistocene secondary contact are also found between subspecies of *Pseudotsuga menziesii* in central Oregon, north central Washington and south central British Columbia (Li and Adams 1989). The congruence in geographic pattern between unrelated codistributed taxa is consistent with a Pleistocene (or earlier) vicariance event with recontact in the Holocene in the Western Rocky Mountains and in British Columbia (Brunsfeld et al. 2001).

Alternatively, there may have been a southern glacial refugium such as the Clearwater River drainage in Idaho (Daubenmire 1952, Delting 1968, Soltis et al. 1997)

for both algal lineages. The current distribution of the two lineages can be explained by a divarication in post-Pleistocene expansion to the east and west based on differing environmental tolerances of the two algae.

Implications for symbiotic mutualists

Conservation efforts in lichens have been focused on rare fungal taxa (California Lichen Society Conservation Committee 2009). Due to their abundance and large geographic range, widespread lichens such as Mountain Wolf lichens are not currently a conservation concern anywhere in North America. However, studies of the geographic structure of algal symbionts may require a rethinking of how common lichens are treated. A caution from this study is that widespread and abundant lichens should not be dismissed as a conservation concern based on field identification of the fungal component alone. Without data on geographic structure in the algal partner, it cannot be assumed that the symbiosis is spatially homogeneous. Geographic structure in the lichen may only become apparent through molecular or physiological work on the algal partner. If algae vary in traits important to the functioning of the symbiosis, traits such as tolerance to ultraviolet light or to sulfur dioxide pollution, then the fungal-algal symbiotic partnership is the unit of biological interest and conservation in lichens, not the fungus alone. An analogous argument has previously been made for the marine coral-algal symbiosis (Ulstrop et al. 2003, Baird et al. 2008), and it is potentially relevant to all obligate symbiotic mutualists.

As the photosynthetic partner in the symbiosis, genetic diversity in lichenized algae may be crucial for adaptation to climate change. As taxa adjust to climate change

through alterations in their geographic ranges (Inouye et al. 2000, Epps et al 2004, Millar et al. 2004), genetic diversity in the "rear edge" of their current distribution may acquire increased importance for the maintenance of long-term biological diversity (Hampe and Petit 2005, Thuiller et al. 2008). For Mountain Wolf lichens, that diverse "rear edge" is demonstrably in Southern California, a region where lichen diversity in general has been decreasing over the last century (Hasse 1913, Sigal and Nash 1983). Much of the small geographic ranges of Alga 2, Alga 5, and Alga 9 occurs in areas of Southern California with high air pollution and habitat reduction associated with growing human populations (FRAP 2003). If other lichens depend on Mountain Wolf lichens to provide their photosynthetic partners, the threat is not just to the genetic diversity of a single weedy lichen, but possibly to a larger symbiotic network.

Table 2.1 Population Names, Collection Site Groups, And Locations

Collection site groups are clusters of collection sites used to delineate populations using SAMOVA. N= the number collections per site.

Population	Collection Site Group	Collection Site	State/ Prov.	Latitude	Longitude	N
British Columbia	British Columbia	Greenstone Mountain	BC	50.35.54	120.36.04	6
British Columbia	British Columbia	One Hundred Mile House	BC	51.36.01	121.17.96	4
British Columbia	British Columbia	Skwaha	BC	50.23.95	121.31.59	8
British Columbia	British Columbia	Stein Valley	BC	50.16.29	121.38.40	5
British Columbia	British Columbia	Trophy Mountain	BC	51.46.78	119.56.15	5
British Columbia	British Columbia	Bachelor Hills	BC	50.48.91	120.26.72	1
British Columbia	British Columbia	15 km NW of Cache Creek	BC	50.53.09	121.25.98	2
British Columbia	British Columbia	Coldwater Creek	BC	49.39.50	121.00.50	1
British Columbia	British Columbia	Edgewood Blue	BC	51.52.12	120.01.32	1
British Columbia	British Columbia	15 km NW of Hedley	BC	49.24.21	120.15.57	1
British Columbia	British Columbia	Kamloops overlook	BC	50.39.58	120.13.55	1
British Columbia	British Columbia	Naramata	BC	49.35.00	119.35.00	1
British Columbia	British Columbia	Near Twin Creeks Ranch	BC	50.45.90	121.35.07	1
British Columbia	British Columbia	Pavilion Lake	BC	50.49.99	121.41.57	1
British Columbia	British Columbia	Monte Creek	BC	50.39.00	119.52.00	1
British Columbia	British Columbia	Niskonlith Lake	BC	50.46.90	119.46.80	3
British Columbia	British Columbia	Oregon Jack	BC	50.38.57	121.32.17	1
British Columbia	British Columbia	Spahats	BC	51.44.31	120.00.84	1
Cascades	Northern Cascades	Odell Butte	OR	43.29.80	121.50.74	5
Cascades	Northern Cascades	Mount Hood	OR	45.19.88	121.42.64	3
Cascades	Northern Cascades	Willamette National Forest	OR	44.19.57	121.59.48	2
Cascades	Northern Cascades	Tumwater Canyon	WA	47.39.16	120.43.38	4
Cascades	Northern Cascades	White Pass	WA	46.38.61	121.22.70	8
Cascades	Northern Cascades	Del Rio Road	WA	47.55.00	119.18.00	2
Cascades	Northern Cascades	McGinnis Canyon	WA	47.36.00	120.08.00	3
Cascades	Southern Cascades	Castle Crags	CA	41.09.62	122.18.84	4
Cascades	Southern Cascades	Lassen National Forest	CA	40.45.00	121.29.00	4
Cascades	Southern Cascades	Horsethief	CA	41.41.25	122.03.22	2
Cascades	Southern Cascades	Siskiyou National Forest	CA	41.54.12	123.38.79	1
Cascades	Southern Cascades	Green Springs Pass	OR	42.08.10	122.29.01	7
Cascades	Southern Cascades	Mt Ashland	OR	42.04.07	122.36.24	3

Population	Collection Site Group	Collection Site	State/ Prov.	Latitude	Longitude	N
Eastern Rockies	Alberta	Bow Lake	AB	51.40.00	116.29.00	2
Eastern Rockies	Alberta	Coleman	AB	49.38.00	114.30.00	1
Eastern Rockies	Alberta	West Jumpingpound	AB	51.03.87	114.40.84	9
Eastern Rockies	Wyoming	Black Hills	SD	44.11.00	103.34.00	1
Eastern Rockies	Wyoming	Dry Fork Ridge	WY	44.60.44	107.31.91	5
Eastern Rockies	Wyoming	Grouse Mountain	WY	44.18.94	106.51.19	6
Eastern Rockies	Wyoming	Inyan Kara Mountain	WY	44.12.82	104.21.30	4
Eastern Rockies	Wyoming	Tongue River	WY	44.44.16	107.28.31	4
Eastern Rockies	Wyoming	East Tensleep Lake	WY	44.13.15	107.10.36	1
Peninsular Range	Peninsular Range	Laguna Mountain	CA	32.50.95	116.25.88	6
Peninsular Range	Peninsular Range	Palomar Mountain	CA	33.19.91	116.52.26	5
Peninsular Range	Peninsular Range	San Bernardino Mountains	CA	34.12.61	116.51.18	5
Peninsular Range	Peninsular Range	San Jacinto Mountains	CA	33.46.08	116.39.62	7
Peninsular Range	Peninsular Range	San Gabriel Mountains	CA	34.17.73	118.00.45	2
Sierra Nevada	Sierra Nevada	Plumas National Forest	CA	39.55.70	120.50.00	8
Sierra Nevada	Sierra Nevada	Stanislaus	CA	38.14.27	120.20.66	8
Sierra Nevada	Sierra Nevada	23 miles east of Placerville	CA	38.49.24	120.22.60	1
Sierra Nevada	Sierra Nevada	Lassen National Forest	CA	40.26.35	120.50.11	1
Sierra Nevada	South Coast	Ventana Wilderness	CA	36.18.64	121.34.11	6
Sierra Nevada	South Coast	Wilsons Corner	CA	35.28.04	120.22.59	3
Transverse Range	Transverse Range	Frasier Mountain	CA	34.46.85	118.59.22	6
Transverse Range	Transverse Range	Tehachapi County Park	CA	35.04.13	118.28.91	7
Transverse Range	Transverse Range	Pine Mountain	CA	34.38.75	119.23.18	2
Blue Mountains	Blue Mountains	Cuddy Mountain	ID	44.44.53	116.48.68	4
Blue Mountains	Blue Mountains	Umatilla	OR	45.43.93	118.01.59	5
Blue Mountains	Blue Mountains	Wallowa	OR	45.09.12	116.52.58	5
Blue Mountains	Blue Mountains	Posy Valley	OR	44.50.01	117.06.42	1
Western Rockies	Idaho	Boise	ID	44.01.47	115.36.39	5
Western Rockies	Idaho	Ketchum	ID	43.41.37	114.24.65	6
Western Rockies	Idaho	Marsh Creek	ID	44.24.93	115.11.15	5
Western Rockies	Idaho	Titus Lake	ID	43.51.77	114.42.46	5
Western Rockies	Western Montana	Bitter Root	MT	45.53.64	113.57.27	4
Western Rockies	Western Montana	Helena	MT	46.57.61	112.40.12	4
Western Rockies	Western Montana	Gallatin	MT	45.28.05	110.56.29	2
None	None	Mount Prevost	BC	48.50.00	123.46.00	1
None	None	Prince George	BC	54.19.65	122.40.85	2

Population	Collection Site Group	Collection Site	State/ Prov.	Latitude	Longitude	N
None	None	White Mountains	CA	37.30.07	118.12.33	2
None	None	Chesley Road	ID	46.20.00	116.33.00	2
None	None	Coeur D'Alene Mountains	ID	47.45.00	115.50.00	2
None	None	Giant White Pine St. Park	ID	47.00.30	116.40.00	1
None	None	Selway Bitterroot Wilderness	ID	46.14.00	115.12.58	1
None	None	Great Basin	NV	39.00.00	114.18.00	1
None	None	Desatoya Mountains	NV	39.17.94	117.45.54	1
None	None	Jarbidge Mountains	NV	41.51.60	115.19.13	1
None	None	Toiyabe National Forest	NV	38.30.57	119.01.01	1
None	None	Cypress Hills Park	SK	49.45.00	109.30.00	1
None	None	WasatchCache Ntl Forest	UT	40.55.95	110.06.67	1
None	None	Colville National Forest	WA	48.52.00	117.24.00	1
None	None	Swanson Lakes	WA	47.39.00	117.17.50	1

Table 2.2 SAMOVA Fct Values For The ITS Locus And Actin I Intron

K = the number of populations. All Fct values are significant ($P < 0.01$).

	Fct values							
K	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
ITS	0.458	0.442	0.417	0.381	0.327	0.304	0.277	0.263
Actin	0.561	0.544	0.529	0.484	0.370	0.346	0.394	0.387

Table 2.3 SAMOVA Collection Site Group Clusters

Collection site group clusters found under nine SAMOVA values of K for the ITS locus and the actin I intron. In addition to the maximum value of Fct in each analysis, clusters of collection site groups that appear under at least two values of K in each locus were used to infer populations. Collection site clusters accepted to infer populations appear in bold. Group abbreviations are as follows: AB=Albert, Blue=Blue Mountains of Oregon, Coast=Central Coast of California, ID=Idaho, MT=Montana, NC=Northern Cascades, Penin=Peninsular Ranges, SC= Southern Cascades, Sierra=Sierra Nevada, WY=Wyoming.

	K							
Locus	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
ITS	**AB-WY**	none	none	none	none	**NC-SC**	NC-SC, ID-MT-Sierra, SC-Coast, Coast-Penin	ID-MT-Sierra, SC-Coast, Coast-Penin
Actin	**AB-WY**	**AB-WY**	none	none	**AB-WY, NC-SC**	**AB-WY**	**ID-MT, NC-SC**	**ID-MT, NC-SC**

Table 2.4 Genetic Diversity Indices For The Combined Algal Lineages

Genetic diversity indices of the ITS and actin I intron loci for the combined algal lineages partitioned by population. N = number of individuals in each population. Haps = number of haplotypes. Poly. sites= number of polymorphic sites. Private alleles are haplotypes found only once in the data set.

		ITS						Actin I intron					
Population	N	Haps	Poly. sites	No. of private alleles	Gene diversity	Mean No. of pairwise differences	Nucleotide diversity	Haps	Poly. sites	No. of private alleles	Gene diver-sity	Mean No. of pairwise differences	Nucleo-tide diversity
Blue Mountains	15	9	17	2	0.924	4.210	0.0067	3	9	0	0.562	1.923	0.0096
British Columbia	45	15	18	6	0.844	3.822	0.0060	6	14	3	0.554	3.200	0.0160
Cascade Range	47	14	14	4	0.863	2.337	0.0037	7	7	4	0.409	0.775	0.0039
Central Coast	9	5	5	0	0.833	1.667	0.0026	3	11	0	0.556	4.556	0.0230
Eastern Rockies	33	9	18	5	0.646	1.830	0.0028	5	11	2	0.481	0.998	0.0050
Peninsular Ranges	17	8	21	5	0.590	2.893	0.0046	11	30	3	0.900	6.750	0.0338
Sierra Nevada	17	6	16	0	0.721	2.632	0.0042	5	13	0	0.507	2.411	0.0121
Transverse Range	15	8	12	2	0.905	5.086	0.0077	9	27	4	0.924	8.895	0.0445
Western Rockies	31	14	21	7	0.860	4.610	0.0073	5	9	0	0.641	3.658	0.0183

Table 2.5 Genetic Diversity Indices For Algal Lineage 1

Genetic diversity of the ITS and actin I intron loci for algal lineage 1 partitioned by population. N = number of individuals in each population. Haps = number of haplotypes. Poly sites = number of polymorphic sites. Private alleles are haplotypes found only once in the data set. The Transverse Ranges and Eastern Rocky Mountain populations were excluded from this table due to small sample size.

		ITS						Actin I intron					
Population	N	Haps	Poly. sites	No. of private alleles	Gene diversity	Mean No. of pairwise differences	Nucleotide diversity	Haps	Poly. sites	No. of private alleles	Gene diversity	Mean No. of pairwise differences	Nucleotide diversity
Blue Mountains	14	8	12	1	0.9121	3.725	0.00589	2	2	0	0.4945	0.989	0.00497
British Columbia	36	10	9	3	0.7714	2.424	0.00384	3	3	1	0.3317	0.627	0.00315
Cascade Range	47	14	14	4	0.8631	2.337	0.00370	7	7	4	0.4089	0.775	0.00388
Central Coast	6	4	4	0	0.8000	1.867	0.00300	1	0	0	0	0.000	0.00000
Peninsular Ranges	16	4	6	2	0.5167	1.150	0.00182	5	5	1	0.7750	2.067	0.01033
Sierra Nevada	15	4	6	0	0.6381	1.486	0.00234	3	3	0	0.3619	0.724	0.00362
Western Rockies	23	8	10	3	0.7510	2.538	0.00402	3	3	0	0.4229	0.846	0.00423

Table 2.6 Unique Haplotypes By Population

Unique combined ITS - actin I intron haplotypes partitioned by population. There are a total of 89 different ITS - actin I intron haplotype combinations found among the *Letharia lupina* lichen algae, most of which (58%) are unique.

Population	Sample Size	Number Unique	% Unique
Blue Mountains	15	3	20
British Columbia	45	10	22
Cascade Mountains	47	9	19
Central Coast	9	1	11
Eastern Rockies	33	7	21
Peninsular Ranges	25	6	24
Sierra Nevada	17	1	6
Transverse Ranges	15	6	40
Western Rockies	31	9	29

Table 2.7 Pairwise Φst By Population, All Algal Lineages

Pairwise Φst for ITS (below diagonal) and actin I intron (above diagonal) by population for the combined algal lineages. Values significantly different from zero ($P < 0.05$) are indicated in boldface. Overall Φst for ITS = 0.272, P = 0.0000. Overall Φst for actin I intron = 0.397, P = 0.0000.

	BC	Casc.	E. Rock	Sierra Nev.	Penin.	Trans.	W. Rock	Coast	Blue
Population	n=45	n=47	n=33	n=17	n=25	n=15	n=31	n=9	n=15
British Columbia	X	**0.127**	**0.661**	0.033	**0.144**	**0.313**	0.014	**0.126**	0.015
Cascade Range	**0.075**	X	**0.892**	0.030	**0.294**	**0.557**	**0.207**	**0.360**	**0.087**
Eastern Rockies	**0.488**	**0.666**	X	**0.801**	**0.592**	**0.496**	**0.620**	**0.775**	**0.823**
Sierra Nevada	0.042	**0.068**	**0.619**	X	**0.108**	**0.293**	0.036	0.072	-0.028
Peninsular Ranges	**0.094**	**0.128**	**0.578**	-0.013	X	**0.127**	**0.111**	0.057	**0.095**
Transverse Range	**0.226**	**0.363**	**0.400**	**0.189**	**0.144**	X	**0.252**	0.093	**0.319**
Western Rockies	-0.011	**0.116**	**0.437**	0.047	**0.096**	**0.186**	X	**0.127**	0.039
Central Coast	0.031	0.020	**0.688**	-0.050	0.0128	**0.249**	0.046	X	**0.156**
Blue Mountains	0.024	0.032	**0.594**	0.032	**0.083**	**0.223**	0.037	-0.027	X

Table 2.8 Pairwise Φst By Population, Algal Lineage 1

Pairwise Φst for ITS (below diagonal) and actin I intron (above diagonal) by population for Alga 1. Values significantly different from zero ($P < 0.05$) are shown in bold. Overall Φst for ITS is 0.069 ($P = 0.0000$). Overall Φst for the actin 1 intron is 0.220 ($P=0.0000$).

	British Columbia	Cascade Range	Sierra Nevada	Peninsular Ranges	Western Rockies	Central Coast	Blue Mountains
Population	n=36	n=47	n=23	n=16	n=23	n=6	n=14
British Colum.	X	-0.010	-0.037	**0.519**	-0.016	0.005	0.049
Cascade Range	**0.070**	X	-0.041	**0.517**	-0.004	-0.029	0.074
Sierra Nevada	**0.101**	**0.057**	X	**0.430**	-0.042	0.000	0.023
Penin. Ranges	**0.178**	**0.103**	-0.007	X	**0.410**	**0.489**	**0.316**
West. Rockies	-0.018	**0.094**	**0.096**	**0.184**	X	0.061	-0.024
Central Coast	0.002	0.005	-0.052	0.079	0.007	X	0.192
Blue Mountains	0.048	0.033	0.058	**0.110**	0.042	-0.033	X

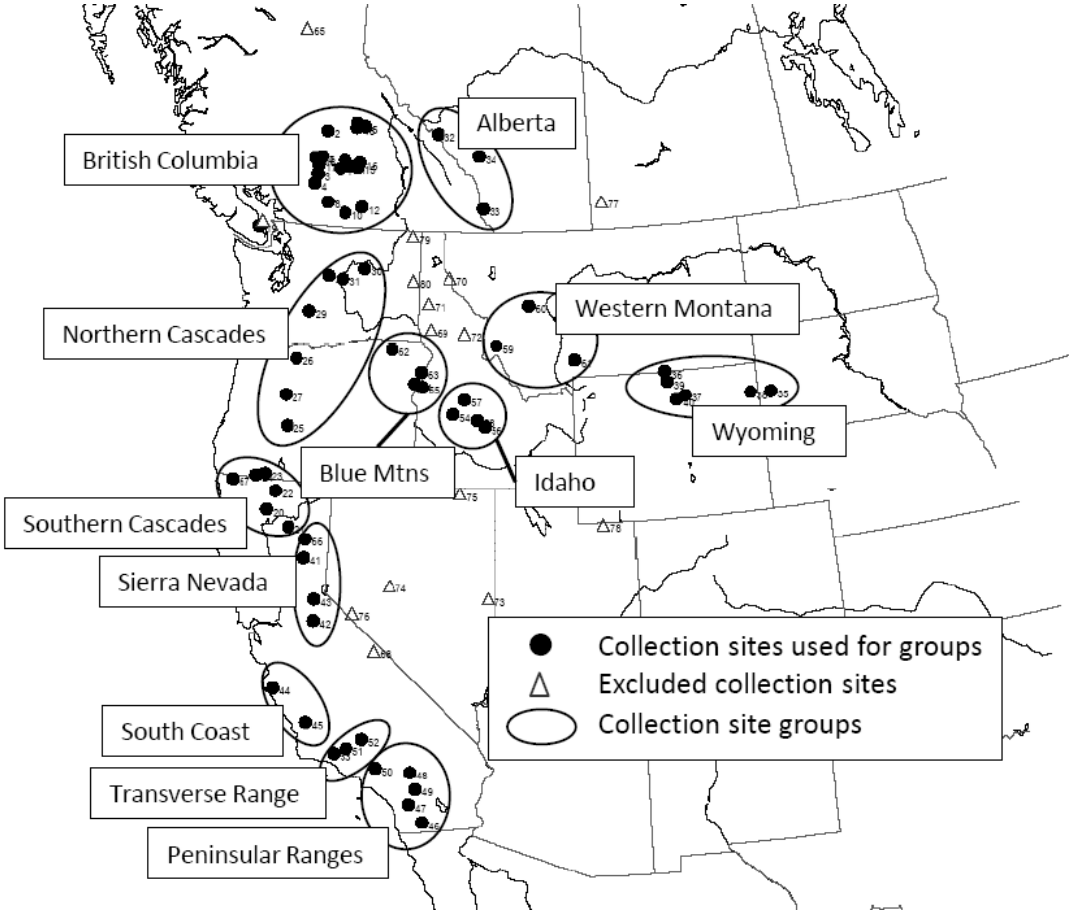
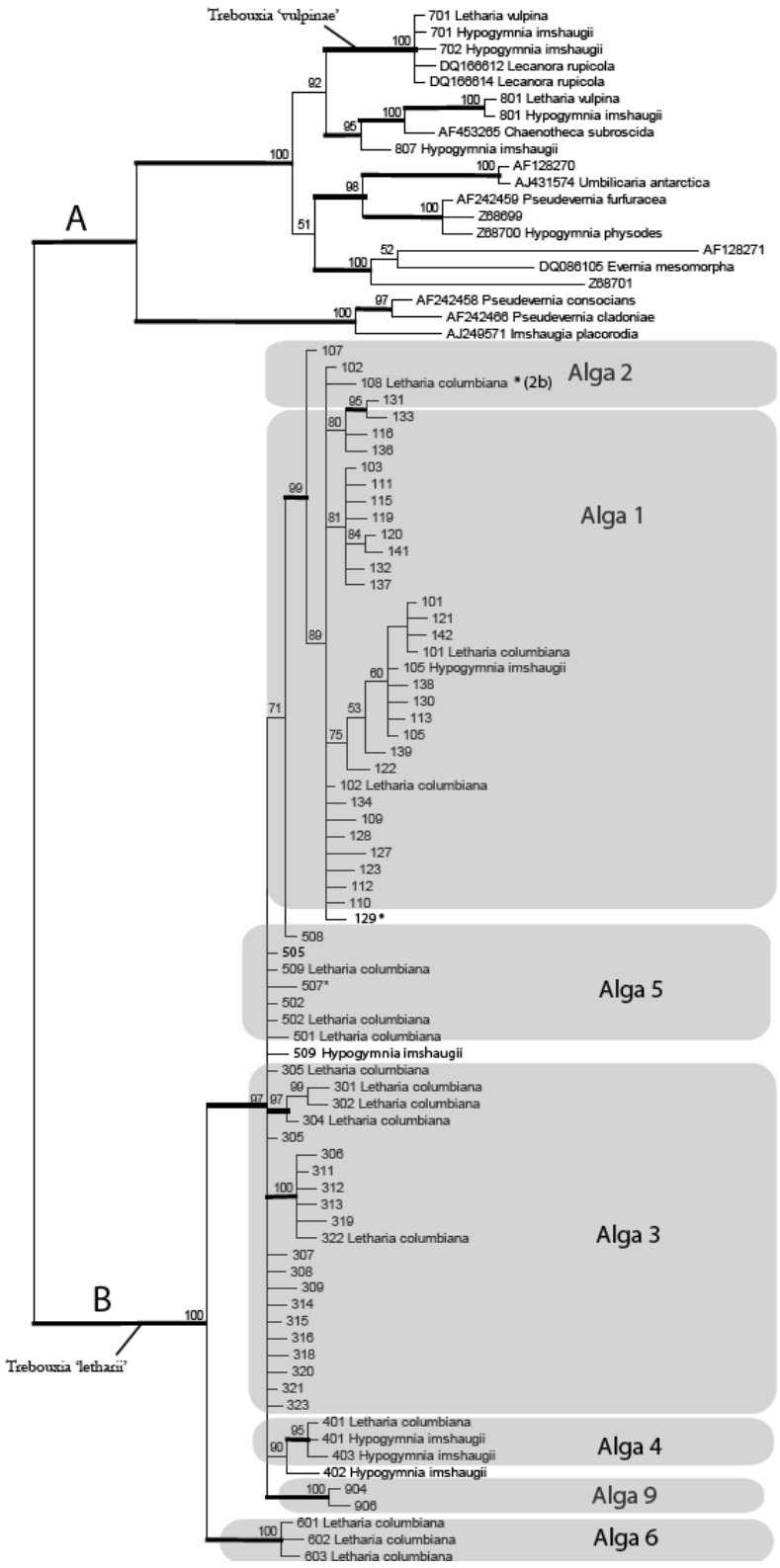


Figure 2.1 SAMOVA Collection Site Groups

Collection sites and collection site groups used to infer populations using SAMOVA. Fifteen hollow triangles mark sites that are too geographically remote or had to few samples to assign them to a collection site group.

Figure 2.2 *T. jamesii* Algal ITS Bayesian Tree
Numbers that appear above branches are Bayesian posterior probabilities. Branches with greater than 94% posterior probabilities appear in bold. Three digit numbers are haplotype codes. Haplotypes found with other fungal taxa are labeled with the fungal taxon name. Note that some haplotypes are found with multiple fungal taxa. Lineage numbers are based on clade congruence with the actin locus (Figure 3). Asterisks mark unique haplotypes (108, 129, 507) of uncertain lineage. The major clades of algae that partner with *Letharia* lichens are labeled "A" and "B" (Chapter 1). The clades previously named by Kroken and Taylor (2000), *Trebouxia 'vulpinae'* and *Trebouxia 'letharii',* are labeled at their respective branches. Individuals with ITS haplotypes 402 and 509 pair with actin I intron haplotypes 1209 or 1212, and likely represent two new lineages thus far only found in Forked Tube lichens.


Trebouxia 'vulpinae'
701 Letharia vulpina
701 Hypogymnia imshaugii
702 Hypogymnia imshaugii
DQ166612 Lecanora rupicola
DQ166614 Lecanora rupicola
801 Letharia vulpina
801 Hypogymnia imshaugii
AF453265 Chaenotheca subroscida
807 Hypogymnia imshaugii
AF128270
AJ431574 Umbilicaria antarctica
AF242459 Pseudevernia furfuracea
Z88699
Z88700 Hypogymnia physodes
AF128271
DQ086105 Evernia mesomorpha
Z88701
AF242458 Pseudevernia consocians
AF242466 Pseudevernia cladoniae
AJ249571 Imshaugia placorodia
A
Alga 2
107
102
108 Letharia columbiana * (2b)
131
133
116
136
Alga 1
103
111
115
119
120
141
132
137
101
121
142
101 Letharia columbiana
105 Hypogymnia imshaugii
138
130
113
105
139
122
102 Letharia columbiana
134
109
128
127
123
112
110
129 *
508
505
509 Letharia columbiana
507*
502
502 Letharia columbiana
501 Letharia columbiana
509 Hypogymnia imshaugii
Alga 5
305 Letharia columbiana
301 Letharia columbiana
302 Letharia columbiana
304 Letharia columbiana
305
306
311
312
313
319
322 Letharia columbiana
307
308
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318
320
321
323
Alga 3
B
Trebouxia 'letharii'
401 Letharia columbiana
401 Hypogymnia imshaugii
403 Hypogymnia imshaugii
402 Hypogymnia imshaugii
Alga 4
904
906
Alga 9
601 Letharia columbiana
602 Letharia columbiana
603 Letharia columbiana
Alga 6

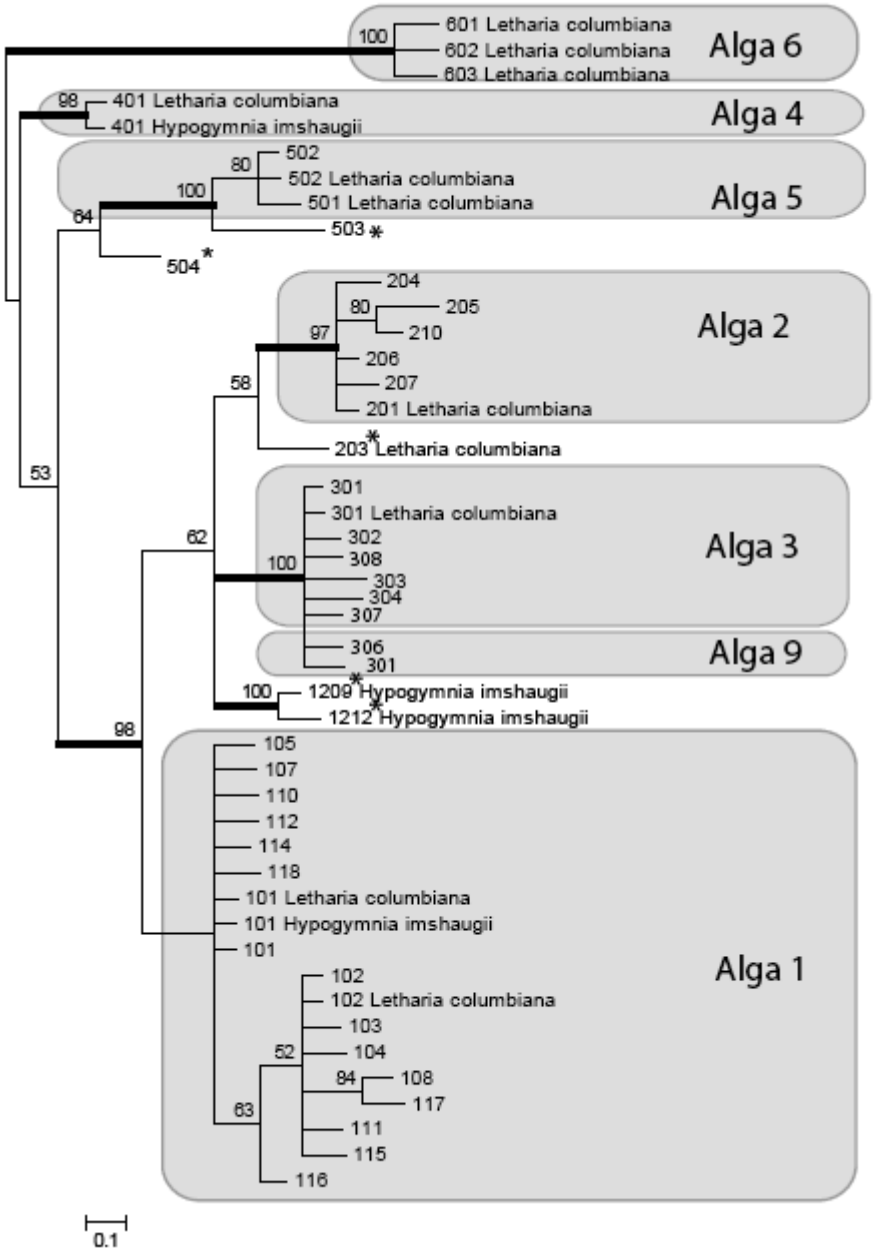
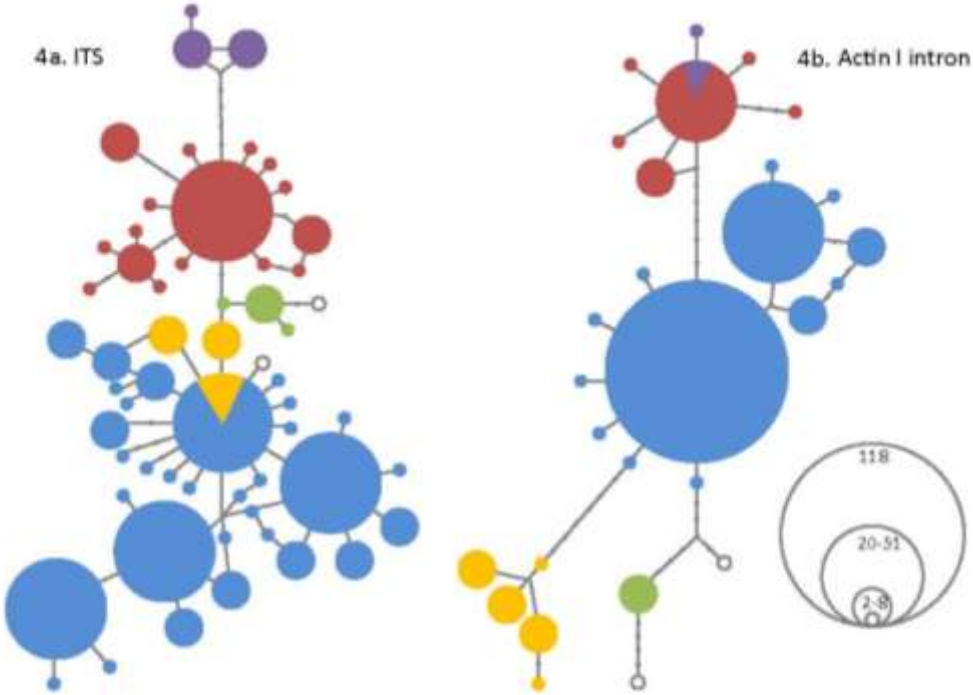


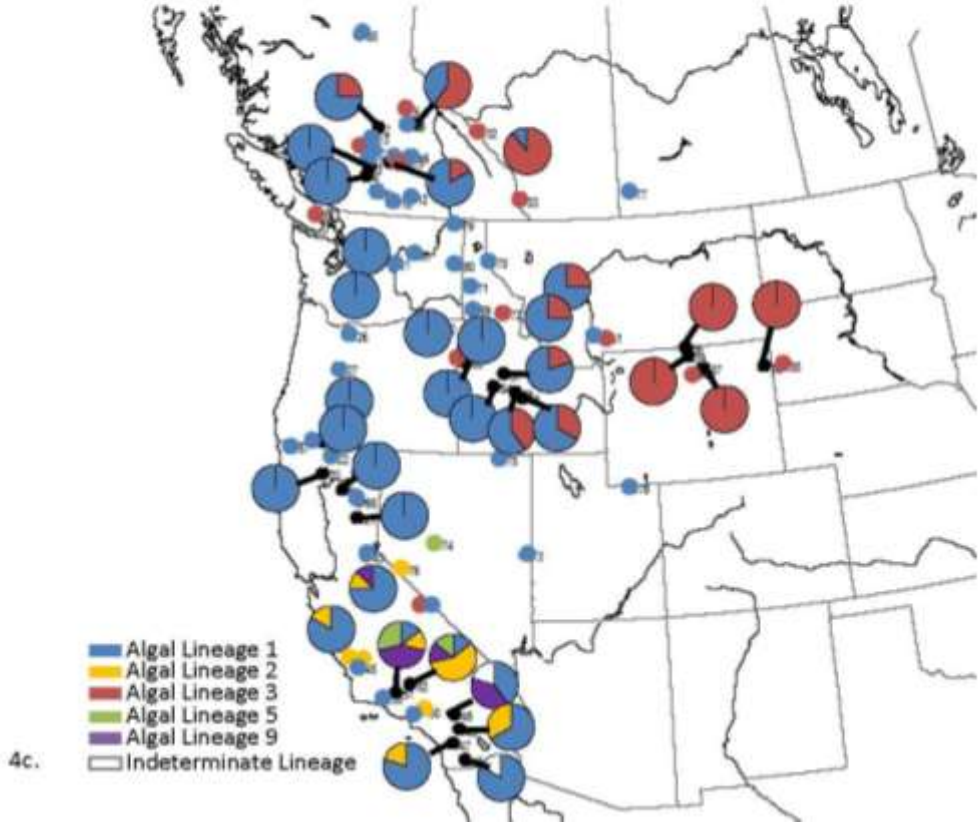
Figure 2.3 Actin I Intron 50% Consensus Bayesian Tree

Numbers above branches are Bayesian posterior probabilities. Branches with greater than 95% posterior probabilities appear in bold. Three and four digit numbers at branch tips are haplotype codes. Haplotypes found with fungal taxa besides *Letharia lupina* are labeled with the fungal species name. Note that some haplotypes are found with multiple fungal taxa and some haplotypes appear under multiple algal lineages. Lineage numbers are based on clade congruence with the ITS locus. Asterisks mark haplotypes of uncertain lineage. Alga 6 was arbitrarily chosen to root the tree for display purposes.

Figure 2.4 Frequency Of Algal Lineages By Collection Site

a. ITS haplotype network. The size of the circle is proportional with the number of individuals with that haplotype (see scale). Small solid grey circles represent missing haplotypes, and lines represent single base pair differences. Haplotypes that are shared by more than one algal lineage are represented as pie diagrams. The network is color coded by algal lineage (see legend). For a color version of this figure, contact the author.
b. Actin I intron haplotype network.
c. Algal lineage pie diagrams show the frequency of each algal lineage at sites with more than 2 individuals. Individuals from sites with 1 or 2 samples are represented by small colored circles.


4a. ITS
4b. Actin I intron
118
20-51
2-8


Algal Lineage 1
Algal Lineage 2
Algal Lineage 3
Algal Lineage 5
Algal Lineage 9
Indeterminate Lineage

4c.

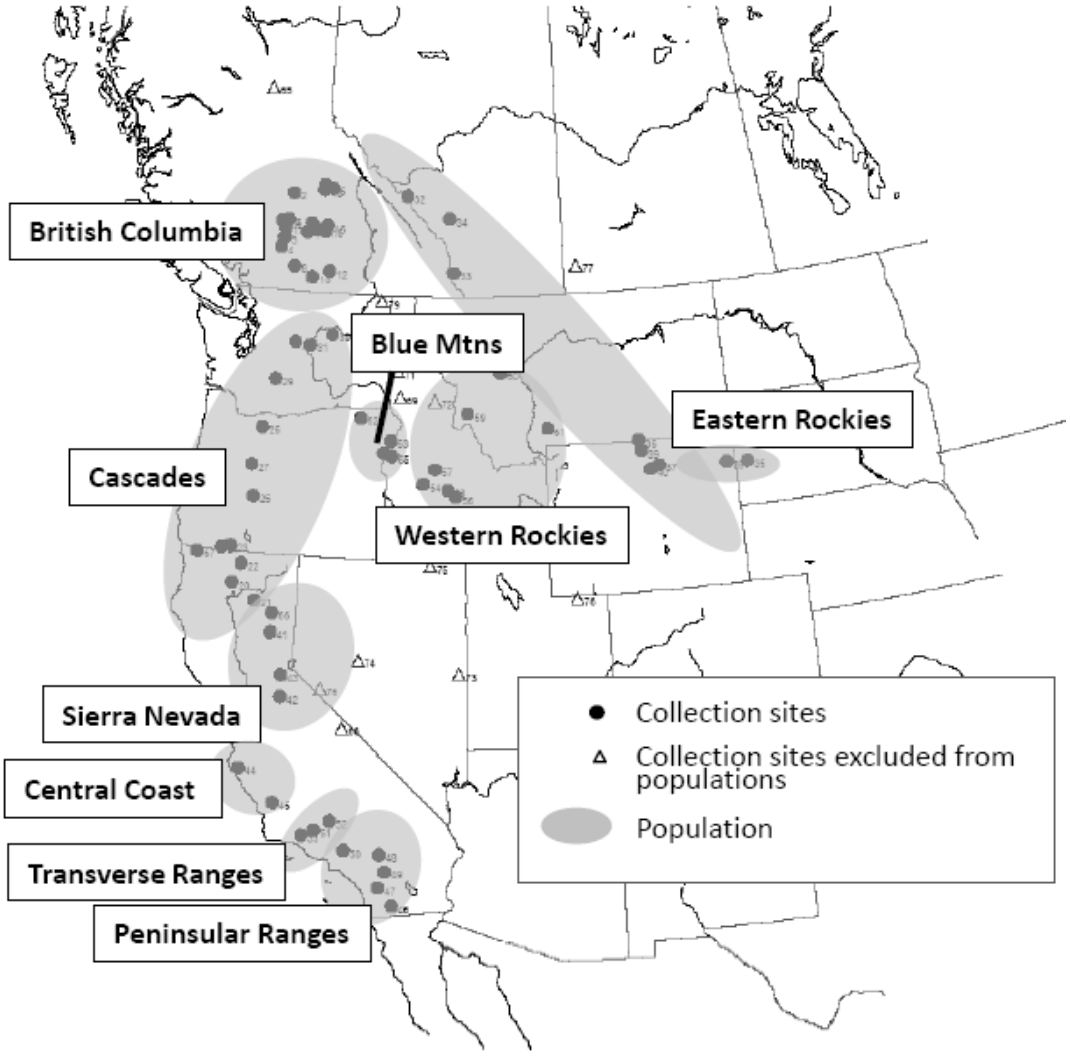


Figure 2.5 Nine Populations Suggested By SAMOVA Analyses

SAMOVA analyses were adapted to identify clusters of genetically and geographically cohesive collection site groups. These populations are the basis for molecular diversity, and Φst analyses.

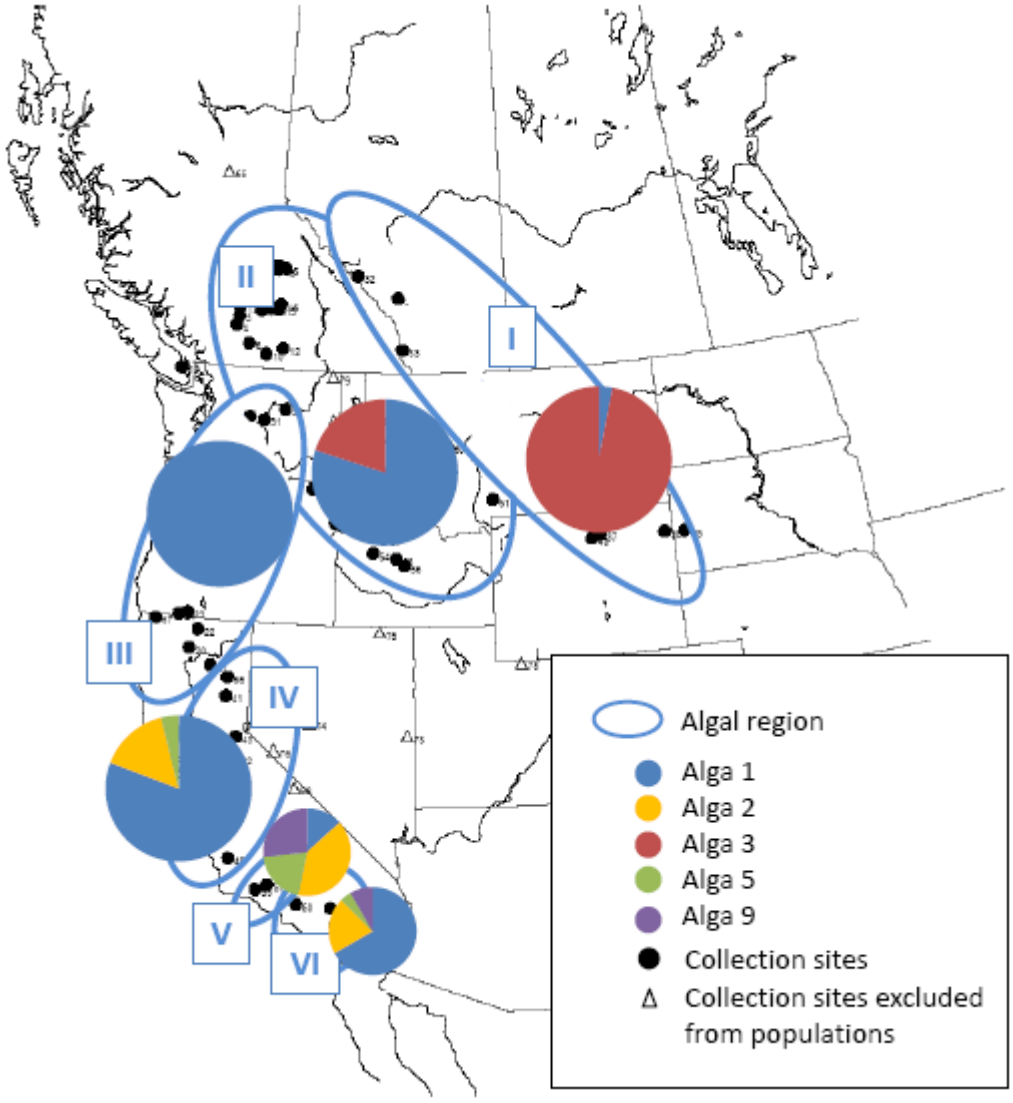


Figure 2.6 Six Regions Suggested By All-Lineage-Inclusive Pairwise Φst

Figure is based on populations defined in **Figure 2.1**. Neighboring populations with no statistically significant genetic differentiation at both loci are pooled together (see **Table 2.7**). The resulting regions each consist of a unique frequency distribution of algal lineages.

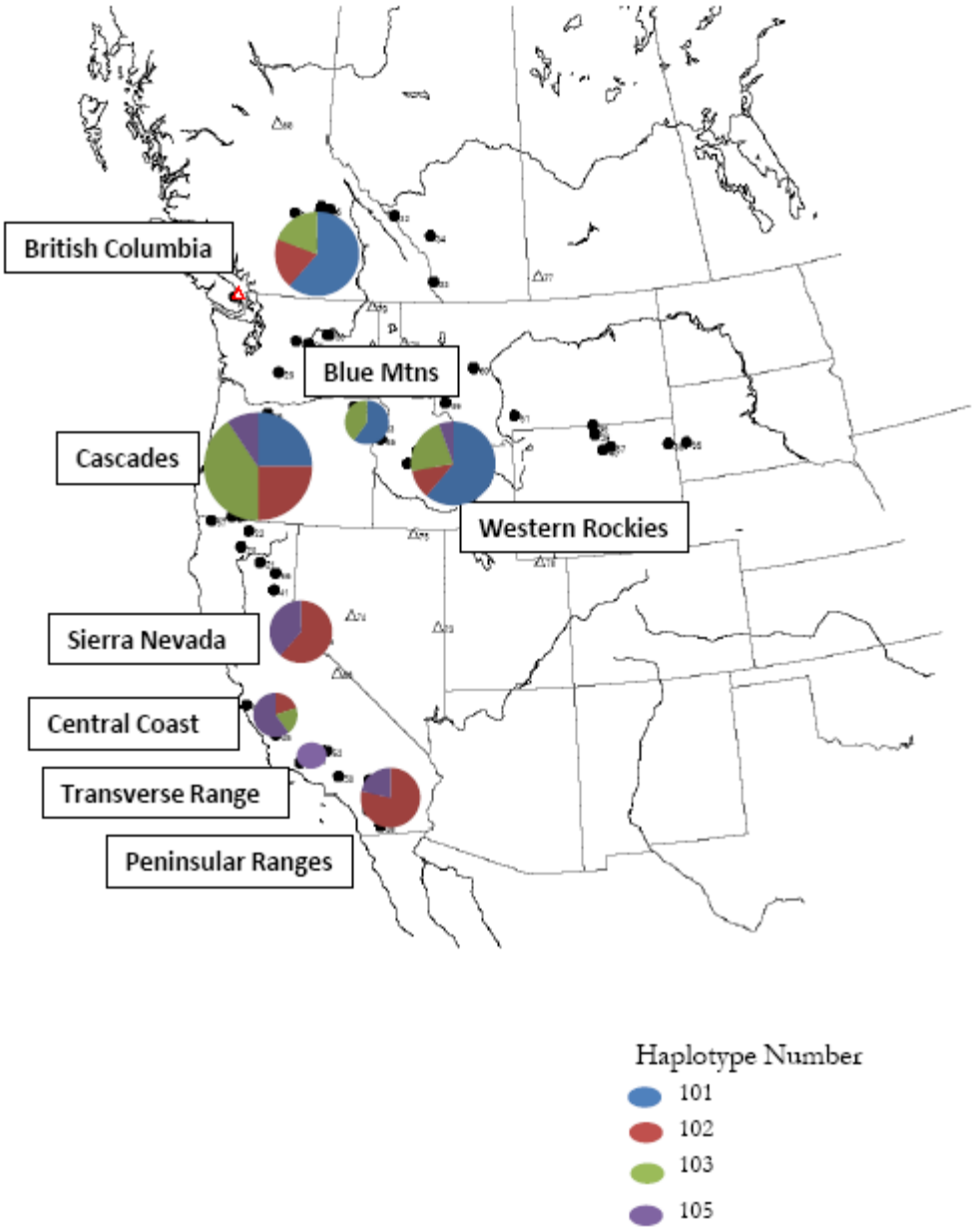


Figure 2.7 Geographic Distribution Of The Most Frequent Alga 1 ITS Haplotypes

Data are partitioned by population. The size of the pie diagram is proportional with the total number of individuals in that population that have any of the four frequent haplotypes.

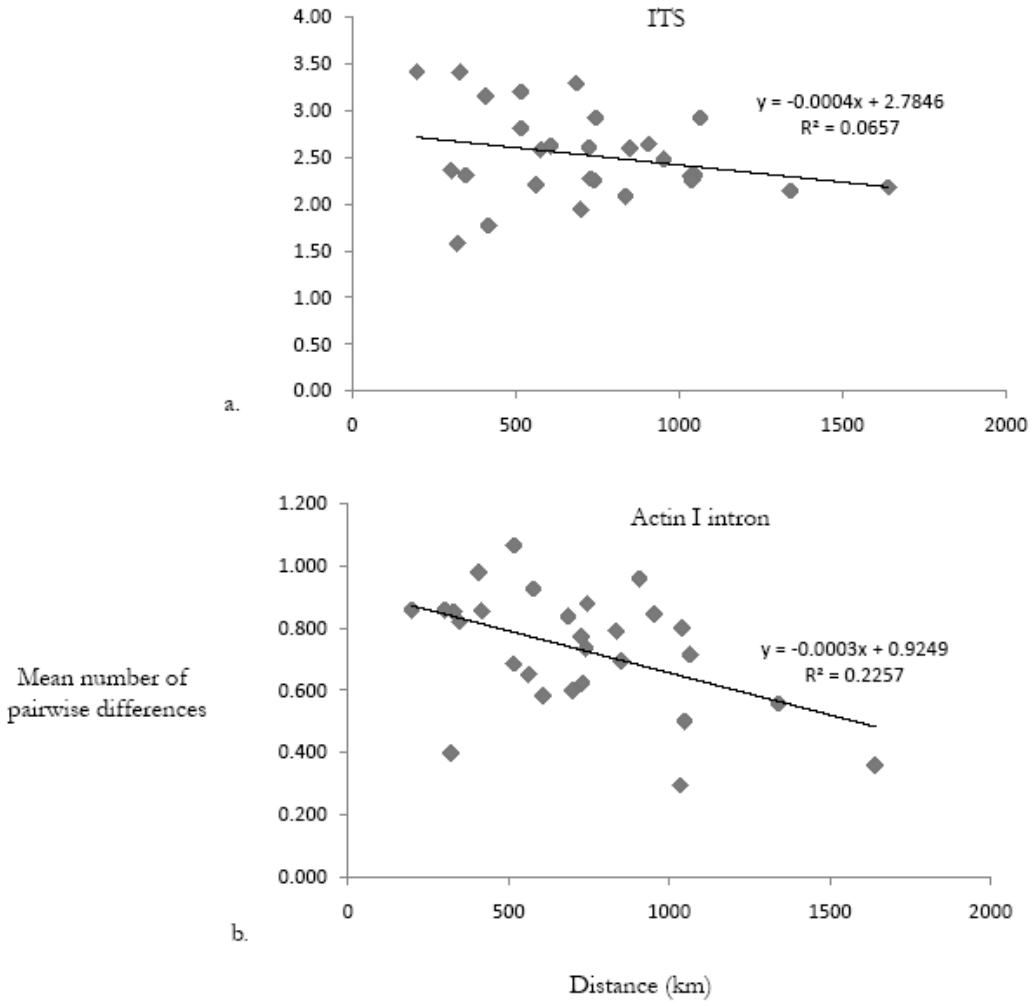


Figure 2.8 Plot Of Alga 1 Mean Pairwise Distance Versus Geographic Distance

Geographic distances are between collection site groups (**Table 2.1** and **Figure 2.1**). The Peninsular Range group was removed from both plots because it created a bimodal distribution in the actin plot due to a single high frequency divergent haplotype. a. ITS locus b. Actin I intron locus.

CHAPTER 3

A COMPARISON OF GEOGRAPHIC STRUCTURE OF MOUNTAIN WOLF LICHEN FUNGAL AND ALGAL SYMBIONTS

ABSTRACT

Genetic structure of the lichenized fungus *Letharia lupina* (Ascomycota) is compared with its symbiotic green algal partners in Mountain Wolf lichens of Western North America. Based on sequencing of five fungal loci, *L. lupina* fungi appear to be less geographically structured than *Trebouxia jamesii* (Chlorophyta) algae. From the perspective of both the fungal and algal symbionts, genetic diversity is highest in Southern California, near the southern extreme of Mountain Wolf lichen distribution. A fungal genetic discontinuity and algal genetic discontinuity between British Columbia and neighboring Western Alberta suggests that the Canadian Rocky Mountains are a barrier to lichen dispersal or a transition zone between distinct environments that select for different genetic types. Recombination has affected the genetic structure of both symbionts, and the fungi appear to have higher recombination in regions where algal lineage diversity is the highest. A distinct fungal population pairs with one of the algal lineages, an indication that the fungi are not pairing randomly with the five algal taxa. Fourteen widely distributed multi-locus fungal clones are inferred to result primarily from recombination, not long distance dispersal. Despite the occurrence of fungal genetic clones, when the algal genome is also considered, no two lichen thalli are genetically identical.

INTRODUCTION

The term symbiosis is used to describe the relationship between taxonomically unrelated organisms that live in physical intimacy (De Bary 1879). Symbioses include mutualistic, antagonistic, and commensalistic relationships. In comparative phylogeography, patterns in geographic structure of co-distributed taxa are analyzed for evidence of common histories (Abrogast and Kenagy 2001, Bermingham and Moritz 1998). Symbioses form a special case among studies of comparative phylogeography because of the expectation that such species interactions would lead to concordance in geographic structure (Funk et al. 2000, Nieberding and Olivieri 2007, Whiteman and Parker 2005, Wirth et al. 2005). Phylogeographic studies of antagonistic symbioses are more common than those on commensalistic or mutualistic interactions. In parasitisms, the level of congruence between symbionts has been related to the degree of vertical transmission of symbionts (Nieberding and Olivieri 2006). Among the limited studies concerning symbiotic mutualisms, horizontal transmission of symbionts is associated with incongruent geographic structures (Anderson et al. 2004, DeChaine and Martin 2006, Jones et al. 2006, Thompson et al. 2005) whereas vertical inheritance is associated with congruent genetic structures (Abbot and Moran 2002, Funk et al. 2000, Hurtado et al. 2003).

Many mutualistic symbioses, such as the intimate relationship between scleractinian coral animals and their algal consorts (Baker 2003), are made up of multiple species participating in a network of symbiotic partnerships. Lichens are the classic example of mutualistic symbiosis (Smith and Douglas 1987) and lichenized

fungal species commonly pair with multiple algal taxa and *vise versa* (although usually not simultaneously in the same thallus). Many lichens have mechanisms for dual fungal-algal dispersal, yet many lichenized fungi retain the ability to reproduce sexually and asexually through alga-independent spores. Such is the case for Mountain Wolf lichens: they have abundant isidia/soredia (dual fungal-algal propagules) and occasionally the fungi bear spore-producing fruiting bodies that are the result of sexual union (Kroken & Taylor 2001a and 2001b). The geographic scale at which vertical symbiont inheritance structures genetic diversity of symbiotic partners is not known. The main objective of this study is to describe the extent of congruence in the geographic structure on both sides of a widespread mutualistic symbiosis that has an unambiguous mechanism for vertical inheritance. The geographic structure of Mountain Wolf lichen *Letharia lupina* fungi are compared with the geographic structure of their algal partners, multiple lineages of *Trebouxia jamesii* s.l. green algae, throughout their geographic ranges in Western North America.

Fungi appear to be underrepresented in phylogeographic studies and appear to be even more poorly represented in comparative studies (Beheregaray 2008). Lichenized fungi uniquely lend themselves to such studies because they form discrete thalli with macroscopic phylogenetically informative morphological characters. With the increasing resolution of molecular tools, population genetic studies of lichenized fungi often show high genetic diversity but offer no generalizations about the geographic scale of gene flow. At a local scale (maximum distance 2 km), Werth and Sork (2008) found high genetic diversity, high gene flow, and no geographic structure

among populations of *Ramalina menziesii*. Lindblom and Eckman (2006) also found high genetic variation but no genetic structure between populations of *Xanthoria parietina* (within the same habitat type) at this small geographic scale. High gene flow was present in a study at a similar geographic scale in *Lobaria pulmonaria*, but in this case fine geographic structure was found (Werth et al. 2007). At a larger scale (10-15 km) Printzen and Eckman (2003) had mixed results studying island populations of *Cladonia subcervicornis*. Only one of the four populations was distinct from the others, suggesting gene flow among the other three populations. At an even larger geographic scale (40-500 km), Walser et al. (2005) found high genetic variation within populations and significant geographic structure between populations of *Lobaria pulmonaria* in British Columbia, but no geographic structure among populations in Switzerland. Even bipolar lichenized fungi provide contrasting examples of both geographic structure (Murtagh et al. 2002) and lack of genetic differentiation between populations (Crespo et al. 2002, Myllys et al 2003). Given this context, membership in the group "lichenized fungi" does not allow any assumptions about the geographic scale of gene flow.

Population genetic studies of lichenized algae are less common than fungal studies, and none cover the full geographic range of either the algae or the host fungi. Few studies offer comparisons of the genetic variability in the fungus with that of the alga because most studies that concern both symbionts are phylogenetic in nature and suffer small sample sizes and low resolution loci (Tibell 2001, Tibell & Beck 2001, Romeike et al. 2002, Piercey-Normore 2004, Summerfield and Eaton-Rye 2006, Nelson

& Gargas 2008). A few studies evaluate the genetic variation in the lichen as a composite organism, including the dominant fungal and algal partners. Ohmura et al. (2006) used the ITS locus on both *Parmotrema tinctorus* and *Trebouxia corticola* s.l. on a geographic scale of 60 km in Japan, and found 28 fungal-algal genetic combinations in 69 specimens. Kotelko et al. (2008) compared presence/absence of group I introns of the small subunit nuclear ribosomal DNA of *Cladonia arbuscula* with a single restriction fragment length polymorphism in the algal ITS locus. With a sample size of 84 individuals at a geographic scale of 30 km, they found variation in the fungus and not in the alga, but this may be an artifact of the low resolution method employed. Robertson and Piercey-Normore (2007) used the same loci for the same lichen with a sample size of 48 thalli and a small geographic scale (2 km). They found some geographic structure on the fungal side of the symbiosis, but none on the algal side of the symbiosis. Using RFLPs, Piercey-Normore (2006) found five algal genetic types from two clades of *Trebouxia jamesii* s.l. paired with two genetic types of the widespread fungus *Evernia mesomorpha* among populations within 11 km of each other. With a sample size of 290 thalli, Piercey-Normore found more evidence for geographic structure in the alga than in the fungus. Her study was complicated by the fact that 45% of thalli contained multiple algal genetic types, an indication of a higher order of genetic variability on the algal side of the symbiosis.

Only one published study compares fungal and algal diversity within a single lichen taxon over a large geographic scale. The Yahr et al. (2006) *Cladonia subtenuis* lichen study included eleven sites in Florida north to New Jersey and west to the Ozark

Mountains. Yahr et al. found geographic structure among four Asterochloris group *Trebouxia* ITS lineages (clades) that pair with the lichenized fungus *C. subtenuis*. Genetic structure of *C. subtenuis* was evaluated with three loci, ITS, EF1alpha and RBP2. In contrast with the algae, no evidence for geographic structure among the fungi was found, and there was evidence that the fungi were recombining.

MATERIALS AND METHODS

Molecular methods

DNA extractions from two hundred fifty-nine specimens previously analyzed for the algal component of Mountain Wolf lichens (Chapter 2) were also used in this study.

The identity of the fungal partner was verified by sequencing three loci (ITS, DO and locus 11) as described in Chapter 1, and the algae were characterized using the algal ITS locus and an actin I intron as described in Chapter 2. Four additional fungal loci were sequenced for this study (**Table 3.1**). Locus BA and locus CS are anonymous loci previously developed by Kroken and Taylor (2001a). Elongation factor 1-alpha (EF1-alpha) is a coding gene known for regions of high variability (Johannesson et al. 2000). The intergenic spacer (IGS) locus is a non-coding region of the ribosomal DNA array that is located in close proximity to the ITS locus. For the polymerase chain reactions, 25 microL reaction volumes were used with 0.625 units Qiagen Taq DNA polymerase (#201205 Valencia, California) according to the manufacturer's instruction. Each reaction included 0.5 microL of extracted DNA. The PCR conditions follow Kroken and Taylor (2001a) for BA and CS and Printzen and Eckman

(2002) for IGS. For EF1-alpha, the conditions were: 95 C for 3 minutes followed by 34 cycles of 94 C for 30 seconds, 56 C for 1.5 minutes, and 72 C for 1.5 minutes. This was followed by 10 minutes at 72 C. PCR products were cleaned with Exonuclease I and Shrimp Alkaline Phosphatase (USB Corporation) in 13 microL reactions according to the manufacturer's instructions. PCR products were sequenced in two directions using an ABI 3730 xl DNA Analyzer.

Sequences were aligned with Sequencher (Gene Codes Corporation) and then corrected by eye. Collapse 1.2 (Posada 1998) was used to identify identical sequences, and duplicate sequences were removed.

Fungal clones

In order to avoid collecting vegetative clones at least 100 meters separated each collection location whenever possible. Individuals that share identical multi-locus haplotype combinations are considered here to be clones although they may not be full genomic clones. Even outside the 100 meter perimeter, most multi-locus fungal genetic clones should be found in close proximity to each other and frequencies should decrease with distance. Local recombination that regenerates haplotype combinations found at other locations should result in a distribution of pairwise distances that is similar to that of the entire data set. Pairwise distances between all collection sites were calculated in Microsoft Excel using the latitude and longitude distance formula supplied online by BlueMM (2009). A distribution between pairwise distances in the entire data set (65,791 pairs) and among all multi-locus clones (18 pairs) was generated in Excel.

Population delineation & structure

The same modified spatial analysis of molecular variance (SAMOVA: Dupanloup et al. 2002) population delineation approach used for the algae in Chapter 2 was employed here with the fungal loci. Data from 64 sites were divided up into 12 groups based on geographic proximity, physiographic barriers, and on trends in haplotype frequencies (**Figure 2.1**). Due to geographic isolation, the fifteen remaining sites representing 18 individual thalli were excluded from the spatial analysis. Excluded sites are located in Nevada (4 sites), British Columbia (2 sites), Saskatchewan (1 site), Utah (1 site), Idaho (4 sites), Washington (2 sites) and California (1 site).

Spatial analysis of molecular variance (SAMOVA: Dupanloup et al. 2002) yields clusters of sampling groups according to the a priori assigned number of populations (K) based on a simulated annealing procedure. The analysis takes into account both genetic and geographic distances to identify genetically and geographically cohesive populations. SAMOVA analyses were carried out for each locus separately. K=2 to K=9 populations with 100 simulated annealing processes were tested for each fungal locus. Collection site groups were pooled into populations based on clusters with high F_{ct} values, clusters found under multiple values of K, and congruence between group clusters at each locus. For further discussion of this analysis, see the methods of Chapter 2.

Overall and pairwise Φs were used with partitions by fungal population (**Figure 3.1**), algal population (**Figure 3.2**), and algal lineage to describe genetic and geographic differentiation using Arlequin 3.1 (Excoffier et al. 2005). Arlequin was also

used to calculate standard indices of genetic diversity for each locus and for each locus partitioned by fungal population.

Mean number of pairwise genetic distances were plotted against geographic distance between collection site groups to estimate isolation by distance within each locus. Linear regressions were carried out in Systat 10.2 (Systat Software Inc.).

Reproductive mode

Fungi. Arlequin 3.1 (Excoffier et al. 2005) was used to test for non-random pairwise association of fungal loci with all data pooled and with the data partitioned by fungal population. This is also known as the exact test of linkage disequilibrium (Slatkin 1994). To test for recombination within and between all loci, the 4 gamete test was employed in DnaSP v. 5.00.07 (Rozas et al. 2009) on an alignment with all five loci combined. Pairs of sites, each with two alleles, are compared for their allele combinations. A single mutation with lineage sorting can lead to any three of the four possible allelic combinations, but the fourth combination can only emerge through an unlikely second mutation or through recombination. The 4 gamete test in DnaSP counts the number of biallelic pairs of sites with 4 gametic types. As a single recombination event can result in multiple pairs of sites with 4 gametic types, Rm is calculated. Rm is the minimum number of recombination events required to explain the data (Hudson and Kaplan 1985), and it is an underestimate. Individuals with missing or short sequences were excluded from the analysis, and the data were partitioned by fungal population.

Algae. To compare geographic structure in reproductive mode of both symbionts, the algal sequences of the ITS locus and an actin I intron from Chapter 2 were used to carry out the exact test of linkage disequilibrium within each algal lineage and within the two most widespread lineages, partitioned by algal population. The 4 gamete test was carried out by algal population for the most widespread algal lineage, Alga 1. The 4 gamete test was employed with both algal loci combined into a single alignment.

Association between fungal and algal genetic types

In order to address the question of whether any fungal and algal genetic types are associated with each other, two approaches were used. Non-random association of fungal and Alga 1 haplotypes was tested with all data pooled and partitioned by algal population. This is an unconventional application of the exact test for linkage disequilibrium in that the loci are from separate genomes (fungal and algal). I also tested for non-random association of fungal and algal haplotypes in Microsoft Excel using 10,000 randomized haplotype combinations for each fungal and algal locus combination.

RESULTS

DNA sequences were generated for all five fungal loci in 240 out of 258 individuals. PCR amplification was unsuccessful for a handful of individuals at the BA and IGS locus (**Table 3.2**). The EF1-α locus did not amplify in 14 individuals. The EF1-α locus had the largest mean number of pairwise differences (5.89) and number of

haplotypes (30) whereas IGS had the smallest mean number of pairwise differences (0.89) and the fewest haplotypes (17). The other three loci were intermediate in diversity. Only 4% of the analyzed specimens were fertile (apothecia present). *Letharia lupina* fungi are genetically diverse: 85% of thalli have unique five-locus fungal genotypes.

Fungal clones

Fourteen multi-locus haplotypes appear more than once in the dataset, and none appear more than three times. Sixteen out of eighteen pairwise geographic distances between clones span over 200 km. The average distance between clones is 861 km whereas the average distance between all collection sites is 897 km. Although long distance dispersal is expected for weedy lichenized fungi, the frequency distribution of pairwise distances between dispersed clones should have a negative slope. In contrast, the histogram for the pairwise clone distances is flat, a pattern more congruent with a random distribution of fungal clones across the landscape (**Figure 3.3**). Long distance dispersal cannot be ruled out for any single pair of clones, but regeneration of multi-locus clones through local recombination is a more likely explanation for the widely separated fungal genetic clones.

Remarkably, nine out of the twelve pairs of long-distance fungal clones pair with a different algal lineage at each location. Even those clones that were found with the same algal lineage multiple times were found with a different algal genetic type within that lineage.

Population delineation

The modified SAMOVA analysis (**Table 3.3**) suggests that the collection site groups can be divided up into six genetically and geographically cohesive populations: Alberta, British Columbia, the Sierra Nevada Mountains, a Cascade-Western Rocky Mountain population (includes the Blue Mountains and South Central Idaho), a Coast–Peninsular Range population, and a disjunct Wyoming-Montana-Transverse Range population (**Figure 3.1**).

A similar SAMOVA analysis was previously applied to the algal data (Chapter 2) and the geographic boundaries of the resultant populations differ from those of the fungi (**Figure 3.4**). Similarities include the pooling of the Northern and Southern Cascades groups into one population, and the fact that British Columbia and the Sierra Nevada groups formed populations on their own, not merging with any of their neighbors. There are additional differences between the geographic boundaries of the fungal populations and algal populations. For example, the fungi of Alberta and Wyoming do not group together, the Cascade fungi were pooled with their neighbors to the east, and the Transverse Ranges fungi were pooled with the Montana and Wyoming collections.

The grouping of the Transverse Ranges collections with the Wyoming and Montana collections into one fungal population stands out as anti-intuitive, especially given the proximity of the Transverse Ranges to the Southern California and Coast collection sites. However, as noted in Chapter 2, there is evidence for continuous Mountain Wolf lichen habitat from the Transverse Ranges to the Rocky Mountains

during the Pleistocene (Wells 1983), and a genetic signature of a dispersal corridor may persist in the Transverse Ranges.

Population structure

Pairwise Φst analyses were carried out with partitions by fungal population, algal population and algal lineage (**Table 3.4**). All the loci were informative in at least two out of the three analyses, except locus BA. Most pairwise comparisons, regardless of partition regime, were not significant or showed only low to moderate population differentiation. In addition, only 6/26 of the significant pairwise comparisons were between neighboring or sympatric (the fungal and algal population boundaries are not always identical) populations. Among the Φst analyses partitioned by fungal population, the Alberta population shows moderate differentiation from other populations at more than two loci. This is congruent with the results for the analysis partitioned by algal population. The Eastern Rocky Mountain algal population includes Alberta (in addition to Wyoming and South Dakota) and was found to have low to moderate differentiation with several other populations, including neighboring British Columbia. Partitioning the fungal data by algal population did not increase the number of significant pairwise comparisons and appears not to be a superior description of fungal population structure. When the data are partitioned by algal lineage, both locus CS and locus EF indicate that *L. lupina* haplotypes partnering with Alga 9 are distinct from those partnering with Alga 1 and Alga 2.

The primary differences between non-contiguous *Letharia lupina* populations are in haplotype frequencies, not in genetic distances. Over a wide geographic range *L.*

lupina fungi exhibit only modest geographic structure. Based on the pairwise Φst, the BA locus indicates that the fungi are panmictic while the other four loci indicate moderate geographic structure between some population pairs, especially between non-contiguous populations (**Table 3.4**). Overall, the fungi have weaker discontinuities between populations than the algae (**Table 2.7** and **Table 2.8**).

Fungal diversity indices

Population-specific genetic diversity was assessed for each locus (**Table 3.5**). There is no consistent, congruent geographic pattern of diversity shared by the five fungal loci. Generally, the two populations restricted to California, the Sierra Nevada and Coast-Peninsular populations, rank highest while the Cascade-Rocky Mountain population ranks the lowest in diversity. In terms of multi-locus genotypes, the Alberta and Sierra Nevada populations have the highest percentage of unique multi-locus genotypes (92%) and British Columbia and Wyoming-Montana Transverse Range populations have the lowest (71% and 72%, respectively).

Geographic distance plays no significant role in genetic distance between collections site groups (**Figure 3.5**), as was also found within Alga 1 (**Figure 2.8**).

Reproductive mode

Fungi. The results of the exact test of linkage disequilibrium are consistent with unlinked fungal loci in seven out of 10 pairwise comparisons (**Table 3.6**). There is evidence for linkage between the ITS locus and IGS locus ($P = 0.000$). There is also evidence for linkage between IGS and EF1-α ($P = 0.006$) and between BA and CS ($P =$

0.016). However, when the data are partitioned by fungal population (data not shown), the signal for significant linkage between the latter two pairs of loci disappears. The linkage between the ITS and IGS loci persisted at the population level except in the two populations exclusively in California: the Sierra Nevada Mountains and the Coast-Peninsula population. In addition, multiple recombinant gametic types were found in each population (**Table 3.7**)

Algae. The results of the exact test of linkage disequilibrium are consistent with unlinked loci in all lineages except Alga 1 (**Table 3.8**). However, when partitioning the data by algal population (**Table 3.9**), a significant signal of linkage emerges only for the Cascade Mountain population. Linkage disequilibrium occurs in the presence of recombinant gametic types there (**Table 3.10**).

Despite no linkage disequilibrium overall in Alga 3, when partitioned by algal population, significant linkage is localized to the Eastern Rocky Mountains (**Table 3.9**).

Association of fungal and algal genetic types

When all sites are pooled together, the exact test for linkage disequilibrium shows significant linkage between six out of ten pairwise fungal-algal locus combinations (**Table 3.11**). When the data are partitioned by algal population, the signal of linkage between lichen bionts disappears except for fungal EF1-alpha and algal ITS in the Sierra Nevada population (n=17, P=.0000) and between EF1-alpha and the algal actin locus in the Western Rockies population (n=37, P=.0018). Using permutation tests, only three out of the ten fungal-algal locus comparisons showed a

significantly smaller number of combinations than expected if the loci were associating randomly (**Table 3.12**).

DISCUSSION

Fungal geographic structure

Across the distribution of *L. lupina*, there is no relationship between geographic and genetic distance (**Figure 3.5**), as was found in the widespread lichenized fungus *Xanthoria parietina* (Lindblom and Ekman 2007). Using ITS and ribosomal large subunit rDNA sequences, Zoller et al. (1999) also found no isolation by distance in *Lobaria pulmonaria* within Switzerland. However, at a much smaller geographic scale of 4 km and using microsatellites, Werth et al. (2007) found clear isolation by distance in *L. pulmonaria*. This result may have as much to do with the reproductive mode of *L. pulmonaria* as it does with the sampling scale of a fungus whose primary reproductive and dispersal mode is clearly asexual (Werth et al. 2006). Frequent sexual reproduction may limit local genetic structure in lichens. Sampling at local geographic scale (within 2 km) in highly fertile *Ramalina menziesii* revealed no isolation by distance, in fact no geographic structure at all (Werth & Sork 2008). No geographic structure or minor geographic structure was also found within four sexually reproducing species of tropical epiphyllous lichens over a geographic scale of 240 km (Baloch and Grube 2009). It would be fruitful to compare the local genetic structure of *L. lupina* in sites with and without sexual reproduction to more fully understand the effect of sexual reproduction on local genetic structure.

The *L. lupina* diversity pattern contrasts with that of *Cavernularia hultenii* (Printzen & Ekman 2002, Printzen et al. 2003), a lichenized fungus that shares a portion of its Pacific Northwest geographic range (Southern British Columbia, Washington and Oregon) with Mountain Wolf lichens but is associated with more mesic environments. *C. hultenii* and *L. lupina* lichens share the same reproductive mode as assessed by morphology: they both reproduce mainly through asexual soredia/isidia. Printzen et al. (2003) found that *C. hultenii* has many rare haplotypes in Oregon and Washington, while in British Columbia it is mostly made up of three widespread haplotypes. From this pattern the authors inferred a Pleistocene refugium south of the ice sheets. Nested within their larger geographic distribution, Mountain Wolf lichens do not share *C. hultenii*'s impoverished genetic diversity in British Columbia as compared with Washington and Oregon. Mountain Wolf lichens are more aggressive, weedy lichens and may have more effective long distance dispersal.

Comparative fungal-algal geographic structure

Fungal genetic diversity (**Table 3.5**) is highest in the California populations. Similarly, the algae enjoy highest lineage diversity in Southern California (Chapter 2). By combining this information, it is clear that the southern extreme of the lichen's distribution is the center of diversity in the Mountain Wolf lichen symbiosis. This finding is congruent with diversity in patterns in a variety of plants and animals. Southern California has the highest number of endemic plants per square kilometer in the California Floristic Province (Stebbins and Major 1965), and it is increasingly cited

as a species diversity hotspot in animals (Davis et al. 2008, Calsbeek et al. 2003, Chatzimanolis and Caterino 2007, Vandergast et al. 2008).

The only neighboring fungal populations with significant differentiation at more than one locus are British Columbia and Western Alberta. This is congruent with the algal side of the symbiosis in that algal lineage 1 is rare east of the Rocky Mountains (**Figure 2.4**), suggesting a barrier to dispersal or an inability to establish. Both explanations are likely for the Canadian Rocky Mountains because they manifest a strong physical barrier and also distinct climatic zones. Growth experiments with reciprocal transplant of thalli of known fungal and algal genetic types would test whether adult thalli respond differently to climate on either side of the Rockies, but testing dispersal and establishment themselves remain experimentally challenging (Werth et al. 2006).

As was previously found *Cladonia subtenuis* and *Evernia mesomorpha* lichens, *L. lupina* fungi are less geographically structured than their algal partners. Each Mountain Wolf lichen algal lineage is likely a reproductively isolated lineage: each is probably a cryptic (undescribed) species (see Chapter 2 and Kroken and Taylor 2000). The individual algal lineages differ in their geographic distributions, and this gives the Mountain Wolf algae more structure than the fungi. However, the algae appear to be more geographically structured than the fungi even when only a single algal lineage is compared with the fungal species. This finding could be based on superior dispersal abilities of the fungi, as might be expected of sexually produced ascospores or conidiospores dispersed by wind or insect vector. Alternatively, the fungi may have no

special dispersal advantage, and the algae are more geographically structured based on their adaptations to local ecological conditions. Comparative dispersal of lichen fungi and algae is only beginning to be studied and would benefit from higher resolution molecular data such as microsatellites for both sides of the symbiosis, studies at multiple spatial scales, and a way to capture and analyze lichen diaspores (Werth et al. 2006, Widmer et al 2008).

Geographic structure in reproductive mode

Fungi. Sexual reproduction within *L. lupina* was previously inferred by Kroken and Taylor (2001b) based on incongruence in gene genealogies as well as paternity analysis on individual spores. Here, with all populations combined, the important exception is that the IGS and ITS loci show linkage disequilibrium, and this is expected based on the proximity of the two loci on the chromosome. When the ITS-IGS analysis is partitioned by fungal population, two California populations (Sierra Nevada and Coast-Peninsula) lack an indication of linkage disequilibrium. The lack of linkage signal is not associated with higher ITS-IGS diversity in these populations (higher diversity facilitates detection of linkage disequilibrium). My result is consistent with more intense local recombination in California, intense enough that loci in close proximity are disassociated, and it may be evidence for geographic structure in reproductive mode. Although fertile thalli were collected in every population except Alberta, it was particularly easy to find them in the Sierra Nevada Mountains (personal observation). The data suggest more fungal sexual reproduction in the geographic areas where there are more than two algal lineages (the Sierra Nevada and Southern

California). Fungal genetic diversity through recombination may be an advantage when partnering with multiple algal lineages in the same geographic region.

Algae. Lichenized green algae are thought to reproduce asexually because meiotic tetrads have not been observed, and gamete fusion has scarcely been observed (Ahmadjian 1967, Friedl and Rokitta 1997, but see Slocum et al. 1980). Kroken and Taylor (2000) found evidence for recombination within Alga 1 through the Partition Homogeneity Test and through the identification of recombinant genetic types. Here with an expanded data set, genetic evidence for within-lineage recombination based on the exact test for linkage disequilibrium is found for all lineages except Alga 1 (**Table 3.8**). However, when the Alga 1 data are partitioned by population, only the Cascade population shows evidence of clonal reproduction (**Table 3.9**), an indication of geographic structure. Another sign of geographic structure to reproductive mode is the Eastern Rocky Mountains population's unique emergence with a signal of linkage between the two loci in Alga 3.

There is no obvious parallel in the geographic structure of fungal reproductive mode and geographic structure of algal reproductive mode, even when the analysis is scaled down to compare the fungi with only one algal lineage at a time. Comparing taxa of similar rank in this way, the difference between the two symbionts is that the fungus demonstrates evidence for more intense local recombination in California whereas the algae suggest clonal reproduction in the Cascade Mountains (for Alga 1) and in the Eastern Rocky Mountains (for Alga 3). In other words, recombination in the fungus is generally evident and appears to be especially intense in California

whereas recombination in the two widespread algal lineages is also generally evident and appears to be especially weak in the Cascade and Eastern Rocky Mountain populations.

Specificity of fungal-algal partnerships

Using the exact test for linkage disequilibrium, it appears that some fungal and algal loci are genetically linked (**Table 3.11**). Similarly, permutation tests with pooled data showed three significant non-random partnership combinations based on comparisons of pairs of fungal and algal loci (**Table 3.12**). These findings may be a result of successful specific associations between fungal and algal genomes. Alternatively, they may be an artifact of geographic structure in the fungi and algae. Fungal loci were generally found to be unlinked with algal haplotypes when the data were partitioned by algal population.

When the fungal data are partitioned by algal lineage, a non-random subset of fungal haplotypes pairs with Alga 9 according to two out five fungal loci (**Table 3.4**). At the algal ITS locus, Alga 9 is separated from the other clades by at least six steps, more than any other lineage (**Figure 2.4a**). It may be that Alga 9 has unique physiological traits and that a distinct subset of *L. lupina* genetic types is adapted to Alga 9. Alternatively, if Alga 9 is a relatively new alga in the Mountain Wolf fungal-algal partnership network, it may be that only a subset of fungal genetic types have tapped into this algal resource. There is no indication of intraspecific fungal specificity for Alga 1 or Alga 3, the two most widespread algal lineages. No fungal haplotype specificity was detected for these lineages, even though both lack sympatric algal

lineages in significant portions of their geographic distributions, a fact of geographic structure that might be expected to favor mutual genetic accommodation. Similarly, Yahr et al. (2006) found no fungal ITS clade specificity to geographically widespread algal ITS lineages in *Cladonia subtenuis* lichens. With the exception of one case of within-species fungal lineage specificity to algal partners (Alga 9), the data suggest that such specificity is not generally the case. However, there may be meaningful genetic links between fungal and algal loci that occur at a spatial scale well within the artificial populations I have delineated.

If the widespread geographic distribution of multi-locus fungal clones betrays long distance dispersal, the unit of dispersal was likely not a dual fungal-algal propagule unless the alga did not persist in the partnership. Algal "switching" among lichenized fungi is rarely documented at short time scales (Ahmadjian et al. 1980, Sanders & Lucking 2002, Schaper and Ott 2003) but it is strongly inferred given phylogenetic evidence within fungal families, genera, and species (Piercey-Normore and DePriest 2001, Yahr et al. 2004, Miadlakowska et al. 2006), including within Mountain Wolf lichens (Kroken and Taylor 2000). It is possible for fungi to switch algal partners, especially at early stages of development (Friedl 1987). Through production of sexual and asexual spores, it appears that the fungi have much more effective dispersal than the algae, and the fungi simply relichenize with algae that are locally available. Werth et al. (2006) showed no dispersal limitation in the *Lobaria pulmonaria* fungi dispersing via soredia, but the algal component was not similarly examined.

Strong asymmetry in dispersal ability is not required to explain the difference in fungal and algal geographic structure. Yahr et al. (2006) used their work on the *Cladonia* lichens to inform a model of fungal-algal association that is similar to the Combes "filter model" describing mechanisms that mediate parasite-host compatibility (Combes 2001). In the lichen model, the frequency of specific fungal-algal partnerships is not only determined by the abundance of compatible fungal and algal lineages present at the site (dispersal) but by selection on the symbiotic marriage. The data from *Cladonia subtenuis* and from *Letharia lupina* lichens indicate that at large geographic scales the algae have more geographic structure than the fungi. Since both lichens have mechanisms for dual fungal-algal dispersal (presumably by fragmentation in *C. subtenuis*) selection can act on the fungal-algal partnership according to local environmental conditions. According to the model, many partnership combinations arrive at a site, algal "switching" takes place, but few partnerships persist. Consistent with this view of ecological filtering, Werth et al. (2006) found that establishment limitation is a better explanation than dispersal limitation for the distribution of *Lobaria pulmonaria* soredia.

Studies of geographic structure in symbiotic mutualisms usually include multiple species on one or both sides of the interaction. Rarely are two species (or taxa of analogous rank) compared for concordance in *intraspecific* geographic structure. Vertical inheritance of *Buchnera* bacterial endosymbionts of aphids has led to concordant geographic structure (Funk et al. 2000). The same was inferred for hydrothermal vent clams and their symbiotic sulfer-oxidizing bacteria (Hurtado et al.

2003). However, among symbiotic mutualisms with horizontal inheritance of symbionts, there is a consistent asymmetry in the geographic structure in the partners, and it is often attributed to differences in dispersal between symbionts. Examples include squid and their light organ bacteria (Jones et al. 2006), gobies and snapping shrimp (Thompson et al. 2005), protocarnivorous plants and hemipterans (Anderson et al. 2004), and plants and pollinating herbivorous butterflies (DeChaine & Martin 2006).

Post-Pleistocene colonization of formerly glaciated northern lands

Because every thallus analyzed in this study is genetically unique, it is clear that Mountain Wolf lichens are not made up of a few highly successful partnership clones that have swept the landscape. The British Columbia population is of special interest because this region was inundated by the Cordilleran ice sheet during the Pleistocene and has only been colonized by plants and animals in the last 14,000-18,000 years (Pielou 1991). Previously glaciated areas are associated with decreased genetic diversity (Broyles 1998, Conroy and Cook 2000, Critchfield 1984, Hewitt 1996, Lewis and Crawford 1995, Printzen et al. 2003, Printzen and Eckman 2002), but an important substrate tree for Mountain Wolf lichens, *Pinus contorta*, lacks depauperate northern populations, a genetic pattern that has been attributed to effective dispersal or cryptic northern refugia (Jerome and Ford 2002). The fact that the mistletoe parasite of these trees has higher genetic diversity in formerly glaciated regions, is consistent with the latter explanation (Jerome and Ford 2002), but fossil and palynological evidence for any coniferous northern refugium is minimal (Anderson et al. 2006, Brubaker et al. 2005). Formerly glaciated regions are also associated with asexual reproduction in plants

(Horandl 2006, Kearney 2005). I had expected to find a signature of the Mountain Wolf lichens' northern colonization through decreased haplotype diversity as a founder effect, and increased linkage disequilibrium due to asexual reproduction. Little of these were found on either side of the symbiosis. Apparently, vertical inheritance of algal partners and relatively recent colonization have not significantly distorted the genetic structure of the symbiosis as it invaded the ice-free zone. Recombination in the fungus, recombination in the alga, effective long distance dispersal, and/or switching between partners all could contribute to dilution of the homogenizing effect of vertical inheritance at the large spatial scale of this study.

Table 3.1 Primers Used To Amplify The Five Fungal Loci

TD = touch down PCR. C = annealing temperature is in Celsius.

Locus	Primer Name	Forward Primer sequence	C	Target Length	Primer Citation
ITS	1F	CTTGGTCATTTAGAGGAAGTAA	60	680	Kroken and Taylor 2001
	4A	ATTTGAGCTGTTGCCGCTTCA			
BA	BAa	GGAGACAAGGAAGGATGGAG	TD 70	430	Kroken and Taylor 2001
	BAb	AGGGAGGAGGTAGTGAAGTC			
CS	CSa	CAAGTGGATCGTCGGGAAGA	56	372	Kroken and Taylor 2002
	CSb	GCCTTCAAGGTTTATATGAC			
EF1-α	EFF	RGACAAGRCTCACATCAACGTSGT	52	600	Johanneson et al. 2000
	EFR	CCAGTRATCATGTTCTTGATGAART			
IGS	IGS 12b	AGTCTGTGGATTAGTGGCCG	TD 40	233	Printzen & Ekman 2002
	SSU-0072R	TTGCTTAAACTTAGACATG			

Table 3.2 Comparison Of The DNA Sequence Diversity In Five Fungal Loci

N = number of individuals. Haps. = number of haplotypes. Poly. sites = number of polymorphic sites minus multi base pair indels. Private alleles are genetic types only found once in the data set.

Locus	N	Haps	Poly. sites	No. of private alleles	Gene diversity	Mean no. of pairwise differences	Nucleotide diversity
BA	257	20	34	8	0.608	5.89	0.0146
CS	258	22	24	9	0.788	2.21	0.0063
EF1-α	244	30	34	9	0.907	2.98	0.0058
IGS	255	17	14	10	0.679	0.83	0.0018
ITS	258	27	26	15	0.803	1.55	0.0028

Table 3.3 SAMOVA Collection Site Group Clusters

Collection site group clusters found under nine SAMOVA values of K for 5 loci. Values of K for which there was no significant result are indicated with "none." Clusters of collection site groups that appear under at least two values of K in at least two loci were used to infer populations. Collection site clusters accepted to infer populations appear in bold. Group abbreviations are as follows: AB=Alberta, Blue=Blue Mountains of Oregon, Coast=Central Coast of California, ID=Idaho, MT=Montana, NC=Northern Cascades, Penin=Peninsular Ranges, SC= Southern Cascades, Sierra=Sierra Nevada, WY=Wyoming.

	K		
Locus	2	3	4
BA	none	none	none
CS	none	none	none
EF	**Blue-ID-WY-NC-MT-[Mt-Trans]**-Penin-BC	**Blue-ID-NC**-Penin-**MT-Trans**-BC	**Blue-ID-NC**-MT-Penin-BC
ITS	none	none	none
IGS	none	none	none

Table 3.3 (cont.)

5	6	7	8	9
Coast-Penin-BC AB-**ID-SC** Blue-**MT-WY-[MT-Trans]**	none	**Coast-Penin** AB-**ID-SC** **MT-WY-[MT-Trans]**	**Coast-Penin** AB-SC **MT-WY-[MT-Trans]**	**Coast-Penin** AB-ID **MT-Trans**
none	none	none	AB-ID-MT Coast-SC-Trans	none
Blue-ID-NC-MT-Penin	Coast-SC-Sierra **Blue-ID-NC**-MT-Penin	Coast-SC-Sierra **Blue-ID-NC**-MT	Coast-SC-Sierra **Blue-ID** MT-NC	Coast-SC-Sierra Blue-MT
Sierra-WY **Coast-Penin** **Blue-ID-NC-SC** AB-**MT-Trans**	**MT-WY** **Coast-Penin** **Blue-ID-SC-NC** AB-Trans	**MT-WY** **Coast-Penin** **Blue-ID-NC-SC**	**MT-WY** **Coast-Penin** **Blue-ID-SC**	**Coast-Penin** **Blue-ID-SC**
none	none	none	**Blue-ID-NC** AB-MT Penin-Trans	none

Table 3.4 Pairwise Φst for Fungal Loci

Φst are partitioned by fungal population, algal population and algal species. Only significant comparisons are shown for each of the five fungal loci.

Fungal Locus	Fungal Population	Algal Population	Algal Species
BA		Blue Wrock 0.07266	
CS	Coast BC 0.04143 Coast Sierra 0.0565	BC Erock 0.05284 BC SoCal 0.06962 Blue Erock 0.0904 Blue SoCal 0.11958 Erock Sierra 0.06850 Sierra SoCal 0.08794	9-1 0.07992 9-2 0.14755
EF	AB BC 0.07905 AB Casc 0.09905 AB MT 0.12705 AB Sierra 0.10493		9-1 0.15412 9-2 0.12794 9-3 0.16700
IGS	Casc AB 0.13284 Casc-MT 0.03879 Coast-MT 0.04967	BC SoCal 0.08111 Casc Coast 0.15244 Casc Erock 0.02932 Erock SoCal 0.14275 SoCal Wrock 0.07229	
ITS	AB BC 0.05294 AB Casc 0.08714 AB Coast 0.09037 Coast MT 0.03071	Erock SoCal 0.04849	1-3 0.01897 3-5 0.13408

Table 3.5 Genetic Diversity Indices Of Five Loci Partitioned By Fungal Population

N = number of individuals in each population. Haps = number of haplotypes. Poly. sites= number of polymorphic sites. Private alleles are haplotypes found only once in the data set.

	BA							CS						
Population	N	Haps	Poly. sites	No. of private alleles	Gene diversity	Mean No. of pairwise differences	Nucleotide diversity	N	Haps	Poly. sites	No. of private alleles	Gene diversity	Mean No. of pairwise differences	Nucleotide diversity
Alberta	12	4	17	0	0.6515	6.6515	0.0165	12	6	11	0	0.8485	2.7727	0.008155
British Columbia	45	9	21	1	0.5465	4.7222	0.0117	45	12	16	1	0.7222	2.2565	0.006484
Casc, Blue, ID	83	11	29	2	0.6197	6.0117	0.0149	83	15	17	4	0.8061	2.2874	0.006592
Coast-Penin	34	12	29	2	0.7130	6.9982	0.0173	34	9	13	2	0.8217	2.0588	0.005916
MT WY Trans	45	6	18	1	0.4505	4.6787	0.0116	46	7	10	0	0.7913	2.1845	0.006277
Sierra Nevada	17	7	19	1	0.7647	7.2206	0.0179	17	6	6	1	0.7059	1.7353	0.004986

	EF							IGS						
Alberta	12	8	10	0	0.9091	2.7424	0.005325	12	5	2	0	0.8333	0.8939	0.001965
British Columbia	39	15	21	2	0.9312	3.3441	0.006609	44	5	3	1	0.6554	0.7114	0.001527
Casc, Blue, ID	81	19	22	1	0.8917	2.6404	0.004127	83	9	6	3	0.6418	0.6101	0.001329
Coast-Penin	31	11	18	2	0.8753	2.8795	0.005591	34	8	9	2	0.7362	1.2603	0.002770
MT WY Trans	46	14	18	3	0.8841	3.1053	0.006030	45	6	4	2	0.6879	0.8262	0.001812
Sierra Nevada	16	12	19	2	0.9500	3.6583	0.007187	16	4	2	0	0.6417	0.6250	0.001344

	ITS						
Alberta	12	6	5	2	0.2083	1.3939	0.002591
British Columbia	45	12	17	2	0.8697	1.9959	0.003662
Casc, Blue, ID	83	14	16	3	0.7543	1.2823	0.002366
Coast-Penin	34	10	11	3	0.6168	1.1854	0.002191
MT WY Trans	46	13	14	5	0.8193	1.5371	0.002836
Sierra Nevada	17	9	19	1	0.8897	2.6323	0.004875

Table 3.6 Exact Test Of Linkage Disequilibrium For Five Fungal Loci

P values for significantly linked loci appear in boldface type.

Locus	BA	CS	EF	IGS	ITS
BA	X				
CS	**0.016**	X			
EF	0.321	0.064	X		
IGS	0.683	0.252	**0.006**	X	
ITS	0.217	0.103	0.311	**0.000**	X

Table 3.7 The 4 Gamete Test on Combined Fungal Loci

Tests are partitioned by fungal population. N is the number of individuals per population, "4 gametes" is the number of pairs of biallelic sites with 4 allele combinations, Rm is the minimum number of recombination events.

Population	**N**	**4 Gametes**	**Rm**
Alberta	12	147	4
British Columbia	39	297	6
Cascades/Blue/ID	77	384	4
Coast-Penin	32	279	5
MT/WY/Trans	43	369	6
Sierra Nevada	14	180	5

Table 3.8 Exact Test Of Linkage Disequilibrium For Each Algal Lineage

Tests between the ITS and actin I intron locus. The statistically significant result ($P < 0.05$) appears in boldface and indicates linked loci within alga lineage 1.

Algal Lineage	Sample Size	P value
1	177	**0.0365**
2	16	0.529
3	52	0.086
5	5	0.598
9	7	1

Table 3.9 Exact Test Of Linkage Disequilibrium By Algal Population

Tests are between the ITS and actin I intron locus for Alga 1 and Alga 3. Hap. Combo. = number of ITS-Actin haplotype combinations. Significant linkage disequilibrium is indicated in boldface ($P < 0.05$).

	Alga 1			Alga 3		
Population	Sample Size	Hap. Combo.	P value	Sample Size	Hap. Combo.	P value
Blue Mountains	14	9	0.390	NA	NA	NA
British Columbia	36	14	0.064	9	6	0.152
Cascade Mountains	47	20	**0.019**	NA	NA	NA
Central Coast	4	4	NA	NA	NA	NA
Eastern Rockies	1	1	NA	32	11	**0.001**
Peninsular Ranges	16	8	0.096	NA	NA	NA
Sierra Nevada	15	6	0.399	NA	NA	NA
Western Rockies	33	10	0.635	9	7	0.585

Table 3.10 The 4 Gamete Test Partitioned By Alga 1 Population

N is the number of individuals, "4 gametes" is the number of pairs of biallelic sites with 4 allele combinations, Rm is the minimum number of recombination events.

Population	n	4 gametes	Rm	Rm/n
British Columbia	36	6	1	0.17
Blue Mountains	14	4	1	0.29
Cascades	47	5	1	0.11
Coast	6	0	0	0.00
Sierra Nevada	15	0	0	0.00
Southern California	15	4	1	0.27
Western Rockies	23	4	1	0.17

Table 3.11 Exact Tests Of Linkage Disequilibrium Between Fungal And Algal Loci

Statistically significant results ($P < 0.05$) appear in boldface and indicate linked fungal-algal loci. N=213 thalli.

		Fungal Locus				
		ITS	IGS	BA	CS	EF
Algal Locus	ITS	**0.000**	0.594	1.000	0.083	**0.000**
	Actin	**0.000**	**0.000**	**0.000**	1.000	**0.000**

Table 3.12 Associations Between Fungal And Algal Loci

Associations between fungal and algal loci based on the number of haplotype combinations found in the data set compared with 10,000 randomized combinations. Statistically significant results (P<= 0.05) appear in boldface.

		Fungal Locus				
		ITS	IGS	BA	CS	EF
SampleSize		258	250	243	252	240
Algal Locus	ITS	0.501	0.130	0.370	0.320	**0.020**
	Actin	**0.002**	0.816	0.345	0.720	**0.037**

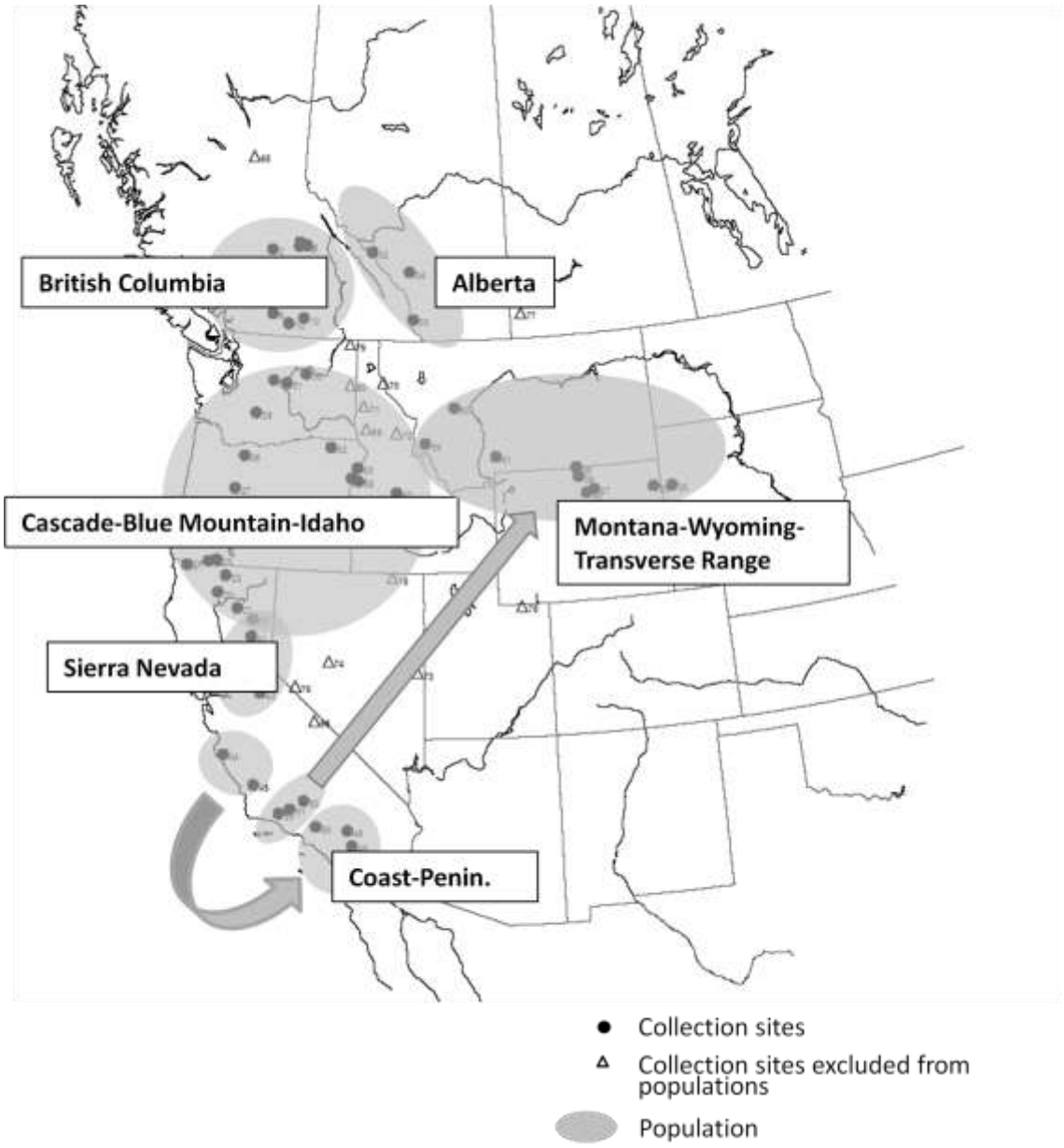


Figure 3.1 Six Fungal Populations Suggested By SAMOVA Analyses

Adapted SAMOVA analyses were used to identify clusters of genetically and geographically cohesive collection site groups. These populations are the basis for molecular diversity and linkage disequilibrium analyses.

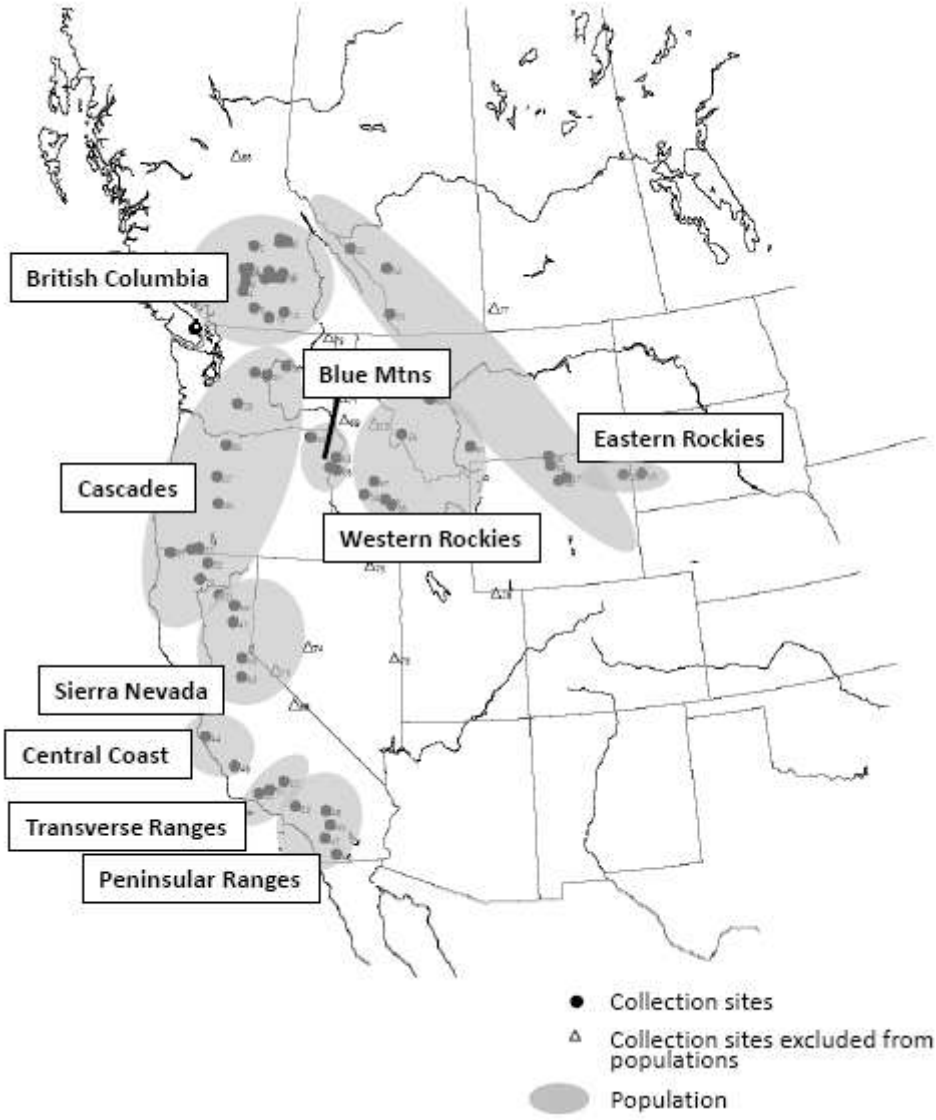


Figure 3.2 Nine Algal Populations Suggested By SAMOVA Analyses

SAMOVA analyses were adapted to identify clusters of genetically and geographically cohesive collection site groups.

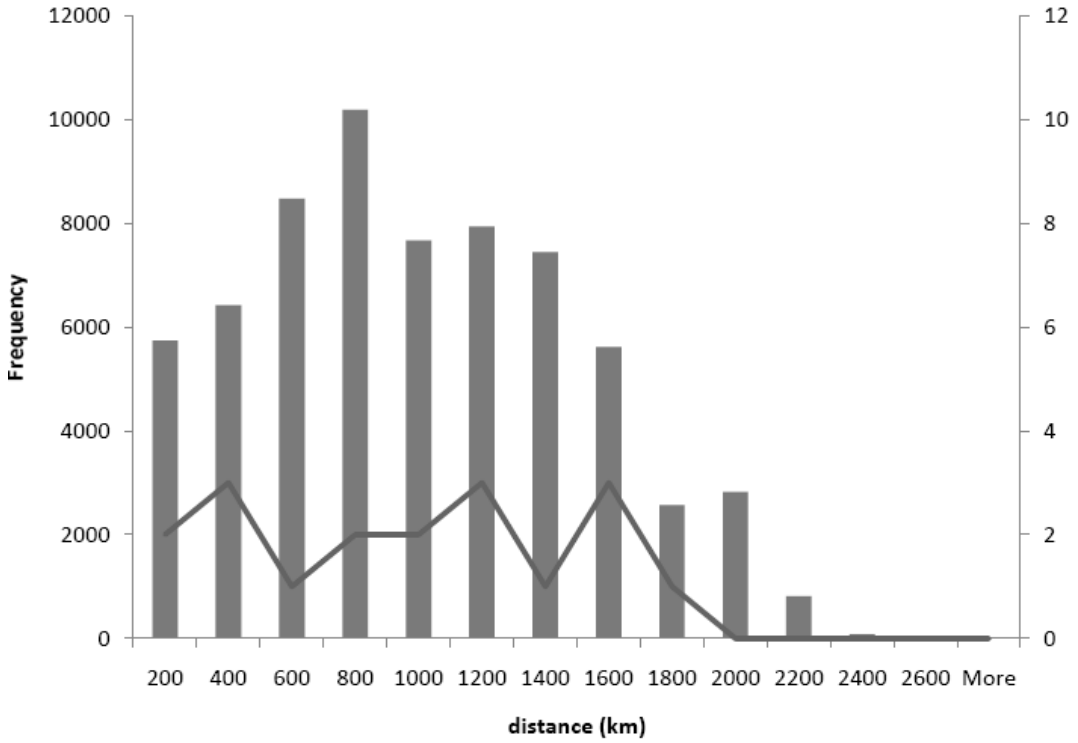


Figure 3.3 Frequency Distributions Of Pairwise Geographic Distances

Frequency distribution of pairwise distances between all collection sites (bars) versus frequency distribution of pairwise distances between all multi-locus fungal clones (line). Note that the left vertical axis corresponds with the bars and the right vertical axis corresponds with the line.

Figure 3.4 Comparison Of Fungal And Algal Populations

Mountain Wolf lichen algal populations (dotted lines) superimposed on fungal populations (grey ellipses). Both sets of populations were derived from a modified SAMOVA analysis. Arrows denote continuity between non-contiguous sub-populations.

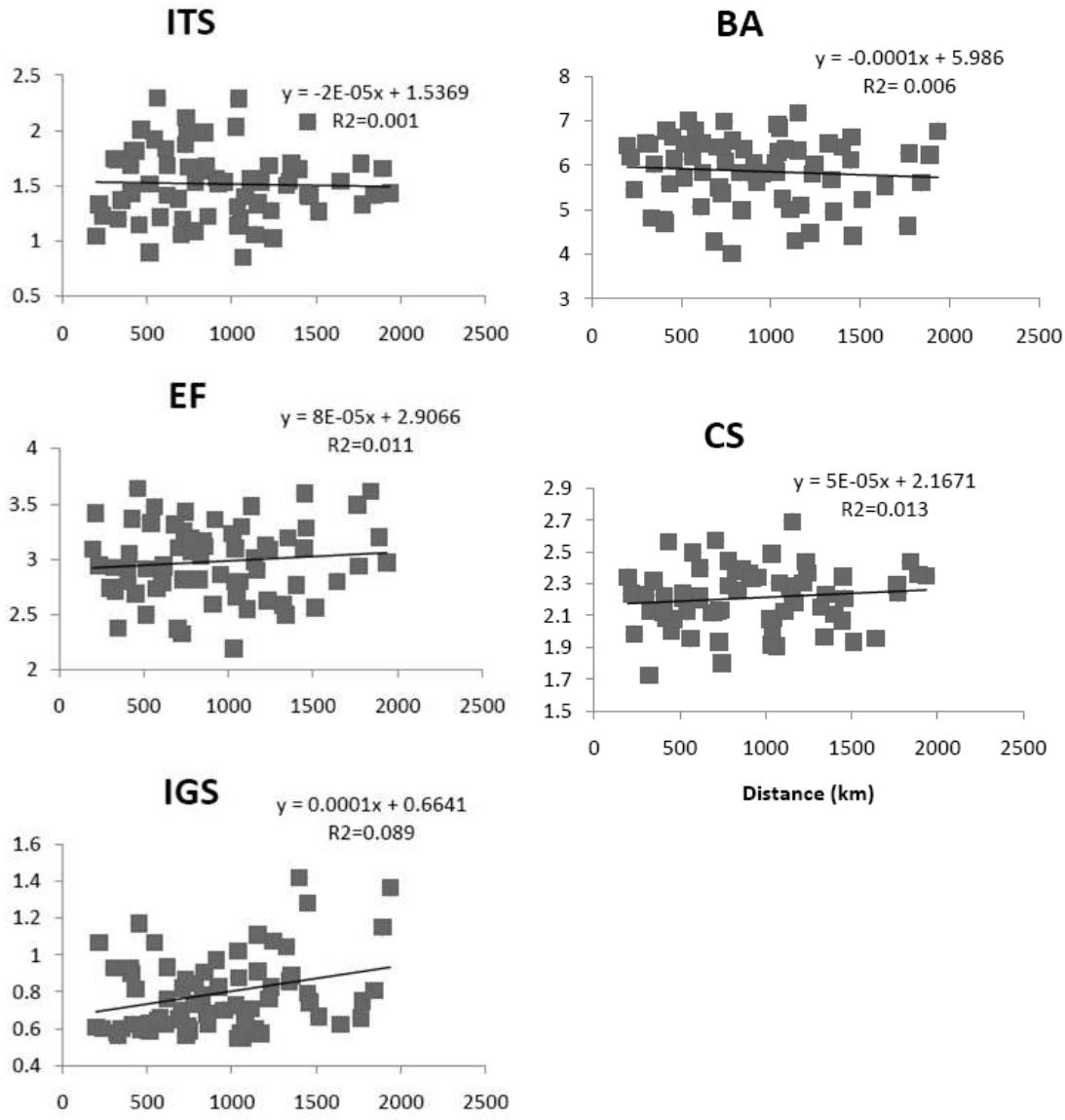


Figure 3.5 Mean Pairwise Distance Versus Geographic Distance

Geographic distances are between collection site groups for each fungal locus. Isolation by distance is not apparent.

DISSERTATION SUMMARY

The whole is greater than the sum of its parts. In this dissertation, I have asserted the primacy of the partnerships between symbiotic mutualists over an atomized taxonomic and phylogeographic understanding of the individual components. And in the course of the study, the "whole" has expanded to include the network of symbiotic relationships in which Mountain Wolf lichens are embedded. Below, I summarize the major points that relate to this gestalt perspective on a widespread symbiotic mutualism.

First, common names of lichens are employed in order to refer to the symbiotic partnership as a unit, as opposed to referring to the fungal taxon plus algal taxon. The classification and naming of taxa fundamentally organizes our thinking, and mutualistic symbioses suffer a disadvantage in the spheres of conservation, ecology and evolution because meaningful, enduring species interactions have no taxonomic currency. Common names are not wholly satisfactory in the naming of symbiotic mutualisms, but they are useful place holders for these important functional biological units.

Second, the description of a new fungal species is partly based on the mutually exclusive set of algal partnerships that the fungus has when compared with its most morphologically similar congener. The argument is made that phylogenetically independent taxa that form distinct symbioses deserve taxonomic recognition.

Third, the partnership patterns of a geographically widespread mutualistic symbiosis are mapped from the perspective of a single partner (the fungus *Letharia*

lupina). Like the lichen common names, maps become place holders that aid in our thinking about complex relationships. Such maps enable visualization of hidden geographic patterns in the symbiotic partnerships around us. What had appeared to be the same lichen from the Mexican border to Prince George, British Columbia, and from the coastal ranges of Oregon to the Black Hills of South Dakota, turns out to be a geographically variable network of fungal-algal partnerships. The constituents of the network as well as the number of taxa available in the network vary geographically.

Fourth, the geographic structure in the symbiosis is asymmetrical, with more geographic structure on the algal side of the symbiosis. There is also an asymmetry in the partnership specificity of the symbionts: *Letharia lupina* fungi are more specific than their algal partners. Where the fungus *L. lupina* pairs with multiple lineages within a single clade of closely related green algae, two of the algal lineages each partner with two unrelated genera of fungi (*Letharia* and *Hypogymnia*).

Fifth, vertical inheritance of symbiotic partners does not appear to shape the geographic structure of the symbiosis, at least not at large geographic scales. Sexual reproduction within each of the symbiotic partners as well as switching of partners provide ample bi-genomic genetic variability, and both likely contributed to the genetic uniqueness of every single symbiotic partnership analyzed in this study. The Mountain Wolf lichen symbiosis is perhaps best viewed as a system in which vertical inheritance is embedded within a network of labile fungal-algal partnerships.

Sixth, the analysis of symbiont geographic structure is placed into a larger comparative context of co-distributed plants and animals of Western North America.

There are parallels in the geographic structure of lichen symbionts and other diverse taxa, especially in Southern California and in the Rocky Mountains. This indicates that some post-Pleistocene phylogeographic patterns may be truly generalizeable across species, phyla and kingdoms.

Lastly, it is shown that the fungus *Hypogymnia imshaugii*, a sexually reproducing species that lacks a discrete mechanism for vertical symbiont inheritance, is able to pair with two distinct clades of *Letharia* lichen algae. Reproductive mode and phylogenetic range of algal partnerships may be related in such a way that strictly sexual fungi have the genetic diversity and ecological imperative to pair with diverse algae. The fate of strictly sexual fungi may be linked to that of certain other lichens, such as Mountain Wolf lichens, that seed the environment with abundant dual fungal-algal propagules. The full phylogenetic and geographic scale of linkages between unrelated taxa, through shared pools of symbiotic partners, is yet to be determined and is a ripe field for ecological investigation in symbiotic mutualisms.

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