# GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE POTENTIAL BIOCONTROL AGENT, *VALDENSINIA HETERODOXA,* AND ITS HOST *GAULTHERIA SHALLON* (SALAL)

by

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**ABSTRACT**

*Valdensinia heterodoxa* Peyronel is an ascomycete fungus currently being considered as a potential biocontrol agent for *Gaultheria shallon* Pursh. (salal). In order to design an effective biocontrol agent and to assess its effectiveness and risks, the population structure of both *V. heterodoxa* and salal must be investigated. Infected salal leaves were collected from three geographically separate populations on Vancouver Island and coastal mainland British Columbia. Uninfected salal was collected from two additional sites. *V. heterodoxa* was cultured from the infected leaves and single spore cultures were obtained prior to DNA isolation. Amplified fragment length polymorphisms (AFLPs) were used to generate individual DNA fingerprints for each isolate. Of the 214 loci analyzed, 30% were polymorphic, suggesting low genetic diversity. There were many shared haplotypes within each population, and as expected, analysis of pairwise kinship coefficients showed that as spatial distance increased, genetic similarity decreased. Analysis of molecular variance (AMOVA) between populations revealed significant genetic differentiation between populations with an $F_{ST}$ of 0.18, perhaps a result of limited gene flow.

Salal DNA was isolated from leaf tissue and AFLPs were used to fingerprint individuals resulting in 230 loci, which were 89.7% polymorphic on average. Within population diversity was high, with an average observed heterozygosity of 0.49. In addition, due to the high ploidy level of salal (octoploid), the results obtained from the dominant AFLP markers likely underestimate the actual genetic diversity in the populations. Populations were poorly differentiated ($F_{ST}$ 0.096), suggesting high gene flow among populations. Within one population (Shawnigan Lake), genetic similarity decreased with increased geographic distance and showed little evidence of clonal population structure.

When the genetic variation in *V. heterodoxa* was compared to that in salal, high correlations of alleles observed between the species suggest that different *V. heterodoxa* pathogenicity groups, or salal varieties with varying levels of susceptibility, could be contributing to the distribution of *V. heterodoxa* in these populations. Overall, the findings from the genetic analyses were used to discuss the potential risks of using *V. heterodoxa* as a biocontrol agent for salal and suggest that with low diversity and high population differentiation, the effectiveness of *V. heterodoxa* as a biocontrol may be limited to use within local salal populations or in combination with other control methods to effectively manage salal in forested areas.

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## CHAPTER 1. Introduction

*Gaultheria shallon* Pursh. (salal) is a densely growing perennial evergreen ericaceous shrub native to the Pacific Northwest, occurring from California to the panhandle of Alaska (Fraser *et al.* 1993). Salal is well suited to its habitat and can adapt to changing conditions by producing both light and shade leaves as well as reproducing sexually and apomictically by seed and vegetative reproduction (Fraser *et al.* 1993). Once established on a site, salal is thought to expand almost solely via vegetative reproduction using rhizomes as well as layering and suckering of roots in both canopied and open habitats (D'Anjou 1990, Fraser *et al.* 1993).

Salal is a predominant understory species, nearly continuous at mid to low elevations in coastal British Columbia forests (Fraser *et al.* 1990, Messier *et al.* 1990). In 1987, it was estimated that salal dominated ~100,000 ha of cedar-hemlock forests in coastal BC (Haeussler *et al.* 1990). In plots set up to compare the performance of tree species, it was found that over half the plots had more than 50% salal coverage. In the Western red cedar plots from this experiment, over 60% had greater than 75% salal coverage (Omule and Krumlik 1987). In areas where the overstory had been removed, Bunnell (1990) showed that after three years, 85% of the space that was available to salal had already been occupied by salal, which has serious economic impacts, as replanting in forested sites often occurs two-to-three growing seasons after harvesting.

For these reasons, salal has become a management problem in forested sites where it competes with conifer regeneration for light and resources. Experiments have shown that in these forest environments, salal competes mainly for water and nutrients resulting in increased tree growth when salal is removed and reduced conifer seedling growth when salal is present. For example, Messier (1992) showed that Sitka spruce

and western red cedar growth increased as the amount of salal decreased and Green (1990) found a 60% improvement in Douglas-fir seedlings when salal leaf area was reduced. Experiments eradicating salal from an area showed that after three growing seasons, spruce, hemlock, and cedar showed more growth when salal was removed than when it was present (Prescott *et al.* 1996). Interestingly, even the ericoid mycorrhizae associated with salal compete with the conifers and aid the establishment of salal. Prescott *et al.* (1996) determined that salal mycorrhizae inhibit the ectomycorrhizae of western hemlock and produce tannins and phenolic acids which interfere with nutrient uptake.

While salal may be considered a weed in forested areas, it also has many important roles in its ecosystems. For example, salal is food for many birds, animals, such as black-tailed deer, and microorganisms (Fraser *et al.* 1993). As well, it is a native plant, and a traditional staple in the lives of the aboriginal people of costal BC (Pojar and MacKinnon 1994). More recently, salal has been used in floral arrangements as greenery, and has generated increased demand for healthy, disease-free plants.

Much research has been focused on trying to determine a reliable, cost-effective method to control salal in order to enhance productivity of the crop trees (Prescott 1999). As of yet, none of the traditional control methods such as herbicide treatment, burning, manual brushing, or other means of scarification have provided an economical, reliable, practical, and ecological solution (D'Anjou 1990, Tirmenstein 1990). Biological control may provide an alternative to these traditional control methods.

A collaborative research venture has recently been established between the Canadian Forest Service – Pacific Forestry Centre (PFC) and Weyerhaeuser Canada Ltd. to develop *Valdensinia heterodoxa* Peyronel (Sclerotiniaceae) as a biocontrol for

salal (Shamoun *et al.* 2000). The imperfect, or asexual, state of *V. heterodoxa, Valdensia heterodoxa,* was first isolated from salal in British Columbia from a leaf spot disease by Redhead and Perrin (1972a, b). *V. heterodoxa* is an ascomycete fungus that exists primarily in its imperfect state, reproducing through large, asexual, star-shaped spores termed staurospores. The perfect, or sexual, stage of *V. heterodoxa* has also been observed in natural populations as apothecia growing from sclerotia (Redhead 1974). Since *V. heterodoxa* is a natural pathogen of salal in British Columbia, the inundative approach to biocontrol can be taken where large amounts of an indigenous pathogen are applied to the target plant, resulting in disease onset and suppression of the host (Wall *et al.* 1992). Included in this approach are mycoherbocides, formulations of a native fungus applied to the target plant in the same manner as chemical herbicides (Templeton *et al.* 1979). This is in contrast to the classical approach, which involves the introduction of an exotic species (Templeton *et al.* 1979, Wall *et al.* 1992).

Considering the many roles of salal in British Columbia forests, the goal of the biocontrol strategy is to suppress the growth of the target plant rather than to completely eradicate it from an area (Wall *el al.* 1992). At the center of discussions on biological control is the manipulation of an existing balance between plants and their pathogens, fueled by the continual evolution of resistance in the host and virulence in the pathogen. This balance is achieved through a reciprocal relationship, with feedback mechanisms in place to maintain a dynamic equilibrium among the host and pathogens, and between them and the abiotic environment (Cook and Baker 1983). In natural forest systems, it has been suggested that the community of pathogens control this relationship, determining plant distributions, landscape pattern, succession, and biodiversity (Castello *et. al.* 1995). However, in order for the pathogen to survive, some host plants

must also survive, and in response to attack by pathogens, plants develop resistance mechanisms. This alternation in the development of virulence and resistance phenotypes is a continual process of coevolution between the plant and its pathogens and ultimately prevents extinction of either (Cook and Baker 1983). In order to understand the balance observed in natural ecosystems and tease apart the evolutionary factors involved, it is essential to understand the population structure and genetic diversity of both the plant and pathogen populations as well as the relationships between resistance and virulence phenotypes in the species.

In Canada, before a mycoherbicide can be released it must conform with Canadian federal regulations, which require that information be obtained about the proposed biocontrol agent using the most up-to-date approaches and methodology (Anonymous 2001). There are also risks to consider before a biocontrol is released into the environment. One of the main risks is gene flow from the biocontrol agent to naturally occurring populations of the pathogen. Introducing a rare virulent gene into the environment could alter the balance of extant evolutionary processes, increasing the ability of local pathogen populations to colonize the host (Templeton *et al.* 1979, Hintz *et al.* 2001). It is also important to investigate the genetic diversity of the host to predict the effects of releasing large amounts of the pathogen especially with respect to host resistance and efficacy of the pathogen. The effects a particular biocontrol will have when applied to natural populations depend in part on the diversity and structure of the existing populations; key factors include the amount of homogeneity among individuals within a population and the rates of gene flow, mutation, and sexual reproduction in natural populations of both the host and pathogen (McDonald and McDermott 1993, Hintz *et al.* 2001).

To adhere to the Canadian guidelines and to understand the potential risks of developing *V. heterodoxa* as a biocontrol agent for the management of salal, this study investigates the genetic diversity and population structure of both species in natural populations on Vancouver Island and coastal mainland British Columbia. In addition, the results from this study will be used to discuss how the genetic variability in *V. heterodoxa* relates to that in salal, and the implications this may have for the use of *V. heterodoxa* as a biocontrol in the context of salal management. More generally, the results will provide basic information about the genetic variability of *V. heterodoxa* and salal populations in British Columbia, since no previous genetic studies have been done on these species.

The main objectives of this study were to:

1). Investigate the genetic diversity and population structure of *V. heterodoxa* within and among three natural populations (Chapter 2);

2). Investigate the genetic diversity of salal within and among five natural populations and assess the level of population structure within one natural population (Chapter 3); and,

3). Discuss the potential risks and efficacy of using *V. heterodoxa* as a biocontrol for salal, focusing on the pertinent evolutionary forces operating in these contrasting host-pathogen systems, and elucidating any patterns in the genetic variation between *V. heterodoxa* and salal (Chapter 4).

## CHAPTER 2. Genetic diversity and population structure of *Valdensinia heterodoxa*

### 2.1 LITERATURE REVIEW

**2.1.1. Life cycle and biology**. *Valdensinia heterodoxa* is a parasitic ascomycete fungus that causes necrotic leaf spot and defoliation of its hosts, which include many ericaceous species. The imperfect state of *Valdensinia heterodoxa*, *Valdensia heterodoxa*, primarily produces multi-celled, star-shaped, asexual macroconidia, termed staurospores, unique to this species (Redhead and Perrin 1972a, b). Rare secondary, single-celled, globular, asexual spores, called phialospores, have also been observed in *V. heterodoxa*, although only in the laboratory (Redhead and Perrin 1972a). Norvell and Redhead (1994) proposed that dispersal of *V. heterodoxa* is primarily achieved through the asexual staurospores. Spore discharge is facilitated by moisture, causing the appendages to swell and form a tear-dropped shape, which presses against the substrate (i.e. leaf) and propels the propagule up to a distance of 20 cm. Germination occurs radially, forming short appressoria (swollen hyphae) that adhere to the host and promote infection, visibly diagnosed by brown, concentrically ringed necrotic leaf spots (Redhead and Perrin 1972a, Norvell and Redhead 1994). Infecting conidia can be found at the center of the necrotic spots for several months. Mature infections are characterized by sclerotia that sclerotize the host veins, followed by leaf death and defoliation (Norvell and Redhead 1994).

The sexual stage of *V. heterodoxa,* is characterized by small, stalked, brown apothecia that arise from the sclerotized veins of fallen leaves. Apothecia are initially concave becoming plane or convex during maturation. The asci are inoperculate (i.e. have no cap or lid that opens to permit spore discharge, Kendrick 1992), cylindrical, thin-walled and tapered at the base. There are eight ascospores per ascus, usually

ellipsoid becoming septate with age. Formation of apothecia (and therefore sexual reproduction) is thought to be rare in nature as it has only been documented three times: in Italy by Peyronel on leaves of *Vaccinium myrtillus* L. in June 1952 (Redhead and Perrin 1972b), in British Columbia by Redhead in March and April of 1974 on *G. shallon*, and more recently in 1996 in Norway on *V. myrtillus* by Holst-Jensen.

An infection cycle has been proposed by Norvell and Redhead (1994). At the beginning of the season (March and April in British Columbia), primary infections are caused by ascospores. These infections then produce staurospores which spread *V. heterodoxa* to neighbouring leaves. Late infections are diagnosed by the presence of sclerotized veins and the absence of new staurospore propagules.

**2.1.2. Taxonomy of *V. heterodoxa*.** The name *V. heterodoxa* originated in Italy in 1923 by Peyronel as the causal agent of a leaf-spot disease on *Vaccinium myrtillus*. Previously, it had been named *Gloeosporium myrtilli* by Allescher in 1897, although subsequent analysis of his cultures determined that *Valdensinia* was the correct genus name due to the characteristic stellate spores (Redhead 1972b). The name was then changed to *Valdensinia myrtilli* with the synonym *V. heterodoxa*. Currently, the accepted name is *V. heterodoxa* Peyr. In British Columbia, *V. heterodoxa* was first named *Asterobolus gaultheriae* Redhead and Perrin in 1972 by Redhead and Perrin (1972a) but once they discovered the preexisting nomenclature, the accepted name was adopted (Redhead and Perrin 1972a, b).

*V. heterodoxa* is an ascomycete fungus in the order Helotiales, and family Sclerotiniaceae, which includes many plant pathogens (Holst-Jensen *et al.* 1997). Phylogeny in the ascomycetes is uncertain due to rapid evolution and lack of reliable morphological or developmental features among the larger clades. *V. heterodoxa* formed a single monophyletic group within the Sclerotiniaceae based on the large-

subunit rDNA sequences (Holst-Jensen *et al.* 1997). When the complete set of rDNA sequences was used, the closest relatives to *V. heterodoxa* were members from four other genera: *Pycnopeziza*, *Stromatinia*, *Ovulinia*, and *Coprotinia*. Interestingly, the two most closely related species based on sequence data, *P. sympodialis* White and Whetzel and *O. azaleae* Weiss, are also plant pathogens of the Ericaceae family (Schumacher 1990, Whetzel 1945).

**2.1.3. *V. heterodoxa* Host Range.** *V. heterodoxa* can infect many different host species and has a wide geographic distribution, identified on at least 33 plant species from 15 plant families in Europe and 17 plant species in North America (Redhead 1979, see Table 2.1). It is a common pathogen of *Vaccinium myrtillus* L. (bilberry) in Italy and Norway, where it significantly influences bilberry coverage in the boreal forests (Aamlid 2000). In Canada, *V. heterodoxa* has predominantly been isolated from salal in British Columbia, *Vaccinium myrtilloides* Michx. (sour-top blueberry) and *V. angustifolium* Ait. (lowbush blueberry) in Quebec (Redhead 1974, 1979), and from *V. ovalifolium* J. E. Sm. (oval-leafed blueberry) in Newfoundland (Parmelee 1988). *V. heterodoxa* has also been isolated from several other plant species including some ferns (Table 1, Ginns 1986). In the United States, *V. heterodoxa* has been observed on salal, *V. alaskaense* Howell (Alaskan blueberry) and *V. membranaceum* Douglas (mountain huckleberry) in Washington, Oregon, and Idaho (Norvell and Redhead 1994).

The most common hosts of *V. heterodoxa* include salal and various *Vaccinium* species such as Alaskan blueberry, oval-leafed blueberry, black huckleberry, and bilberry, which are all shrubs that prefer moist to wet conditions and grow at low to subalpine conditions. The leaves of these host species share several similarities such as an alternate leaf pattern and finely toothed leaves, although the thickness of the leaves varies. For example, salal leaves (particularly mature sun leaves) are thick and

leathery, while black huckleberry leaves are thin. One major difference between the hosts is that salal is evergreen while the *Vaccinium* species are deciduous (Pojar and Mackinnon 1994).

Table 2.1. Host species of *V. heterodoxa* in Canada*.

| Host Species | Common Name | Family | Province Isolated From |
|---|---|---|---|
| *Achlys triphylla* | Vanilla leaf | Berberidaceae | British Columbia |
| *Acer macrophyllum* | Big leaf maple | Aceraceae | British Columbia |
| *Diervilla duham* | Honeysuckle | Caprifoliaceae | Quebec |
| *Dryopteris austriaeca* | Spinulose woodfern | Polypodiaceae | British Columbia |
| *Gaultheria shallon* | Salal | Ericaceae | British Columbia |
| *Malus fusca* | Pacific crabapple | Rosaceae | British Columbia |
| *Menziesia ferruginia* | False azalea | Ericaceae | British Columbia |
| *Polystichum munitum* | Swordfern | Polypodiaceae | British Columbia |
| *Pteridium aquilinium* | Bracken | Polypodiaceae | British Columbia |
| *Rubus parviflorus* | Thimbleberry | Rosaceae | British Columbia |
| *R. pedatus* | Trailing raspberry | Rosaceae | British Columbia |
| *R. spectabilis* | Salmonberry | Rosaceae | British Columbia |
| *R. ursinus* | Pacific dewberry | Rosaceae | British Columbia |
| *Vaccinium alaskaense* | Alaskan blueberry | Ericaceae | British Columbia |
| *V. membranaceum* | Black huckleberry | Ericaceae | British Columbia |
| *V. angustifolium* | Lowbush blueberry | Ericaceae | Quebec |
| *V. myrtilloides* | Sour-top blueberry | Ericaceae | Quebec |
| *V. ovalifolium* | Oval-leafed blueberry | Ericaceae | British Columbia, Newfoundland |
| *V. parvifolium* | Red bilberry | Ericaceae | British Columbia |

* All data is from Ginns (1986), except for information on *V. ovalifolium* in Newfoundland, from Parmelee (1988), and *A. triphylla* and *R. spectabilis* (S. Shamoun, personal communication.).

**2.1.4. Fungal host infection mechanisms.** Considering the many common features of the host plants, they may share some important characteristics that enables *V. heterodoxa* to infect them, such as susceptibility, tolerance, or lack of various resistance genes. Environmental factors such as moisture could also limit the range of *V. heterodoxa* (Norvell and Redhead 1994). No published information is available on the mechanisms of infection and variability of virulence in *V. heterodoxa*; however, work is currently being conducted at the PFC in Victoria, BC. While very little is known about the specific molecular interactions between *V. heterodoxa* and its hosts, comparison to other species in the Sclerotiniaceae (Kendrick 1992, Vidhyasekaran 1997) suggests

that *V. heterodoxa* could produce a range of enzymes, toxins, and possibly suppressors, which enable it to disable or avoid host defenses.

**2.1.5. Population genetics of plant pathogenic fungi**. In the last ten years there has been considerable research on the population genetics of plant pathogenic fungi. This has been due in part to the increased availability of molecular techniques suitable for plant pathogens and also to the shift in agriculture from traditional resistance breeding to population genetic studies and marker-assisted selection to gain a broader understanding of pathogen populations and how they will evolve and respond to changes in the host and different control strategies (McDonald 1997). The following is an overview of the common genetic markers used in genetic diversity studies and the major genetic factors involved in establishing and maintaining pathogen populations, with a focus on predominantly clonal, ascomycete fungi pertinent to *V. heterodoxa*.

*Genetic markers*. Genetic markers are used to quantify the genetic variation present in a population and the type of marker chosen can greatly affect the type of information obtained and the analyses that can be performed. Genetic markers can be separated into two general groups: those that are under selection, such as pathogenicity and resistance phenotypes, and those that are considered selectively neutral. The former are highly relevant to breeding systems and are more likely to provide biased estimates of the potential for genetic change due to selection, and the results can be confused by linkage disequilibrium between loci and hitchhiking alleles (McDonald and McDermott 1993). Selectively neutral markers are typically chosen for population genetic studies as they are the most suitable for answering questions about overall diversity, population structure, and gene flow. When choosing a marker system, markers should be selectively neutral, highly informative, reproducible, technically simple, and cost efficient (McDonald 1997). There are many reviews that discuss the

advantages and disadvantages of various genetic markers in plant pathogen population genetics (e.g. Burdon 1993, McDonald and McDermott 1993, Weising *et al.* 1995, Brown 1996). Rather than discussing how each technique works, this review will include the characteristics of some genetic markers that can be used and the method that was chosen for this study.

Ideally, codominant markers in which heterozygous individuals can be distinguished from homozygous individuals should be used, such as allozyme variation in isozymes. However, because they are functional enzymes, some loci may be under selection and the amount of variation observed is limited. In fungi, it has been reported that allozyme variation is low within and among geographically isolated regions (Burdon 1993), which makes them unsuitable for studies comparing individuals within a geographic region (McDonald and McDermott 1993).

Restriction fragment length polymorphisms (RFLPs) are also codominant markers. While they are generally more variable than allozymes as they survey both coding and noncoding regions of DNA, they are more technically difficult and expensive to perform (McDonald and McDermott 1993). As well, RFLPs are most informative when controlled crosses can be done and parents are known (Milgroom 1995), which is not readily feasible for *V. heterodoxa* since sexual structures and spores have not been produced in the laboratory and are rarely observed in nature.

Microsatellites (SSRs) are another codominant marker becoming increasingly popular in molecular studies. These markers are often highly polymorphic and informative as SSRs are usually in highly variable regions of the genome. However, the primers are mainly species-specific, so unless they have been previously developed, SSR markers are often too expensive as primers must be developed and genomic

libraries sequenced (Mueller and Wolfenbarger 1999). This technique is just beginning to be used for pathogenic fungi (Tenzer *et al.* 1999).

Conversely, dominant markers can also be used to study genetic diversity; however, with dominant markers, heterozygotes cannot be distinguished from dominant homozygotes, which is often considered a drawback. For haploid fungi like *V. heterodoxa*, each individual has only one allele per locus, so there are no heterozygotes (individuals either have the marker or not) and allele frequencies can be directly determined from presence or absence of an allele at each locus (McDonald 1997) so dominance is not an issue. One dominant technique is random amplified polymorphic DNA (RAPD) markers, which uses short (ten base pair) primers that amplify arbitrary regions throughout the genome, requiring no prior sequence knowledge (Williams *et al.* 1990). RAPD markers are usually highly variable, easy to use, and inexpensive; however, they also have many drawbacks, which include difficulties in reproducibility and relatively few loci per primer (McDonald 1997).

The other popular dominant genetic marker technique uses amplified fragment length polymorphisms (AFLPs) to genotype individuals (Vos *et al.* 1995). In this case, retriction enzymes are used to digest the DNA and long primers are used to amplify the fragments. As a result, AFLPs also require no prior sequence knowledge and are much more reliable and reproducible than RAPDs. Although AFLPs are technically more challenging and expensive, a large number of loci are generated from each primer set so fewer primers are needed compared to RAPDs (McDonald 1997, Mueller and Wolfenbarger 1999).

Several factors were important in choosing a molecular marker for *V. heterodoxa*. Based on previous research *V. heterodoxa* was expected to have predominantly clonal reproduction (Redhead and Perrin 1972), so the markers needed to be highly variable in

order to detect polymorphisms. There has been no other population-based genetic work done on *V. heterodoxa* or any of its relatives, so there were no previously developed microsatellite markers. As it has haploid mycelia, dominant markers would not obscure heterozygosity, so AFLPs were chosen as a reproducible and reliable method to generate many loci that could be used for determining genetic variation.

*Genetic diversity.* The focus of genetic diversity research on plant pathogens is to determine and assess the likelihood of the pathogen to evolve in response to changes in the host or environment, and to determine factors influencing populations. These can be used to control the pathogen or to develop an efficient and safe biocontrol. Genetic diversity can be assessed using markers and genetic structure determined. Two different types of genetic diversity contribute to genetic structure and are important to distinguish when populations undergo sexual and asexual reproduction. Gene diversity refers to the diversity at individual loci and is determined by the number and frequency of alleles. Genotype diversity (haplotype diversity in haploids) refers to the frequency of unique genotypes or genetically distinct individuals. It is particularly relevant for clonal reproduction since there will be fewer genotypes than in a sexual population, even though both the asexual and sexual populations could have the same number of genes and allele frequencies (Milgroom 1996, McDonald 1997). Diversity can also be used to infer the age of the pathogen population and whether it was introduced or native. For example, low diversity measures in a fungus that reproduces sexually can indicate a recent introduction of the pathogen to the area (Wang and Szmidt 2000).

Mating system and mode of reproduction, as well as population size, can greatly affect the diversity and population structure of a species. Evolutionary forces that affect population genetic diversity and structure include: genetic drift, mutation, gene flow

(referred to as migration among populations and dispersal within populations), and natural selection (McDonald and Linde 2002). These forces act individually and in concert to determine the evolutionary potential of populations and therefore species. They can also be used to predict how a species may respond to changes in its environment and are important to assess in considering the potential of a plant pathogen for biocontrol of a specific host.

*Clonal population structure.* Most ascomycete fungi produce haploid asexual spores and have predominantly clonal populations (Taylor *et al.* 1999). In clonal populations the offspring is identical to the parent except in the event of a mutation, and is a product of asexual reproduction or selfing. Mutations may create new, more virulent alleles, which, in the presence of selection, may increase in frequency in the population disrupting the present equilibrium of resistance and virulence (McDonald and Linde 2002); however, they can also increase the number of deleterious alleles in the pathogen population over time reducing its overall fitness (Taylor *et al.* 1999). Without recombination to remove deleterious alleles and shuffle genotypes, it is thought that strictly clonal populations will eventually go extinct, due to the lethal combination of deleterious alleles and drift (Kohn 1995). It was predicted by Lynch and Gabriel (1990) that the maximum persistance of clonal populations is $10^4$ to $10^5$ generations. Clonal populations often have small effective population sizes, increasing the relative impacts of genetic drift and population bottlenecks due to extinction. Assuming finite populations, genetic drift will eventually result in allelic loss or fixation, decreasing heterozygosity and reducing evolutionary potential (McDonald and Linde 2002).

Selection also plays an essential role in shaping pathogen populations, particularly for virulence and resistance alleles. Natural selection can alter the frequency of certain genotypes within a population, resulting in local adaptation.

Selection can also act to maintain variation in the population by favouring novel genotypes introduced via gene flow, recombination, and mutation (Ennos and McConnell 1995). The balance between local adaptation and the homogenizing effect of gene flow between populations is important when considering the potential of a population to respond to the introduction of novel genotypes, for example with introduction of a biocontrol.

*Population differentiation.* Gene flow increases the level of diversity within a population by distributing haplotypes uniformly throughout the population. Gene flow between populations (referred to as migration), spreads haplotypes or alleles over a larger geographic scale, reducing the amount of differentiation between populations and disrupting local adaptation (Neigel 1997). Populations with high levels of gene flow are expected to have greater genetic diversity and increased effective population size. In asexual populations, gene flow facilitates exchange of genotypes among populations, which in some cases could be detrimental if they introduce linked, co-adapted alleles that have been selected for in a different environment (McDonald and Linde 2002). There are many methods to estimate gene flow in plant pathogens (reviewed by Neigel 1997) where McDermott and McDonald (1993) proposed that gene flow detected from neutral genetic markers may provide the best indication of the overall success of plant pathogen populations.

Analysis of gene flow can be further extended to determine if the level of differentiation among populations is correlated with geographical distance (Wright 1943, Slatkin 1993). Clonal asexual species typically display more spatial structure than sexually reproducing species (Wang and Szmidt 2000). If a genetic distance measure is correlated with geographic distance, it suggests that closer populations exchange more migrants. For plant pathogens, the presence of isolation by distance can indicate

the likelihood of virulence genes spreading across populations, and whether or not quarantine measures will be effective in controlling the dispersal of the pathogen (McDonald 1997).

**2.1.6. Research objectives**. There were three main objectives for studying the population genetics of *V. heterodoxa*: 1) to investigate the level of genetic diversity in coastal British Columbia populations, 2) to determine the population genetic structure and assess the evolutionary forces affecting these populations, and 3) to assess the level of population differentiation and discuss how it relates to gene flow and geographic distance. The results from these investigations will provide genetic information on natural populations of *V. heterodoxa*, and will be used in Chapter 4 to evaluate the potential of using *V. heterodoxa* as a biocontrol for the management of salal.

## 2.2. MATERIALS AND METHODS

**2.2.1. Site description**. Preliminary fieldwork was done to locate populations of salal that were severely infected by *V. heterodoxa* based on suggestions from R. Countess (pers. comm.). Three different sites were selected for this study. All samples collected from one site were considered a population. *V. heterodoxa* and salal were collected from Mesachie Lake and Port Hardy on Vancouver island, and Deak's Peak on the west coast of mainland British Columbia (Figure 2.1).

The Mesachie Lake site is adjacent to a recent TimberWest clearcut. The site was shady with filtered light from second growth trees. Salal was fairly ubiquitous throughout both the forested and clearcut areas. *V. heterodoxa* infections were found in shady, protected microsites. The Port Hardy site was 2 km along the Tex Lyon trail, south of Port Hardy. The site is mainly flat to slightly rolling at sea level and is dominated by western red cedar and western hemlock. Salal was found throughout the forest and *V. heterodoxa* was scattered over large areas. Most of the trail was shaded, although offshore exposure varied. The Deak's Peak site is just off the Howe Sound Crest Trail, beginning at Highway 99 north of Lion's Bay. The site was relatively flat and at a higher elevation than the other two populations. It was predominantly shaded although some spots had more direct light exposure. The most extensive *V. heterodoxa* infections were observed at this site.

**2.2.2. Sample collection**. Due to the patchy distribution of *V. heterodoxa* at these sites, moving along a transect, samples of infected salal leaves and uninfected salal leaves from each of five salal stems were collected within a one meter radius in infection centres (patches, Figure 2.2). Two to three infected and uninfected salal leaves were collected from each stem. Salal stems sampled were at least 20 cm apart, greater than the documented spore dispersal distance for *V. heterodoxa* to ensure that

different individuals were sampled (Redhead and Perrin 1972a). Between infected patches, one sample of healthy salal was also collected. For each population, ten patches of infected salal were sampled and within each patch, five different salal stems were sampled, resulting in a total of 50 samples each of infected and uninfected salal leaves and ten samples of healthy salal from between patches (Figure 2.2). Infected leaf samples were stored in individual paper bags to prevent spores from spreading and to prevent mould and other secondary pathogens from growing. In the field, all samples were kept on ice and then in a 4° refrigerator.

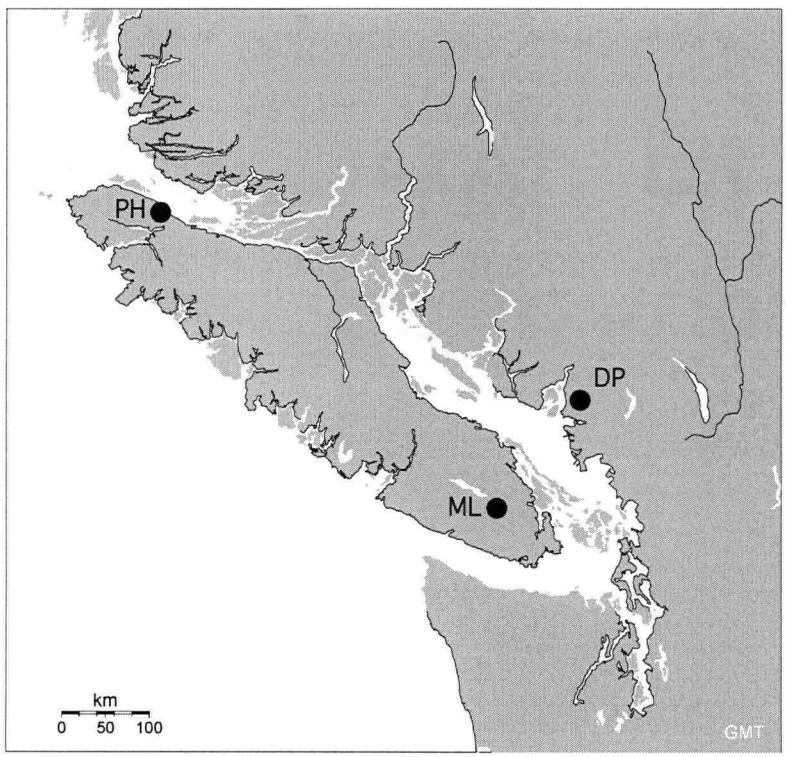


Figure 2.1. Map of Vancouver Island and mainland British Columbia showing locations of the three *V. heterodoxa* populations sampled for this study including Mesachie Lake (ML), Port Hardy (PH), and Deak's Peak (DP).

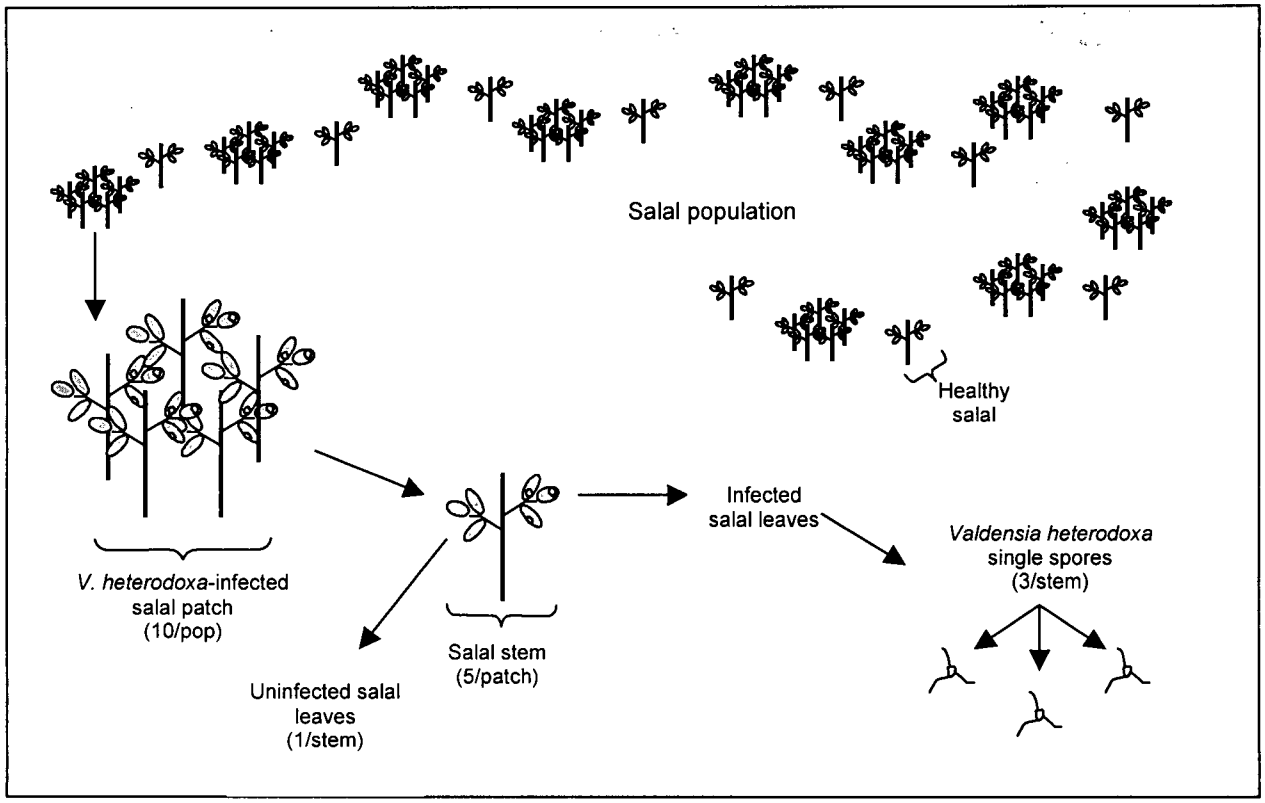


Figure 2.2. Diagram of *V. heterodoxa* and salal sampling scheme. The same scheme was used in each of the three populations sampled, showing that from each salal stem, samples of infected and uninfected salal leaves were collected and that three *V. heterodoxa* individuals, single asexual spores, were isolated from each sample of infected leaves.

**2.2.3. Fungal cultures**. *V. heterodoxa* was isolated from the infected leaves and cultured to confirm its identity and obtain material that could be used to isolate DNA. Leaf pieces were cut from the edges of the lesions, surface sterilized, and incubated on rich solid medium to allow the fungus to grow. For surface sterilization, the leaf pieces were submerged in 95% ethanol for 45 seconds, transferred to 10% bleach for an additional 45 seconds, and then transferred through three successive rinses of sterile distilled water for one minute each. The leaf pieces were briefly dried on autoclaved filter paper and then placed onto potato dextrose agar (PDA, 39 g PDA/L dH2O, Difco, Becton Dickinson, MD, USA) plates with sterilized tweezers. Three plant pieces were placed on each 9 cm Petri-plate, and three plates were made for each sample. The plates were incubated at 20°C in the dark for at least five days until *V.*

*heterodoxa* growth was observed. Scalpels were then used to cut out agar plugs from the growing edge of the fungus which were then transferred to salal-PDA (SPDA, 39 g/L PDA in 15 g/L salal extract [15 g salal leaves, pureed in blender with dH2O, filtered through cheesecloth to remove tissue, final volume adjusted to 1 L with dH2O]) plates. If further secondary pathogens persisted on these plates, then *V. heterodoxa* plugs were transferred to new SPDA plates to further isolate the *V. heterodoxa*. Cultures were incubated at 20°C in the dark until sufficient *V. heterodoxa* growth was observed in order to inoculate sporulation plates.

Once putative *V. heterodoxa* cultures had been attained showing typical discolouration of the media and slow-growing white mycelium, sporulation was induced by growing the cultures on weak-oatmeal agar (WOA, 15 g/L oatmeal agar, 12 g/L agar, Sigma Aldrich, MO, USA), (Vogelgsang and Shamoun 2002). As cultures sporulated, spore discharge plates were set up as described by Vogelsang (personal communication), such that sporulating cultures would discharge spores which would stick to the WOA media on the top of the plate. However, this did not occur so a dissecting microscope was used and single spores were transferred from the sporulating WOA cultures to SPDA plates. Up to five single spores were transferred, paying special attention to spore morphology and selecting the most isolated spores, removing any mycelium. Germination of these single spores on SPDA was very successful, and from these, hyphal tips were cut out of the agar and transferred to individual plates. For each culture that sporulated, three single spores were used to establish the individuals used in this study. Each single spore isolate was considered an individual, so there were effectively three individuals obtained from each infected leaf sample collected in the field. Since 50 infected leaf samples were collected from a population, this would result in 150 *V. heterodoxa* individuals per population. However,

not all samples collected sporulated, so the actual number of individuals obtained per population was much less than expected. Choosing three single spore isolates per sample was a way to increase sample size and to study localized *V. heterodoxa* diversity.

**2.2.4. Fungal mycelium preparation and DNA isolation**. Isolating DNA from *V. heterodoxa* was extremely challenging and required experimenting with many different culturing methods to obtain mycelia and many different DNA isolation protocols. Liquid culture and subsequent isolation procedures resulted in large, gel-like, clear DNA pellets with high levels of polysaccharide contamination, and young colonies with less polysaccharides did not have enough material due to slow fungal growth. This problem is not uncommon in fungal DNA isolation, particularly with members of the Sclerotiniaceae. Minimizing agitation and growing cultures as short a time as possible can reduce polysaccharide production (L. Kohn, pers. comm.). The most effective way to generate mycelia for use in DNA isolation, was to grow the cultures on cellophane laid overtop of SPDA plates, similar to the protocol used by Nielsen *et al.* (2001) and Arroyo Garcia *et al.* (2002). Semi-permeable cellophane (BioRad, Hercules, CA, USA) was cut into squares and autoclaved prior to being placed overtop SPDA plates using 500 μl of sterile dH2O to help the cellophane adhere to the plates. The plates were then dried and stored for at least 24 hours prior to use to ensure that no contaminants had been introduced. Plugs of the single spore isolates were then inoculated into the center of these plates and incubated in the dark at 20°C. This enabled the fungus to extract resources from the media but limited its growth to the top of the cellophane so mycelia could easily be harvested. The mycelial layer was very thin, dry, and papery. The fungus grew faster on SPDA than PDA, so that mycelia could be harvested after

five days. 10-40 mg was transferred to a 1.5 ml Eppendorf tube and immediately frozen in liquid nitrogen prior to storage at –80°C.

It was very difficult to isolate DNA from *V. heterodoxa*. Many different isolation protocols were tested and modified, including: Zolan and Pukilla (1986), Doyle and Doyle (1987), and Weising *et al.* (1995). In addition to these CTAB-based isolation protocols, variations of the DNeasy Plant Mini Kit (Qiagen Inc, Valencia, CA, USA) were tested. Unfortunately none of these methods, or other post-isolation clean-up methods, consistently yielded DNA preparations with low polysaccharide contamination. Success was finally achieved with a modified Zolan and Pukilla (1986) isolation protocol that had been successfully used for Sclerotineacous fungi (Holst-Jensen *et al.* 1997, S. Shamoun pers, comm..., and T. Schumacher, pers. comm.).

Optimizing this protocol for *V. heterodoxa* resulted in a fast, efficient, and consistent method yielding high quality DNA with low contamination. Liquid nitrogen was added to the 1.5 ml Eppendorf tubes containing approximately 20 mg of harvested mycelia and a micropestle was used to lightly crush the mycelia once. CTAB isolation buffer was then added to each sample (600 µl of: 2.5% hexadecyltrimethyl-amonium bromide (CTAB), 1% PVP-40, 1.4 M NaCl, 0.02 M EDTA, 0.1 M Tris-HCl, 0.5% β-mercaptoethanol, Sigma Aldrich, Oakville, ON, Canada) and the tubes were vortexed for 15 sec. Samples were then incubated for 30 minutes at 60°C. An equal volume of CHH-IAA (24:1 chloroform:isoamyl alcohol) was added. Tubes were vortexed until the two solutions were mixed and then spun at room temperature for 5 minutes at 12000 rpm to separate the layers. Approximately 450 µl of the top aqueous layer was transferred to a new Eppendorf tube using a cutoff pipette tip taking care to not disturb the middle protein layer. An equal volume of ice-cold isopropanol was then added and

mixed by inversion and spun at maximum speed for 1 minute to precipitate the DNA. The supernatant was pipetted off trying to remove as much liquid as possible from the diffuse pellet as the pellet was often associated with a viscous liquid partially suspended in the supernatant (most likely polysaccharides), such that the pellet was lost if the supernatant was poured off. The pellet was then resuspended in 300 μl TE buffer (10 mM Tris-CL, pH 8.0, and 1 mM EDTA).

RNA was digested by adding 3 μl of RNase A (10 mg/ml, Sigma) and incubating the tubes for 30 min. at room temperature. The chloroform extraction was then repeated, using 305 μl CHH-IAA. Using a cutoff pipette tip, 250 μl of the aqueous layer was removed and transferred to a new 1.5 ml Eppendorf tube. Next, 100 μl of 5 M ammonium acetate was added followed by 725 μl of ice-cold 95% ethanol. The tubes were mixed by inversion and spun at high speed for one minute to pellet the DNA. The pellets were very clear and large so the supernatant was very carefully poured off and pellets were rinsed for 5 minutes in 500 μl ice-cold 70% ethanol. The supernatant was then poured off leaving a small pellet, which was air-dried in the flow hood (or dried under vacuum for wetter pellets) and then resuspended overnight in 40 μl TE at 4°C. Prior to storing the samples at –20°C, the concentration and quality of the samples was checked using agarose gel electrophoresis by adding 2.5 μl sample to 1.2% agarose gels in 1X TBE buffer using λ/HindIII as the molecular weight marker and staining in ethidium bromide, viewed under UV light. Isolations were repeated for any samples of very low concentration or moderate contamination.

**2.2.5. AFLP analysis**. To find primers for *V. heterodoxa*, the literature was surveyed for ascomycete fungi AFLP primers (i.e. Majer *et al.* 1996, Gonzalez *et al.* 1998, and Cilliers *et al.* 2000). A list of primers was generated and tested on *V.*

*heterodoxa*; primers producing clear banding patterns and polymorphism were selected for use. To ensure reproducibility, each *V. heterodoxa* sample was analyzed twice and only highly repeatable loci were included in the analysis. For *V. heterodoxa* samples, 5 μl of total DNA was used in each AFLP reaction. The DNA was digested with EcoRI and MseI and AFLP was performed following the protocol outlined by C. Ritland (GDC, pers. comm.). The final PCR amplification was performed with +2 EcoRI/+3 MseI primers. Four different primer combinations were used as follows where the first primer refers to EcoRI and the second refers to MseI: AC/CAC, AC/CAG, AG/CAC, and AG/CAG. The LI-COR gel-imaging system was used to electrophorese PCR-generated fragments (LI-COR Inc., Lincoln, NE, USA). Gels were scored using SAGA-MX for AFLP bands (LI-COR Inc.). Bands were scored as present or absent and in two cases, samples had highly differentiated banding patterns and did not share the same monomorphic loci as all the other samples. These two samples were excluded from analysis..

**2.2.6. Data analysis**. *V. heterodoxa* mycelia and asexual spores are haploid, so allele frequencies can be estimated directly (McDermott and McDonald 1993). The haploid AFLP fingerprints were analyzed in programs such as Arlequin Version 2.000 (Schneider *et al.* 2000) and SPAGeDi Version 1.1 (Hardy and Vekemans 2002). In order to compare fingerprints between gels and among populations, it was assumed that comigrating fragments of the same size were identical in sequence.

Pearson correlations from principal components analysis (PCA) in SAS Version 8.0 was used to select only those loci that contributed to the observed variation. Allele frequencies were then calculated for each locus as the frequency of the present allele over all samples. Average gene diversity (the probability that two randomly chosen individuals have different alleles), equivalent to expected heterozygosity, was

determined correcting for sample size (Schneider *et al.* 2000). In clonal populations it is also useful to measure the genotypic diversity, or number of genetically distinct individuals in a population (McDonald 1997). Genotype diversity is equivalent to haplotype diversity in this species and is the number of unique haplotypes relative to the number of individuals in each population. Haplotype diversity was used to asses diversity both within and between *V. heterodoxa* populations, and also on a smaller scale, to indicate the level of diversity among single spore isolates from a *V. heterodoxa* sample.

The distribution of haplotypes and linkage disequilibrium analysis were used to assess the level of clonality. Linkage disequilibrium analysis between pairs of loci was performed in Arlequin for each population separately and for pooled samples. The number of pairwise comparisons was calculated as n(n-1)/2, where n is the number of loci. To establish clonal population structure, the null hypothesis of random association of loci must be rejected for the majority of loci pairs (Kohli and Kohn 1998) based on a modification of Fisher's exact probability test with Bonferroni's correction for multiple comparisons. Clonal population structure may be falsely detected if recombination is masked by the sampling scheme. As a result, clone-corrected data sets, where each haplotype was represented only once, were used to control for this.

The mean number of observed and expected pairwise differences between haplotypes was measured using Arlequin, where the expected difference was calculated as $\pi = \sum_{i=1}^{k} \sum_{j<i} p_i p_j d_{ij}$ where *p* refers to the frequency of each haplotype in the population, and *d* is an estimate of the number of mutations that has occurred since divergence of the two haplotypes. The variance is calculated assuming no recombination between sites and selective neutrality (Schneider *et al.* 2000). The

number of pairs is n(n-1)/2 where n is the number of haplotypes in each population. Observed and expected pairwise differences were compared where significant p-values indicate that recombination has occurred in these populations as more differences between haplotypes were observed than expected from mutation alone.

Analysis of molecular variance (AMOVA), which partitions the observed variation, was used to investigate the genetic structure of the *V. heterodoxa* populations (Excoffier *et al.* 1992) using Arlequin. Comparisons were made among the three populations, which were nested to examine group structure (imposed by the sampling scheme) to determine where the majority of variation was contained. Differentiation among populations was estimated from $F_{ST}$, which represents the portion of variance responsible for differences between populations (Weir and Cockerham 1984).

In addition, the first two principal components from PCA were plotted to investigate differentiation between populations. Pairwise population $F_{ST}$ values were also compared. To estimate migration among all three populations, assuming Wright's island model and that the populations are at equilibrium, migration (m, per generation) can be estimated from $Nm = 1/2[(1/F_{ST})-1]$ for haploid populations (Slatkin 1987, Arroyo Garcia 2002), where two populations of size N are considered to have diverged $\tau$ generations ago from a population of identical size. Correlations between genetic variation and geographic distance were also tested. Using SPAGeDi, matrices of population pairwise $F_{ST}$, Nei's D (1972), and spatial distance were calculated and tested using the Mantel option in Arlequin. Within populations, individual pairwise spatial and genetic distances (i.e. kinship coefficients, Ritland 1996) were calculated in SPAGeDi and Pearson correlations were used to test if genetic similarity within populations is related to spatial distance.

## 2.3. RESULTS

The four primer pairs used in this study yielded 214 scorable loci, of which 65 were polymorphic, resulting in low overall diversity of 30.3% (Table 2.2). This is exemplified by Figure 2.3 showing a typical *V. heterodoxa* AFLP gel with many strong, repeatable, monomorphic loci. The percentage of polymorphic loci in each population using all 214 loci was 25.2% (54/214) in Mesachie Lake (ML), 23.8% (51/214) in Port Hardy (PH), and 24.7% (53/214) in Deak's Peak (DP) compared to when only polymorphic loci were used: 83.1% (54/65) in ML, 78.5% (51/65) in PH, and 81.5% (53/65) in DP.

Table 2.2. Summary of the repeatable and polymorphic loci[1].

| Primer Pair | Number of Loci | No. of Polymorphic Loci | % Polymorphic Loci |
|---|---|---|---|
| 1. AC/CAC | 54 | 13 | 24.1 |
| 2. AC/CAG | 49 | 15 | 30.6 |
| 3. AG/CAC | 61 | 23 | 37.7 |
| 4. AG/CAG | 50 | 14 | 28.0 |
| Total | 214 | 65 | 30.4 |

[1] Only loci with allele frequencies greater than 0.05 or less than 0.95 were included in the analysis to reduce statistical bias introduced by very rare or very frequent loci.

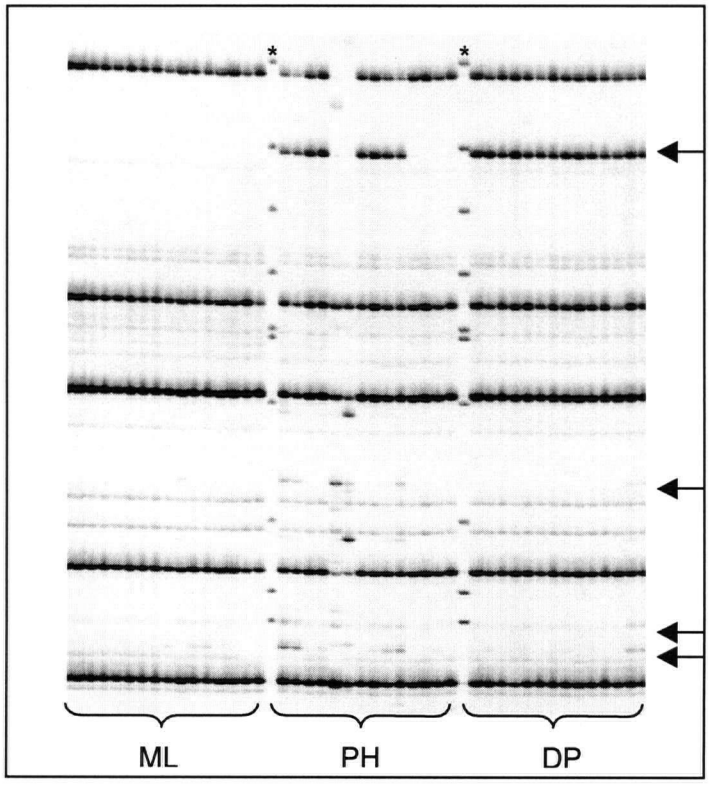


Figure 2.3. Section of a typical AFLP gel emphasizing the strong, monomorphic loci observed in all three populations where ML indicates individuals from Mesachie Lake, PH from Port Hardy, and DP from Deak's Peak. Lanes between populations are 50-700 bp molecular size markers (*). Arrows indicate polymorphic loci.

### 2.3.1. Within sample genetic diversity.

Each lesion on a salal leaf was formed from one spore, evidenced during microscopy where spores remained attached to the center of the lesions throughout the infection process (S. Zhao, pers. comm.). As only three *V. heterodoxa* spores were obtained per salal stem sampled, they may not reflect all the haplotypes actually present on one host. Also, since many of the *V. heterodoxa* isolates did not sporulate, the selected individuals may only represent the most prolific haplotypes.

For the 21 different salal stems sampled from ML, on average, the three *V. heterodoxa* haplotypes from each stem were 68.3% similar. All three individuals had the same haplotype in eight stems sampled while only four stems shared no common *V. heterodoxa* haplotypes (Table 2.3). The same trends were observed for within-sample and within-population analyses: PH had the highest within-sample diversity (only 19.5% shared haplotypes) and ML had the lowest diversity (68.3% shared haplotypes, Table 2.3).

Table 2.3. Shared haplotypes between *V. heterodoxa* single spore isolates within each salal stem sampled.

| Population | No. salal stems sampled | No. *V.heterodoxa* individuals | No. shared haplotypes (/3) | | | | Ave. % shared haplotypes |
|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | |
| ML | 21 | 60 | 4 | 0 | 9 | 8 | 68.3 |
| PH | 12 | 36 | 9 | 0 | 2 | 1 | 19.5 |
| DP | 17 | 52 | 6 | 0 | 8 | 3 | 53.0 |

### 2.3.2. Within population analyses.

***2.3.2.1. Genetic diversity***. PCA was performed on the 65 polymorphic loci for each population and a matrix of Pearson correlations between the principal components and loci was generated. Loci with high loadings (correlations >0.55) on principal components with eigenvalues >1 contributed the most to the variation described by the principal components and were selected for use in analyses. The Pearson correlations from PCA reduced the 65 loci to 39 in ML, 40 in PH, and 42 in DP (Table 2.4). These loci were then used for separate population analyses.

Different alleles were used for within population analyses so as not to underestimate the level of gene diversity by including less informative loci. The allele frequencies of the loci used for within population analyses are listed in the appendix, (Appendix Table 1) and Figure 2.4 shows the distribution of allele frequencies in each population. Within each population, the average gene diversity, which is the probability of obtaining different alleles at randomly chosen homologous loci, was low to moderate. PH had the highest average gene diversity (Ave $H_e$=0.410, Table 2.5), which was expected as PH also had the highest haplotype diversity. The average gene diversity was lowest for ML (Ave $H_e$=0.314) and only slightly higher for DP (Ave $H_e$=0.338).

Table 2.4. Results of principal components analysis of 65 loci on each *V. heterodoxa* population. Loci with Pearson correlations greater than 0.55 were selected from each principle component with eigenvalues > 1 for use in further analyses.

| Population | No. principle components | % Variation explained | No. loci contributing to variation |
|---|---|---|---|
| ML | 14 | 92.8 | 39 |
| PH | 10 | 90.0 | 40 |
| DP | 10 | 87.1 | 42 |

Table 2.5. Number of loci, average allele frequency and gene diversity within each *V. heterodoxa* population.

| Population | No. Loci | Ave. allele frequency (Ave. p) | Ave. gene diversity (Ave. $H_e$) |
|---|---|---|---|
| ML | 39 | 0.399 | 0.314±0.160 |
| PH | 40 | 0.425 | 0.410±0.208 |
| DP | 42 | 0.349 | 0.338±0.171 |

[a] Average frequency of the dominant allele, p at locus i, given by $p_i=x_i/n_{pop}$ where x is the number of individuals with a band and $n_{pop}$ is the total number of individuals in the population averaged over all loci.
[b] Ave. $H_e$ is average gene diversity: the probability of obtaining different alleles at randomly chosen homologous loci, shown with standard deviation.

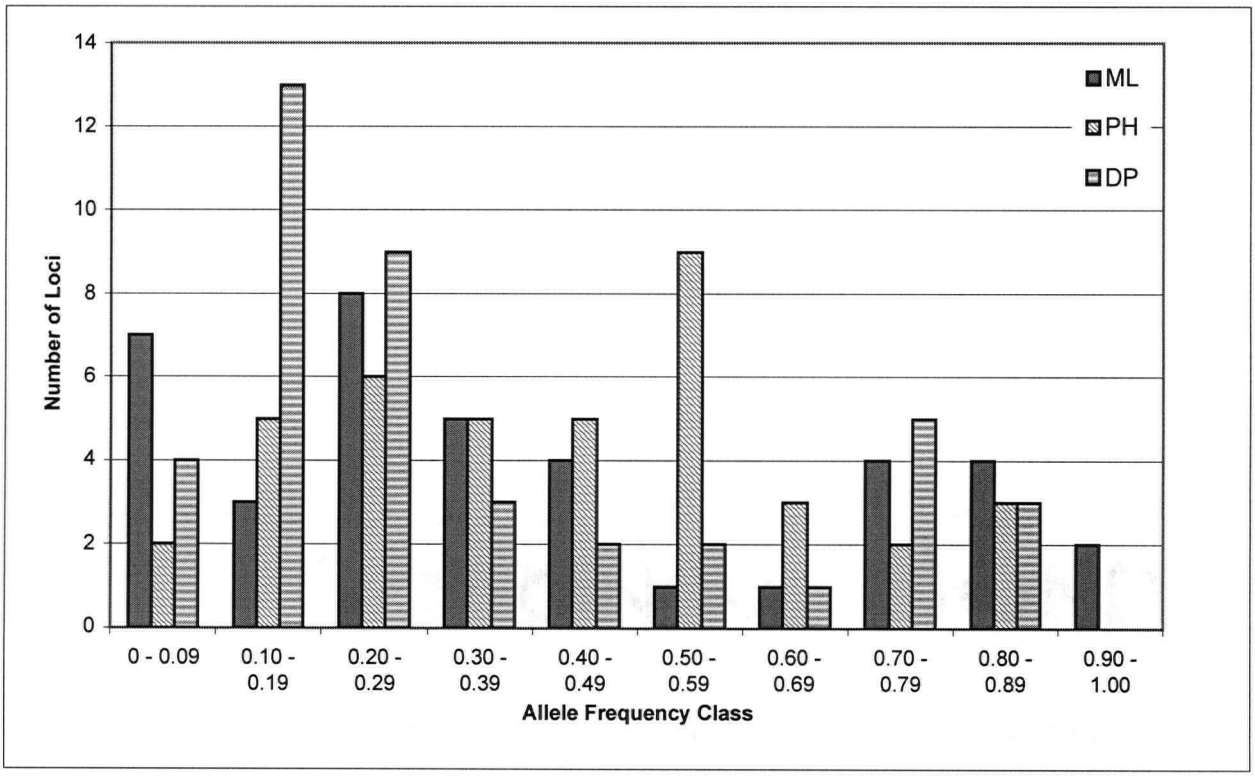


Figure 2.4. Distribution of allele frequencies used for within-population analyses where 39 loci were used for Mesachie Lake (ML), 40 loci for Port Hardy (PH), and 42 loci for Deak's Peak (DP).

**2.3.2.2. Associations among loci indicate some recombination.** The number of significant associations between pairs of loci (i.e. linkage) was greater when all samples were used than in the clone-corrected sub-set. Table 2.6 compares the number of pairwise associations observed in each of the three populations. Less than 20% of comparisons were significant (i.e. linked); ML had the fewest unlinked loci

(Table 2.6). Tests on the pooled data set showed that the majority of loci were linked across all populations (Table 2.6).

Table 2.6. Association between pairs of loci within populations.

| Sample | No. Isolates | No. Loci | No. pairwise comparisons | % Significant comparisons[a] |
|---|---|---|---|---|
| ML | | | | |
| All | 60 | 39 | 741 | 12.7 |
| Clone-corrected | 32 | 39 | 741 | 7.4 |
| PH | | | | |
| All | 36 | 40 | 780 | 13.7 |
| Clone-corrected | 32 | 40 | 780 | 10.3 |
| DP | | | | |
| All | 52 | 42 | 861 | 18.1 |
| Clone-corrected | 37 | 42 | 861 | 14.3 |
| Pooled | 148 | 32 | 496 | 46.4 |

[a] The corrected *p* values were 0.0000675, 0.0000641, 0.0000581 for ML, PH, and DP, respectively.

The mean number of pairwise differences between haplotypes observed in ML was 12.3, 16.4 in PH, and 14.2 in DP (Table 2.7). Variance exceeded the 95% confidence interval implying more differences between these haplotypes than expected. Figure 2.5 shows the distribution of haplotype mismatches. Only a small fraction of individuals in the populations differed by less than five mismatches, which could be explained by mutation (Kohli and Kohn 1998). This indicates that the observed level of differences are likely caused by some other factor.

Table 2.7. Number of mismatched loci between haplotypes and average gene diversity within populations.

| Population | No. haplotypes[1] | No. Loci | No. of comparisons | # Pairwise Differences | *p*-value | Observed Variance |
|---|---|---|---|---|---|---|
| ML | 32 | 39 | 496 | 12.3 | 0.31 | 28.2 |
| PH | 32 | 40 | 496 | 16.4 | 0.53 | 45.9 |
| DP | 37 | 42 | 666 | 14.2 | 0.63 | 59.1 |

[1] Number of haplotypes determined based on the loci used for within population analyses

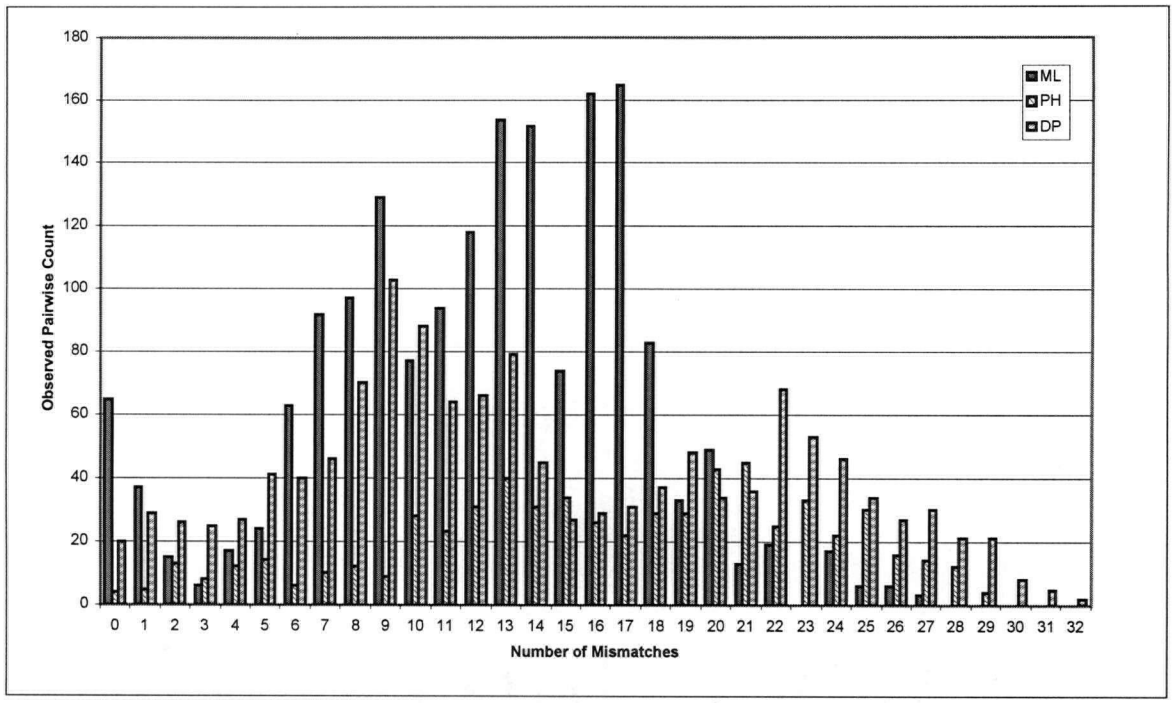


Figure 2.5. Number of mismatches for each pairwise comparison between haplotypes.

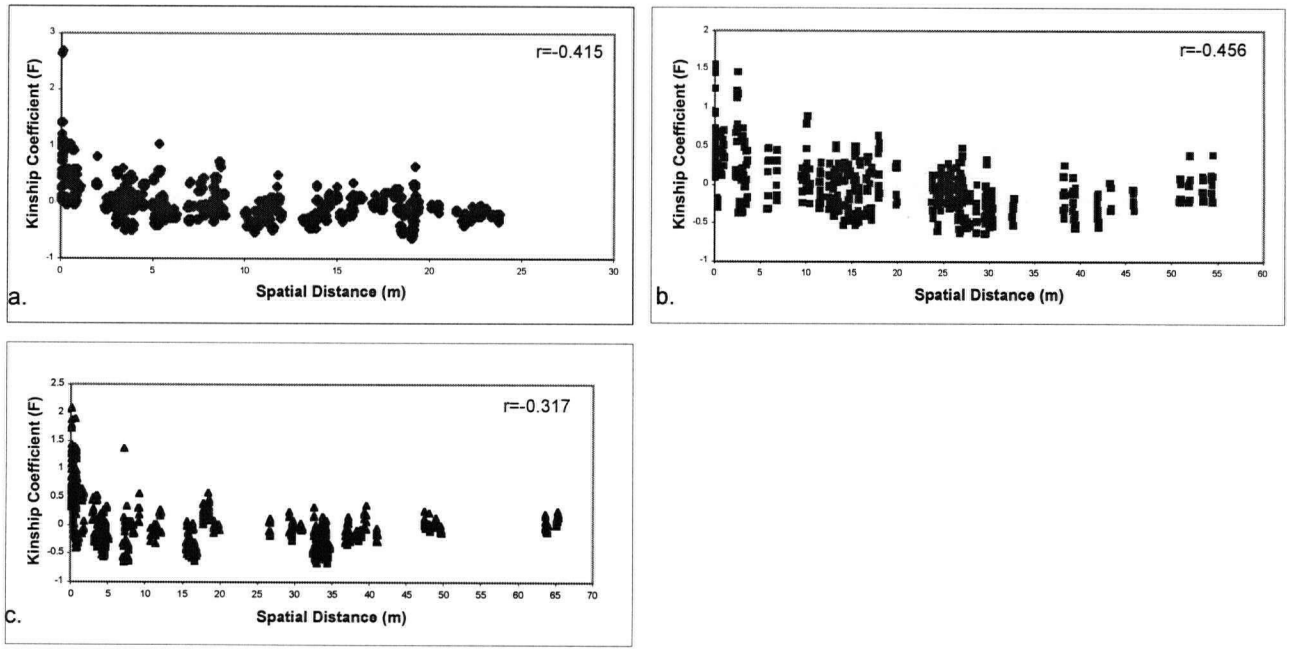


Figure 2.6. Scatterplots and Pearson correlations ( r) of pairwise kinship coefficients and geographic distance for (a.) Mesachie Lake, (b.) Port Hardy, and (c.) Deak's Peak. All correlations were significant ($p$<0.0001).

In all three populations, geographically close individuals had high kinship coefficients (Ritland 1996, Figure 2.6). The steep drop off in correlations past 1.5 m, particularly in DP, shows that individuals within groups are more genetically similar than individuals between groups. Within populations, as spatial distance increases, genetic distance also increases.

The distribution of haplotypes within a population can indicate the degree of population structure and further clarify the relationship between genetic similarity and geographic distance. For each population, individuals were plotted on X and Y coordinates according to their locations within each population and each unique haplotype was given a number (Table 2.10). Haplotypes were usually shared among isolates from the same salal stem and within sampling patches (Figures 2.7, 2.8, 2.9). In ML, all individuals in the last group shared haplotype 28 (Figure 2.7), compared to the first group in DP where all individuals had different haplotypes (Figure 2.9). Haplotypes were sometimes present in more than one group. For example, haplotype 50 in DP was shared among seven individuals in three groups: two in the center of the population, and one at the edge (Figure 2.8). Some haplotypes were also shared among populations, such as haplotype 11, which was present in all three populations, and haplotype 50, which was present in PH and DP (Table 2.10).

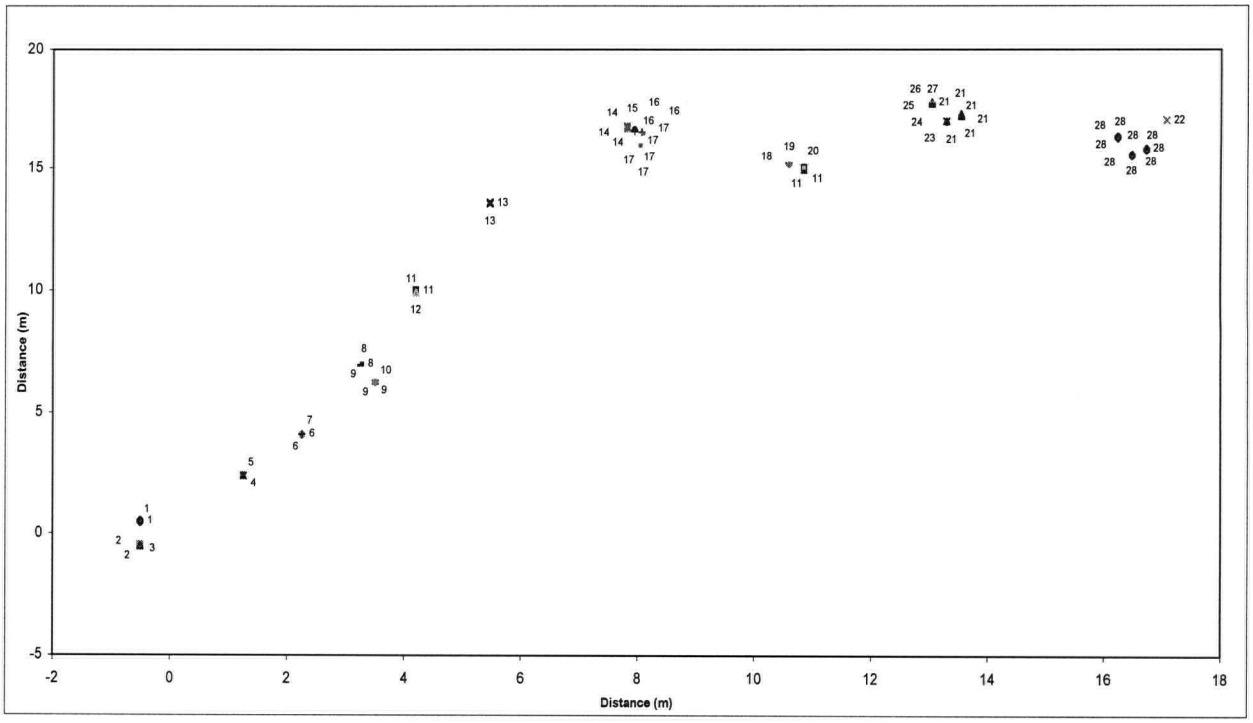


Figure 2.7. Map of the Mesachie Lake population, showing the location of each haplotype. Haplotypes were based on the 32 loci used to analyze all populations.

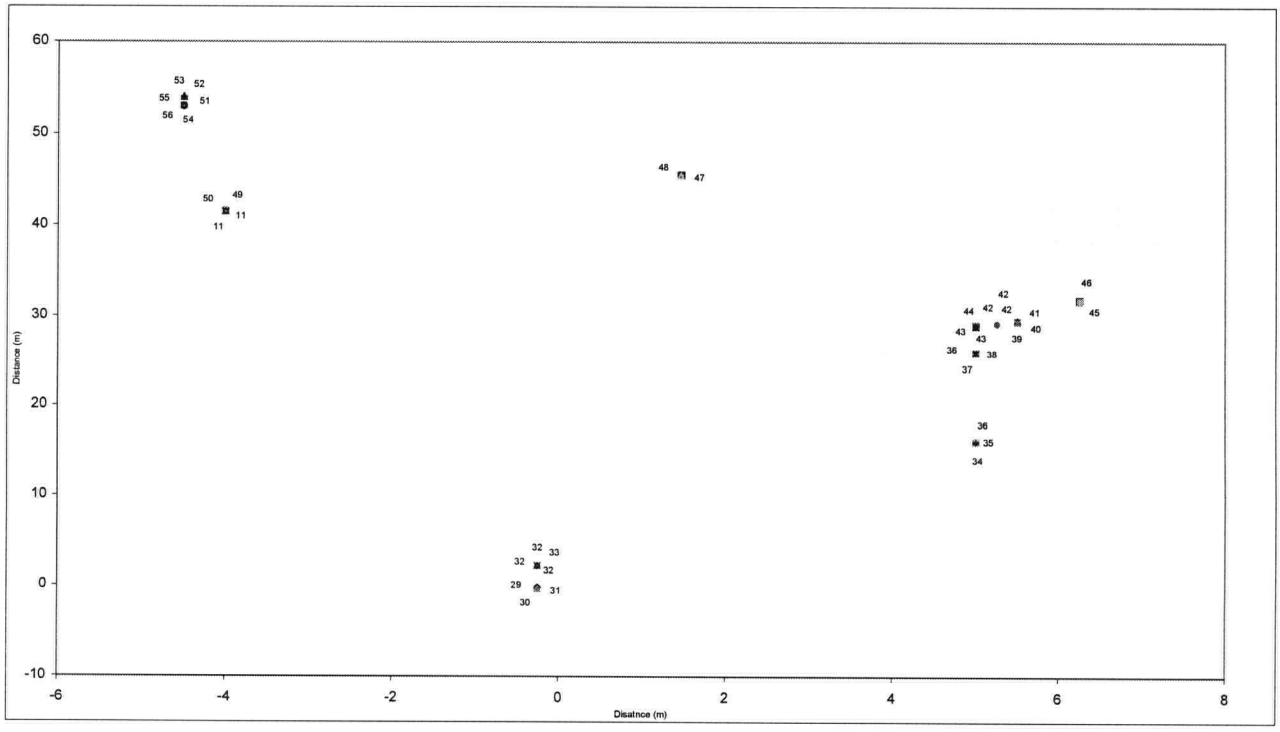


Figure 2.8. Map of the Port Hardy population, showing the location of each haplotype. Haplotypes were based on the 32 loci used to analyze all populations.

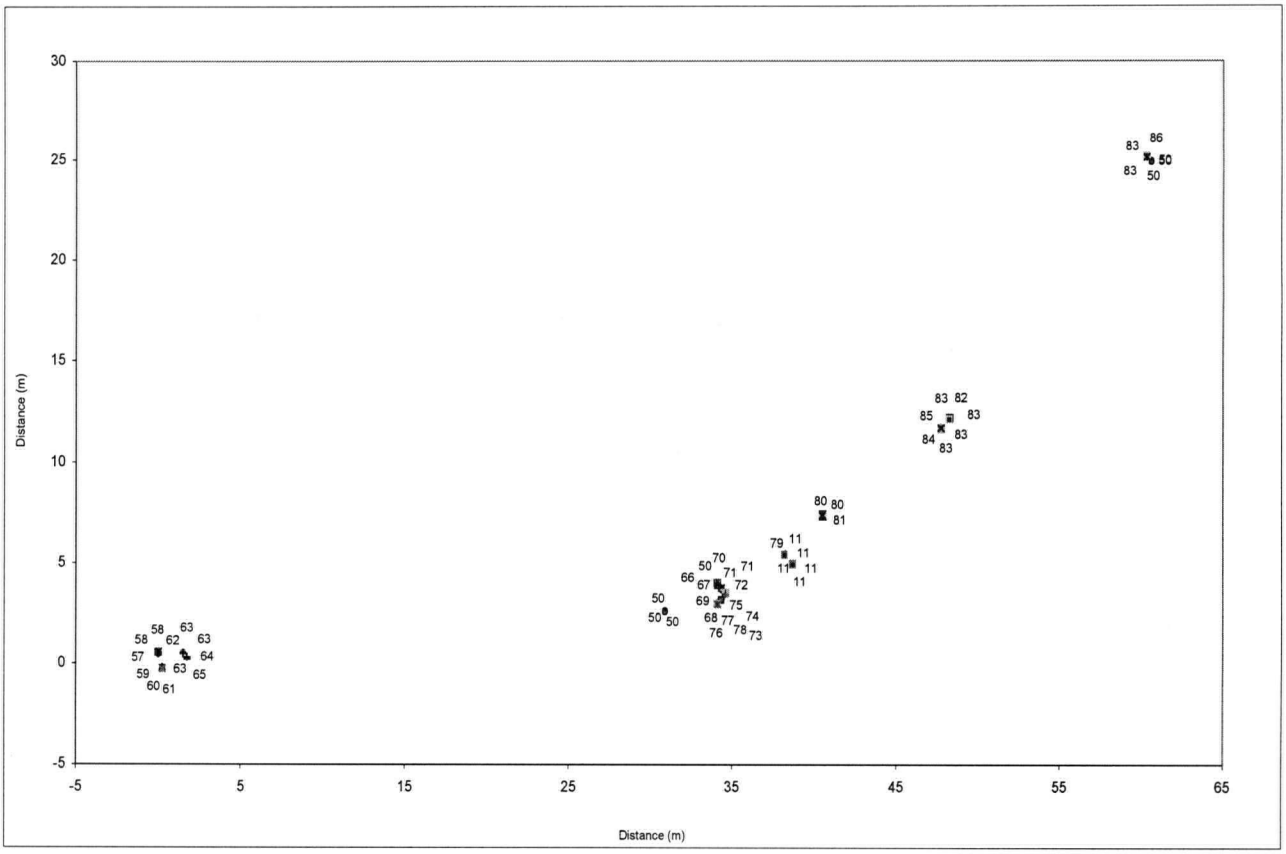


Figure 2.9. Map of the Deak's Peak population, showing the location of each haplotype. Haplotypes were based on the 32 loci used to analyze all populations.

### 2.3.3 Among population analyses.

**2.3.3.1. Genetic diversity**. PCA on the 65 polymorphic loci resulted in 15 principle components with eigenvalues >1 and explained 83.6% of the variation. Pearson correlations between the 15 principle components and the 65 polymorphic loci showed that 32 of these loci had loadings greater than 0.55, and therefore contributed the most to the variation observed in the principle components. These 32 loci were used in all population-level analyses unless otherwise specified.

Allele frequencies varied considerably among loci (see Appendix Table 2 for table of allele frequencies). Most loci had frequencies <0.3 and relatively few loci had frequencies >0.7, particularly for DP (Figure 2.10). PH had the most alleles with

moderate frequencies (0.4 to 0.6). The average gene diversity over all loci was low where ML had the lowest (0.231 ± 0.121) and PH had the highest (0.288 ± 0.150).

Table 2.8. Average gene diversity and standard deviation of alleles used in population-level analyses of all *V. heterodoxa* populations (see Appendix Table 2 for actual allele frequencies).

| Population | ML | PH | DP | Overall |
|---|---|---|---|---|
| Ave. Gene diversity | 0.231 | 0.288 | 0.263 | 0.293 |
| SD | 0.121 | 0.150 | 0.137 | 0.150 |

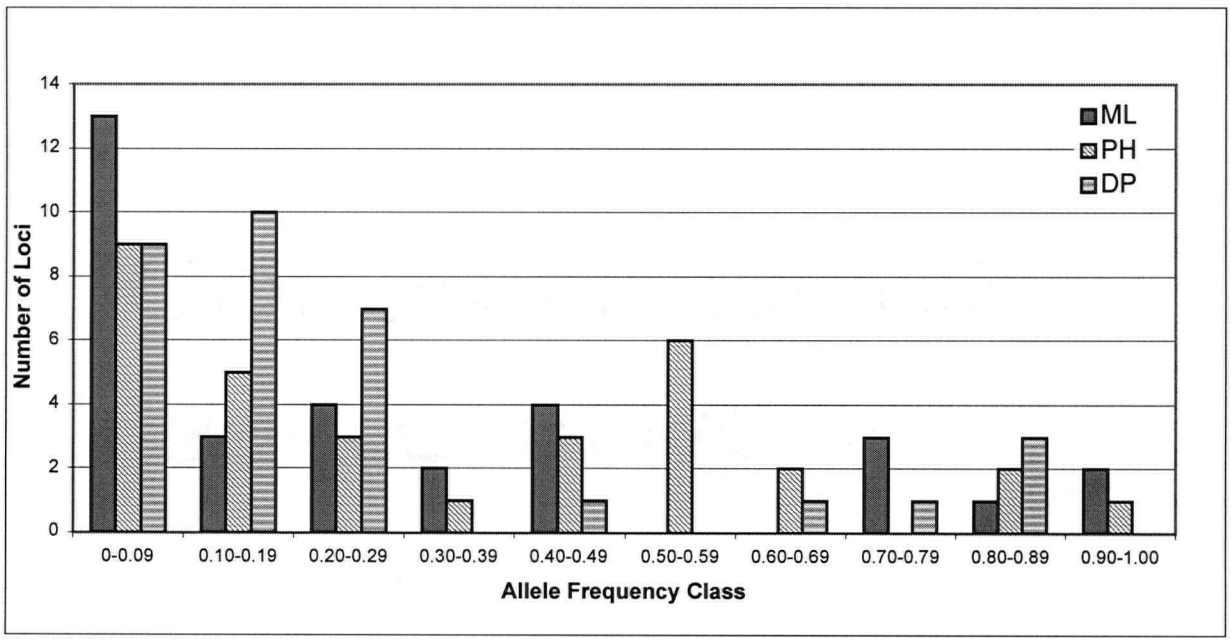


Figure 2.10. Distribution of allele frequencies in each population for the 32 loci used in the population-level analyses. Average standard error jackknifed over all loci was 0.0263.

Low haplotype diversity, or high similarity, was observed within and among populations. The number of shared haplotypes increased when the number of loci was reduced from 65 to 32, indicating sensitivity declining with fewer loci (Table 2.9). With 65 loci no haplotypes were shared among populations; however, when 32 loci were analyzed, haplotype 11, was found in all three populations (Table 2.10). ML had the lowest haplotype diversity (46.7%, Table 2.9). PH, while it had a comparable number of haplotypes to the other populations, had the highest haplotype diversity (80.6%, Table

2.9). The difference between the observed and expected haplotype diversities was significant ($\chi^2$ test, $p<0.05$). Two haplotypes were shared between populations (Table 2.10).

Table 2.9. Number of unique haplotypes and haplotype diversity in all three *V. heterodoxa* populations comparing results from 65 AFLP loci and 32 AFLP loci.

| | | **65 Loci** | | | **32 Loci** | |
|---|---|---|---|---|---|---|
| Population (n) | # Unique haplotypes | Obs. Haplotype Diversity | Exp. Hap. Diversity | # Unique Haplotypes | Obs. Haplotype Diversity | Exp. Hap. Diversity |
| ML (60) | 38 | 0.633 | 0.976 ± 0.008 | 28 | 0.467 | 0.953 ± 0.013 |
| PH (36) | 33 | 0.917 | 0.995 ± 0.008 | 29 | 0.806 | 0.986 ± 0.011 |
| DP (52) | 41 | 0.788 | 0.989 ± 0.006 | 32 | 0.615 | 0.961 ± 0.014 |
| Total (148) | 112 | 0.757 | 0.995 ± 0.002 | 86[a] | 0.581 | 0.987± 0.003 |

[a] The sum of unique haplotypes over all three populations is 89, but to account for the two haplotypes shared between populations, the actual total number of haplotypes was 86.

Table 2.10. List of haplotypes and their frequencies observed in each population based on 32 loci. Haplotypes in italics occur in more than one population.

| **Mesachie Lake** | | **Port Hardy** | | **Deak's Peak** | |
|---|---|---|---|---|---|
| Haplotype ID | Frequency | Haplotype ID | Frequency | Haplotype ID | Frequency |
| 1 | 3 | ***11*** | 2 | ***11*** | 5 |
| 2 | 2 | 29 | 1 | ***50*** | 7 |
| 3 | 1 | 30 | 1 | 57 | 1 |
| 4 | 1 | 31 | 1 | 58 | 2 |
| 5 | 1 | 32 | 2 | 59 | 1 |
| 6 | 2 | 33 | 1 | 60 | 1 |
| 7 | 1 | 34 | 1 | 61 | 1 |
| 8 | 2 | 35 | 1 | 62 | 1 |
| 9 | 3 | 36 | 2 | 63 | 3 |
| 10 | 1 | 37 | 1 | 64 | 1 |
| ***11*** | 4 | 38 | 1 | 65 | 1 |
| 12 | 1 | 39 | 1 | 66 | 1 |
| 13 | 2 | 40 | 1 | 67 | 1 |
| 14 | 3 | 41 | 1 | 68 | 1 |
| 15 | 1 | 42 | 3 | 69 | 1 |
| 16 | 3 | 43 | 3 | 70 | 1 |
| 17 | 5 | 44 | 1 | 71 | 2 |
| 18 | 1 | 45 | 1 | 72 | 1 |
| 19 | 1 | 46 | 1 | 73 | 1 |
| 20 | 1 | 47 | 1 | 74 | 1 |
| 21 | 6 | 48 | 1 | 75 | 1 |
| 22 | 1 | 49 | 1 | 76 | 1 |
| 23 | 1 | ***50*** | 1 | 77 | 1 |
| 24 | 1 | 51 | 1 | 78 | 1 |
| 25 | 1 | 52 | 1 | 79 | 1 |
| 26 | 1 | 53 | 1 | 80 | 2 |
| 27 | 1 | 54 | 1 | 81 | 1 |
| 28 | 9 | 55 | 1 | 82 | 1 |
| | | 56 | 1 | 83 | 6 |
| | | | | 84 | 1 |
| | | | | 85 | 1 |
| | | | | 86 | 1 |
| Total: 28 | 60 | 29 | 36 | 32 | 52 |

**2.3.3.2. Population differentiation and gene flow**. Ordination of the first two principle components (from the PCA on 65 loci), which explained 25.8% of the variance, revealed somewhat isolated clusters from each population as well as a cluster comprised mainly of individuals from populations PH and DP (Figure 2.11). Significant variation was detected among populations with an $F_{ST}$ value of 0.182 (AMOVA, $p<0.000$) where 81.8% of the variation was within populations (Table 2.11). AMOVA nested to include the spatial structure imposed by the patchy distribution of *V. heterodoxa* was also performed (Table 2.11). Samples from 27 patches were obtained

over all three populations. When AMOVA was repeated to include this population structure, only 11.7% of variation was due to differences among populations. 50.3% of the variation occurred among groups within populations, and 38.0% of the variation was within groups ($p<0.000$, Table 2.11). While there are significant differences among populations, most of the variation is due to differences among groups within populations.

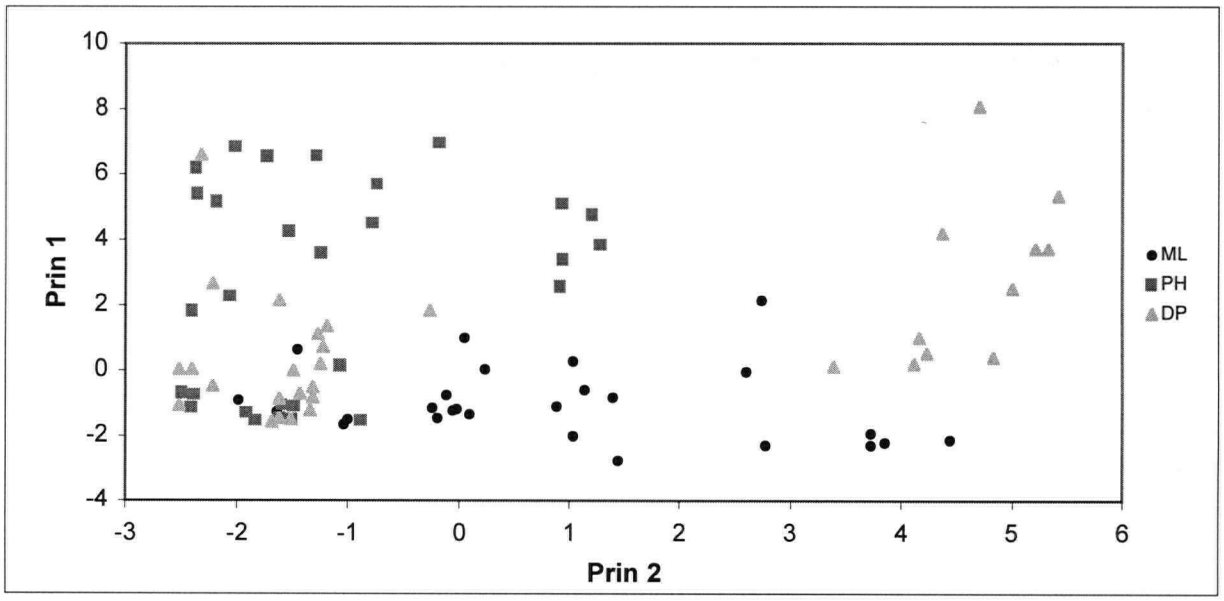


Figure 2.11. Ordination of the first two principle components on 32 polymorphic loci over all three populations, explaining 38.2% of the observed variation.

Table 2.11. AMOVA results for all three populations[a]. For the nested analysis, populations were divided into groups based on patches observed in natural populations.

| Source of Variation | d. f. | Sum of Squares | Variance Components | % of Variation |
|---|---|---|---|---|
| Population level | | | | |
| Among populations | 2 | 96.108 | 0.910 | 18. 2 |
| Within populations | 145 | 593.622 | 4.094 | 81.8 |
| Total | 147[a] | 689.730 | 5.004 | 100.0 |
| | | | | |
| Nested level | | | | |
| Among populations | 2 | 96.108 | 0.587 | 11.7 |
| Among groups within populations | 24 | 363.724 | 2.518 | 50.3 |
| Within populations | 121 | 229.898 | 1.900 | 38.0 |
| Total | | 689.730 | 5.004 | 100.0 |

[a] All $p<0.0001$ based on 1023 permutations.

Pairwise differences between $F_{ST}$ and Nei's D showed there were significant differences between all populations ($p$<0.0001). The greatest differences were between ML and PH ($F_{ST}$ = 0.266, D = 0.129) whereas PH and DP were the most similar ($F_{ST}$ = 0.116, D = 0.047, Table 2.12). Comparing spatial distances, ML and DP are the closest (1.152°), followed by ML and PH (3.843°), and PH and DP, which are the furthest (4.412°, Table 2.12). However, ML and PH are both on Vancouver Island, and are separated from DP by the Straight of Georgia. Assuming that a large waterway could be a physical barrier to gene flow, it was expected that ML and PH would be most similar to each other. However, this was not the case, suggesting that populations are more differentiated north to south than east to west. A Mantel test comparing spatial distances to pairwise $F_{ST}$ values over 1000 permutations was not significant (p=0.678).

Table 2.12. Matrix of population pairwise comparisons of genetic distances: $F_{ST}$ above diagonal, Nei's D above diagonal in brackets, and spatial distance[a] below diagonal.

| Population | ML | PH | DP |
|---|---|---|---|
| ML | -- | 0.2657 (0.1029) | 0.1615 (0.0891) |
| PH | 3.8417 | -- | 0.1156 (0.0628) |
| DP | 1.1521 | 4.4121 | -- |

[a] Spatial distance measured as latitude and longitude in degrees.

## 2.4 DISCUSSION

The *Valdensinia heterodoxa* isolates were 30.3% polymorphic for the 214 AFLP loci obtained from the four primer combinations used in this study, conversely, for 149 of 214 loci, all the individuals were identical. Allele frequencies at polymorphic loci spanned a wide range and average gene diversities were low to moderate. Low levels of both gene and haplotype diversity were observed at all levels of the hierarchical analysis. In general, Mesachie Lake (ML) showed the least amount of diversity and Port Hardy (PH) had the most. Most variation occurred among groups within each population. Haplotypes were spatially distributed within populations (Figure 2.5).

**2.4.1. *Valdensinia heterodoxa* exhibits a predominantly clonal population structure**. In natural populations, *V. heterodoxa* has been observed to reproduce primarily asexually with rare, seasonal sexual reproduction (Norvell and Redhead 1974). This type of life cycle has been characterized by Maynard Smith *et al.* (1993) as an epidemic model, which lies between the extremes of strict clonality and random mating: annual sexual cycles provide frequent recombination, followed by an asexual epidemic resulting in a few successful individuals reproducing clonally and occur at high frequencies in the population. The diversity, distribution, and linkage within and among *V. heterodoxa* haplotypes can be used to test whether the epidemic model applies.

Identical haplotype abundance within a population is a strong indication of clonal population structure (Taylor *et al.* 1999). Of the 148 individuals genotyped in this study, 86 different AFLP fingerprints were observed, suggesting that 42% of individuals are derived from asexual reproduction (based on the 32 polymorphic loci used in the population-level analyses). The analysis was sensitive to the number of loci used: 65 polymorphic loci resulted in 112 haplotypes, indicating 25% of the population was clonal. Fewer pairwise differences between haplotypes were observed at 32 loci

compared to when 65 loci were used. Considering the relatively few loci distinguishing among haplotypes, individuals may have arisen from asexual reproduction and differences caused by mutation; since it has been suggested that mutation rate is elevated in predominantly clonal populations (Milgroom 1996). Some level of clonal population structure was also inferred from the level of association between loci as there were significantly fewer differences observed between haplotypes than expected. On average, 15% of loci formed nonrandom associations (were linked), which indicates that the populations are not completely randomly mating. When populations were corrected for asexual "replicates," the number of linked loci was only slightly reduced suggesting that most loci were linked not only within clones but throughout the population as a whole.

When using linkage disequilibrium to infer population structure, there are other possible mechanisms which may cause nonrandom associations within a population that should be discussed (Milgroom 1996). One factor is that sampling may include more than one natural population or the population may be subdivided by ecological or biological barriers. For example, if alleles were fixed in each subpopulation, populations would appear clonal even though there could be recombination within each subpopulation not detected by the sampling (Taylor *et al.* 1999, Maynard Smith *et al.* 1993). This study included fine-scale sampling over a smaller area, so it is unlikely that there would be barriers not identified in the sampling process. Also, while *V. heterodoxa* growth is dependent on a host, salal was ubiquitous so any structuring is not due to barriers imposed by host discontinuity. Resistance-virulence associations between *V. heterodoxa* and salal may create the patchy distribution observed in natural populations; however, since the sampling scheme included several patches within each population, this would likely have been detected through the hierarchical analyses.

Significant associations between loci were detected in all three populations when they were pooled and analyzed separately so associations were not likely due to population barriers.

Genetic drift and selection can also lower genetic diversity and increase linkage disequilibrium (Milgroom 1996, Kohli and Kohn 1998). For example, if the effective population size was small and gene flow among populations was limited, genetic drift could cause allele frequencies to move toward fixation or loss resulting in localized genotypes (Slatkin 1987). With *V. heterodoxa* it is possible, given the large size and limited dispersal of the spores, that gene flow between populations is restricted. If populations are primarily clonal, effective population size is quite small, making populations more susceptible to genetic drift. However, if drift was a major force acting in these populations, common haplotypes would be localized within each population, and with *V. heterodoxa*, not only are there many haplotypes shared by only one or two individuals, the common haplotypes are distributed throughout the populations as well as localized to certain regions, suggesting that drift, while it may be present, is not likely a major factor contributing to associations among loci. The effects of drift in *V. heterodoxa* populations may be difficult to distinguish from structure imposed by the short-range dispersal of the asexual staurospores. If selection was the major force responsible for the observed population structure, certain haplotypes would be favoured within populations and their frequencies would higher (Kohli and Kohn 1998). While some haplotypes were considerably more frequent than others, there were also many haplotypes that occurred only once or twice,

The distribution of haplotypes within populations is also influenced by environmental factors that effect spore dispersal within the populations. For example, since moisture is essential for spore dispersal (Norvell and Redhead 1994), rainfall,

canopy cover, and the amount of animal and human traffic in the area could impact spore distribution. For example, animals eating or harvesting the leaves could dislodge spores and transport them. *V. heterodoxa* survival could also depend on ambient environmental conditions as it is only observed in shady, protected areas. Limits to *V. heterodoxa* growth imposed by environmental conditions have been observed in both field and greenhouse experiments (Shamoun *et al.* 2000, Vogelgsang and Shamoun 2002).

**2.4.2. Evidence for recombination in *V. heterodoxa* populations.** Since the hypothesis of random mating could not be rejected for all pairwise associations between loci, there is likely some random mating, indicative of sexual reproduction and recombination. This is also evidenced from the sexual structures observed in natural populations (Redhead 1974), which suggest that meiosis occurs and indicates that the potential for recombination exists. The low similarity among some haplotypes suggests that differences were due to recombination as there were more than expected from mutation alone. These factors together provide strong support for the presence of recombination in *V. heterodoxa*, despite its apparent clonal population structure. While the overall structure of *V. heterodoxa* populations may be shaped by clonal asexual reproduction, genetic exchange must also happen in order to generate both the moderate gene diversities and some of the unique haplotypes that were observed. As well, recombination resulting from rare sexual events can be sufficient to generate considerable diversity in populations (Burt *et al.* 1996).

Other than recombination, the relatively large differences among haplotypes may be caused by chromosome rearrangements where for example, transposable elements may move or 'jump' within the genome. This has been demonstrated in some fungal cultures even after just a few transfers, mainly in response to stress (Anderson and

Kohn 1995). However, following the logic of Kohli and Kohn (1998) since there were cases where all *V. heterodoxa* individuals in a group had identical haplotypes, it seems unlikely that chromosomal rearrangements in the regions of the AFLP loci used in this study were major contributing factors to the variation observed between haplotypes. In order to assess the possible contribution of mitotic recombination or chromosomal rearrangements in *V. heterodoxa*, linkage maps and sequence data could be used to identify markers at specific chromosomal positions.

Another explanation for the amount of variation observed is a high mutation rate; although the likelihood of mutation explaining the differences among haplotypes decreases as the number of pairwise differences increases simply due to the number of mutations that would have to occur to create these two haplotypes. Mutation can be used to explain cases where there are only a few differences between haplotypes (Milgroom 1996). For *V. heterodoxa*, there were on average 14 pairwise differences between haplotypes. Given the distribution of the pairwise mismatches (Figure 2.4), only a small fraction of the haplotypes differed by just a few mismatches explainable by mutation. This result is also supported by other studies (Kohli and Kohn 1998 and Arroyo Garcia *et al.* 2002). To minimize the chance of inducing new mutations or chromosomal rearrangements fungal cultures were transferred as little as possible. While many different mechanisms may contribute to the variation observed among haplotypes within *V. heterodoxa* populations, the most likely is that sexual reproduction is more frequent than initially thought and that *V. heterodoxa* populations do follow an epidemic population model.

**2.4.3. Genetic differentiation and limited gene flow.** PCA showed groups of differentiated individuals in each population and also indicated that individuals from PH and DP were more similar to each other than to individuals from ML. $F_{ST}$ values were

moderate relative to other fungi for both pooled (0.182) and hierarchical (0.117) AMOVA (Arroyo Garcia *et al.* 2002). In an AFLP study on *Epichloe festucae* Leuchtm., Scharal, and Siegel, an haploid ascomycete fungus, had an $F_{ST}$ of 0.197 between two populations 41km apart (Arroyo Garcia *et al.* 2002).

Interpopulation gene flow is likely responsible for maintaining the level of differentiation observed among these populations. Genetic drift, mutation, selection, and a predominantly clonal mating system all contribute to local adaptation and differentiation among populations. Gene flow disrupts local adaptation and promotes the genetic exchange among populations (Slatkin 1987). Slatkin (1987) also remarked that in parasites, which can include plant pathogens like *V. heterodoxa*, gene flow and local adaptation are likely to have the greatest evolutionary roles in shaping populations due to their unstable population structure. In *V. heterodoxa*, dependence on the distribution of the host (mainly salal), as well as seasonal life cycle fluctuations greatly affect the population size and range, which in turn, can lead to an unstable population structure with recurring extinctions and recolonizations. It therefore becomes important to estimate the amount of gene flow among *V. heterodoxa* populations to gain a better understanding of the evolutionary potential of *V. heterodoxa*, particularly regarding its spread as a biocontrol agent among isolated populations.

Gene flow can be estimated directly from the number and breeding success of migrants, and also from the geographic distribution of variation among populations (Slatkin 1987). Ideally, populations should be monitored over time to determine the average values, which may be more evolutionarily relevant (McDermott and McDonald 1993), but this is not usually feasible. The results of this study represent a snapshot in time, which provide an indication of gene flow in the species and how it contributes to population structure. Considering the similarities among all populations studied, there

was likely considerable historic gene flow among populations. Alternatively, they could have been founded more recently by similar individuals, in which case it would be interesting to obtain isolates from more distant populations, such as those in Eastern Canada or Europe where *V. heterodoxa* has also been observed, to see how they compare to the isolates from British Columbia. To obtain more definitive evidence of direct gene flow among *V. heterodoxa*, genetic markers that could identify individuals or private alleles found in only one population should be used and monitored over time to determine if the private alleles or individuals appear in other populations.

Gene flow can also be estimated indirectly based on the distribution and frequencies of alleles or genotypes in populations. Using Wright's island model (1943), which considers populations to be made up of many discrete subpopulations, where gene flow among subpopulations occurs via migration, an average of 2.25 migrations per generation would be needed to maintain this level of differentiation. Theoretically, if N*m* is >1, gene flow is strong enough to homogenize differentiation caused by genetic drift. Therefore, limited amount of gene flow among *V. heterodoxa* populations is sufficient to prevent the populations from becoming completely differentiated. The higher $F_{ST}$ value between ML and PH could indicate very low gene flow. *V. heterodoxa* populations in the field were discontinuous and not all suitable habitats were colonized which may promote some local adaptation.

The age of *V. heterodoxa* populations in BC is not known. The species was not observed in BC until 1972, so populations may be relatively young, which may explain the high degree of monomorphism and moderate degree of differentiation. Very little migration would be necessary among populations to account for these patterns, such that estimated levels of gene flow would be inflated as a result of insufficient divergence among the populations. If monitoring over time revealed limited gene flow, depending

on the level of recombination, the populations are expected to become more differentiated. This was also hypothesized by Linde *et al.* (2002) who suggested that due to relatively recent colonization (in this case centuries ago) populations of *Mycosphaerella graminicola* have not yet reached an equilibrium between gene flow and drift, so the source and temporal scale of the observed gene flow could not be determined. This alternate explanation becomes particularly pertinent when considering the dispersal mechanisms in *V. heterodoxa*. Since reproduction in *V. heterodoxa* is asexual for most of the life cycle (Redhead and Perrin 1972a), the predominant dispersal mode would be asexual spores, which are very large, leading to localized structure within populations. It is likely that animals and birds facilitate spore dispersal, but this does not explain the degree of similarity between island and mainland populations. Redhead and Perrin (1972a) observed smaller secondary asexual spores in the laboratory, although they have not been observed by other researchers nor is their contribution in natural populations known. The greatest potential for gene flow and migration in *V. heterodoxa* is via the small sexually produced ascospores, but since the frequency of sexual recombination is likely low and their dispersal distance unknown, it remains to be seen if they could account for the estimated levels of gene flow between populations.

**2.4.4. Isolation by distance**. A Mantel test was not significant ($p$=0.7), suggesting that the differences between populations could not be explained by geographic distance. It may be that geographic separation between populations was not great enough to result in a significant correlation; although the low correlation could also be a function of the low power of the Mantel test with only three populations, since qualitative comparison of the matrices (Table 2.12) suggests there is some relationship between genetic and spatial distance, particularly when the geographic barriers are

considered. For example, the strong genetic differentiation between ML and PH reflects their latitudinal separation, despite the fact they are both on Vancouver Island. If wind disperses *V. heterodoxa* spores, the prevailing wind patterns would facilitate gene flow between the island and mainland populations. In this case, if the prevailing winds are from southwest to northeast, ML and PH would be expected to have many unique haplotypes compared to DP, which would have more shared haplotypes. As well, PH and ML may be expected to be more similar to DP than to each other.

The expected relationship of genetic distance increasing with spatial distance was observed within each of the three *V. heterodoxa* populations (Figure 2.4). The patchy distribution of *V. heterodoxa* in the field reflects that individuals within the same group (less than 1.5 m apart) were more genetically similar than individuals in different groups, and that groups from the same section of the population were more similar to each other than to more distant groups. This result was also supported by the nested AMOVA analysis.

## 2.5 CONCLUSION

In the three populations studied, *V. heterodoxa* had a predominantly clonal population structure based on haplotype diversity and distribution within and among populations. The level of diversity in the populations suggested at least some recombination, most likely from rare or even seasonal sexual recombination. Genetic differentiation may be a reflection of a recent founder event. There was no statistically significant relationship between pairwise genetic and geographic distances, suggesting other factors facilitate differentiation among the populations. Within populations, genetic distance was correlated with spatial distance, leading to population structure and reflecting the patchy distribution of *V. heterodoxa* genotypes in natural populations.

## CHAPTER 3. Genetic diversity and population structure of *Gaultheria shallon*

### 3.1 LITERATURE REVIEW

**3.1.1. Life history characteristics.** *Gaultheria shallon* Pursh. (salal) is a densely growing evergreen perennial shrub native to the Pacific Northwest coast occurring from California to the panhandle of Alaska (Fraser *et al.* 1993). Salal ranges from 0.2 - 5 m in height with thick, shiny, ovate, leathery leaves (Pojar and Mackinnon 1994). From March through July, salal produces many perfect, white to pinkish, urn-shaped flowers in terminal groups of five to 15 (Banerjee *et al.* 2001) pollinated by bees, flies, and hummingbirds (Pojar 1974). Fruits are purple with fine hairs and often remain attached to the plant for several months containing on average, 126 seeds, dispersed by birds and animals that feed on the fruit (Haeussler *et al.* 1990, Fraser *et al.* 1993). Salal produces far more fruit under open stands than in stands with greater than 30% coverage (Pojar 1974, Bunnell 1990). Seedling establishment and survival is poor in both clear cuts and closed canopies (Huffman *et al.* 1994). In these areas, salal relies predominantly on vegetative growth through an extensive root system and ericoid mycorrhizal associations (Xiao and Berch 1993).

Salal stems are connected via underground mats of rhizomes, thought to create a clonal population structure (Bunnell 1990). To study how salal colonizes an area and how vegetative rhizomes shape understory populations, Huffman *et al.* (1994) excavated 51 clonal fragments. Following the definition of Cook (1983), clonal fragments include all stems connected by rhizomes. Ramets are stems capable of independent survival and genets include all genetically identical clonal fragments and ramets, which combined, make up a clone. Huffman *et al.* (1994) found that as clones age they become physically fragmented. The largest clonal fragment excavated contained 218 m of total rhizome length, 292 ramets, and covered an area of 29 $m^2$.

Clonal expansion was greatest in open canopies compared to covered stands (Huffman *et al.* 1994).

**3.1.2. Phylogeny**. Salal is a member of the Ericaceae family along with many other shrubs in coastal BC forests. Within the Ericaceae, salal is part of the Vaccinioideae, which includes the Vaccinieae, Gaultherieae, Andromedeae, and Lyoneae clades. Salal has recently been placed in the Gaultherieae clade by Kron *et al.* (2002) although it is also closely related to the Andromedeae. The *Gaultheria* genus has approximately 115 species, primarily in Latin America, and was first described by Linnaeus in 1751 in the original edition of *Species Plantarum* (Luteyn 1995). *Gaultheria* species share many similar characteristics including leaf, flower, berry, and seed morphology. Most have chromosome base numbers of 11, 12, or 13, and somatic chromosome numbers ranging from 22 to 96. In South America, most of the *Gaultheria* are diploid, (2n=22), with one tetraploid species (Luteyn 1995). Salal also has a base chromosome number of 11, but is octoploid (2n=88) derived from two duplication events, evidenced by multivalent chromosome associations during meiosis (Callan 1941). From phylogenies of morphological and sequence information, salal consistently grouped with *Pernettya tasmanica* Hook (Kron *et al.* 2001) and has been documented hybridizing *in situ* with *P. mucronata* Gaudich, a South American Ericaceous species (Callan 1941).

**2.1.3. Population genetics and polyploidy**. Population genetic studies using molecular markers have not previously been done on salal, although there have been studies on other ericaceous plants, such as *Vaccinium* (Albert *et al.* 2003), *Rhododendron* (Pornon *et al.* 2000), *Elliottia racemosa* Muhlenberg (Godt and Hamrick 1999), and *Calluna vulgaris* L. (Meikle *et al.* 1999). As the main mode of growth and expansion in salal is vegetative (Bunnell 1990), it is expected that salal will display a

predominantly clonal population structure. Traditionally, clonal plants were thought to have low genetic diversity. More recently, clonal plants have been shown to have a high number of genotypes with moderate levels of diversity and an even distribution of genotypes (Ellstrand and Roose 1987). This diversity is thought to originate from founder seeds and continual seedling recruitment (Eriksson 1993). As well, competition among clones, diversifying selection, and frequency-dependent selection can all result in high local heterogeneity, which in turn leads to high genotypic diversity across the populations (Ellstrand and Roose 1987). Diversity within clonal populations can be affected by the type of growth, such as phalanx or guerilla; however, Huffman *et al.* (1994) showed that clonal expansion of salal is more dependent on overstory cover and competition among clones than a particular clonal growth strategy.

Polyploidy in salal may affect the level of diversity observed within populations. In studies comparing polyploids to their diploid progenitors, polyploids have higher heterozygosity and genetic variation than diploids (Soltis and Soltis 2000). Polyploidy is very common in plants (up to 80% of angiosperms are polyploid) and has significantly contributed to their evolution (Leitch and Bennett 1997, Soltis and Soltis 2000). Based on their abundance alone, it is apparent that polyploids are very successful, and likely have some advantages over diploids (Stebbins 1971). In particular, polyploids may have higher selfing rates due to their colonization success and reduced inbreeding depression because of the additional genome copies, which act as a source of variation and buffer the effects of deleterious alleles (Soltis and Soltis 2000). Wendel (2000) has proposed three major fates of duplicated genes in tetraploids: maintaining two sets of functional copies, gaining a new function (depending on the source of the 'new' genome), and gene silencing, where the polyploid eventually reverts to a diploid state by silencing extra gene copies. Being an octoploid, salal may have up to eight copies of

every gene. If they arose from duplications, the amount of variation would depend on the alleles from the original parents and the amount of mutation and recombination since duplication.

Population genetic analyses often analyze overall diversity to assess the genetic and genotypic diversity within and among populations. The level of clonality and distribution of clones can also be used to assess population structure. Clonal population structure can greatly affect the dynamics of plant populations and can influence a species' ability to compete with other plants and fight disease (Godt and Hamrick 1999). Understanding the population structure of salal can thus help predict the possible effects of releasing a biocontrol agent on natural populations.

As described in Chapter 2, there are many molecular markers available to study genetic diversity and population structure. In choosing a marker system for salal, it was important to consider what, if any, previous research had already been done, the time and resources available, and the goals of this study, one of which was to compare salal with *V. heterodoxa* to discuss interactions and the effects these may have on implementing a biocontrol for salal. Several isozyme studies have been done on ericaceous species; however, the level of diversity can be underestimated as isozymes are functional proteins, which may not always conform to neutral marker assumptions. As well, unpublished preliminary work by Y. Berube (pers. comm.) suggested that isozymes were difficult to use and interpret in salal. No previous genetic studies using microsatellites have been done on salal, so no SSR primers were available for use. As well, Hardy (2003) showed that in some cases, dominant markers can be equivalent or better than SSRs at estimating spatial genetic structure within populations. RFLPs can be informative as they are codominant, but are often used in phylogenetic and mapping studies, where parent and offspring are known. RFLPs are technically challenging and

do not always have high reproducibility. Dominant RAPD markers were tested on salal, but were unreliable, difficult to reproduce, and few loci were obtained for each primer (data not shown).

AFLP markers were chosen to generate salal DNA fingerprints. With no prior sequence knowledge of salal, random dominant techniques such as AFLP are useful to obtain a snapshot of the diversity in the system. AFLPs are reproducible, reliable, and many loci are produced per primer pair so few primers are needed (Mueller and Wolfenbarger 1999). AFLPs have been successfully used on many species of the Ericaceae and other clonal species with similar growth strategies, such as *Rubus armeniacus*, which is tetraploid (Kollmann *et al.* 2000). One drawback to AFLP is that since salal is an octoploid, AFLP may not be as informative as codominant markers. However, considering that originally one of the goals of the research was to correlate the results from salal with those from *V. heterodoxa* to determine if the same patterns of diversity and population structure were observed within and among the populations studied, it was an advantage to use the same marker system for both species, as exemplified by Jerome and Ford (2002) on Lodgepole pine dwarf mistletoe.

**3.1.4. Objectives**. There were three main objectives of this study: 1) to assess the level of genetic diversity in natural salal populations on Vancouver Island and coastal mainland British Columbia, 2) to investigate the fine-scale population structure within one salal population, and 3) to discuss how the genetic diversity and population structure of salal could contribute to the efficacy of a biocontrol agent (in particular, *V. heterodoxa*), which will be discussed in Chapter 4. Based on what is known about salal and other clonal plants, it is expected that salal will have high overall diversity, particularly considering its high ploidy level, and that predominantly vegetative reproduction will result in a highly segregated, clonal population structure.

## 3.2 MATERIALS AND METHODS

**3.2.1. Site description**. Five salal populations were used in this study: the three used for *Valdensinia heterodoxa* in Chapter 2, and two additional populations, Sayward and Shawnigan Lake. Four of the populations were from Vancouver Island: Shawnigan Lake (SL), Mesachie Lake (ML), Sayward (SA), and Port Hardy (PH). The fifth population was from Deak's Peak (DP) on the mainland of British Columbia.

The ML, PH, and DP sites have previously been described in Chapter 1. The SA site is located in the underbrush 8.5 m off Highway 19 north of Sayward. The salal at this site was found in a two-metre wide strip under alder and Douglas-fir trees that bordered a mature cedar-hemlock forest. The site was flat at low elevation. Swordfern and huckleberry were also dominant in the understory. The SL site is in a Douglas-fir plantation used previously for a thinning and fertilizer experiment established by Diggle (1972). The stand is mainly Douglas-fir with some lodgepole pine, western hemlock, and western redcedar. Stand age was 55 years when sampled and the site is flat to gently rolling at an elevation of 355 m (Trofymow *et al.* 1997). Salal cover was most recently reported to be 85% (He and Barclay 2000). Figure 3.1 shows the locations of all five salal populations used in this study.

**3.2.2. Sample collection**. ML, SA, PH, and DP, were all sampled as described in Chapter 2, such that for each salal stem sampled, every time *V. heterodoxa*-infected leaves were collected, uninfected salal leaves were also collected (see Figure 2.2). *V. heterodoxa* was distributed in patches, so ten patches, with five salal stems sampled from each patch, plus an additional ten samples from between each patch, results in a total of 60 samples from each of these populations. The SL population was sampled more intensively in order to study fine-scale population structure. In this case, a 5X5 m

grid covering an area of 2500 $m^2$ was set up in a continuous salal population. A salal sample was collected at each intersect on the grid, resulting in 121 samples.

For each salal sample, 1-3 leaves were collected from a single salal stem, equivalent to one plant in this study, and placed in Zip-Lok freezer bags and stored on ice. Salal samples were considered healthy if there were no visible lesions or scars. Discs of leaf tissue were then cut and dried in silica gel to reduce enzyme degradation of DNA and stored at 4°C.

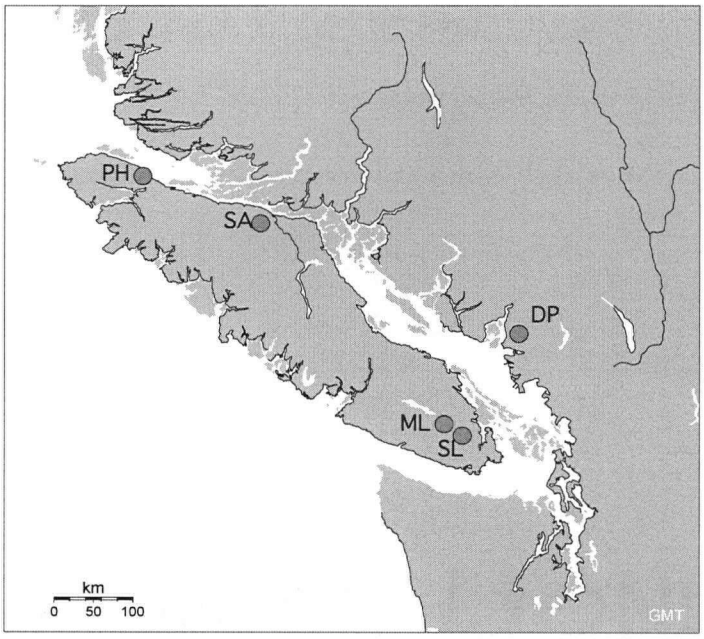


Figure 3.1. Map of Vancouver Island and mainland British Columbia, showing the locations of the five salal populations used in this study: Shawnigan Lake (SL), Mesachie Lake (ML), Sayward (SA), Port Hardy (PH), and Deak's Peak (DP).

**3.2.3. DNA isolation**. Prior to DNA isolation, samples were ground with liquid nitrogen and small mortar and pestles or in 1.5 ml Eppendorf tubes with micropestles. Ground material was transferred to 2.0 ml Eppendorf tubes and stored at –80°C. Four half-inch leaf discs were used in each tube (~100 mg) and two to three tubes were prepared for each salal sample. The protocol for this study is a modification of that suggested in Weising *et al.* (1995) for isolation of plant and fungal DNA. CTAB buffer

(2.5% CTAB, 1% PVP-40, 1.4 M NaCl, 0.02 M EDTA, 0.1 M Tris-HCl, 0.5% β-mercaptoethanol added just before use) was pre-heated to 65°C and 800 μl was added to each sample, vortexed thoroughly, and incubated for 90 minutes at 65°C, gently mixing every 15 minutes. To extract DNA, an equal volume of CHL:IAA extraction buffer (24:1 chloroform:isoamyl alcohol) was added and mixed on a rocking shaker at 70 rpm (Metabios Rocking Shaker, Victoria, BC, Canada), or by inversion for 10-12 minutes. Layers were separated by centrifuging tubes at room temperature for 5 minutes at 10,000 rpm (Eppendorf Centrifuge 5417R, Hamburg, Germany). Aqueous phase (~650 μl) was transferred to new tubes and the extraction was repeated. This second aqueous phase (~400 μl) was transferred to 1.5 ml Eppendorf tubes and incubated at room temperature for 30 min with 10X RNase A (1 mg/ml, Sigma, Oakville, ON, Canada). DNA was precipitated with 0.6X vol. ice-cold isopropanol, mixed gently by inversion followed by 30 min incubation at 4°C, and centrifugation at 14,000 rpm for 5 min at 4°C to obtain pellet. The supernatant was poured off and the pellet washed with 1 ml of wash buffer (10 mM ammonium acetate and 70% ethanol) followed by a final spin at 14,000 rpm for 5 min at 4°C. Supernatant was poured off and tubes were inverted to drain remaining liquid off pellets. Pellets were air dried in the flow hood or by vacuum and resuspended overnight at 4°C in 25 μl TE buffer (10 mM Tris-HCL, pH 8.0, and 1 mM EDTA). DNA was isolated from two replicates of each salal sample and then combined for a final volume of 50 μl TE. Concentration and quality of DNA was tested with 1.2% agarose gel electrophoresis in 1X TBE and also using a spectrophotometer (Pharmacia Biotech, Amersham, Piscataway, NJ, USA). DNA was diluted to 100 ng/μl for the AFLP reactions.

**3.2.4. AFLP analysis.** *Primer and reproducibility testing.* Primers from previous AFLP work on *Rhododendron* (Escaravage *et al.* 1998, Pornon *et al.* 2000), and variations thereof were screened. Of the 36 primer pairs tested, four that produced clear banding patterns and polymorphisms were selected for use. To ensure reproducibility, the procedure was independently repeated on a reduced sample set from three populations. 500 ng DNA was used in each reaction, and the AFLP protocol, including digestion, ligation, PCR amplification, and visualization were as described in Chapter 1. For the final selective amplification, one +3/+4 and three +3/+3 primer pairs were used, where the first sequence refers to the EcoRI primer and the second refers to the MseI primer: ACC/CCTT, ACC/CGT, AGG/CTC, and ACA/CGT.

*Assumptions and scoring of AFLP gels.* When scoring the AFLP fingerprints, it was assumed that comigrating bands on a gel were identical in sequence and that repeatable loci represented stable fragments. Given the predominantly vegetative growth and selfing capabilities of salal, Hardy-Weinburg (H-W) equilibrium could not be assumed so the Lynch and Milligan (1994) correction factor for diploid species with dominant markers was not used to calculate allele frequencies or heterozygosity. Instead, the dominant allele frequency was calculated as $p=x/N$ where x is the number of individuals with the marker and N is the total number of individuals. This inflates the frequency of the dominant allele by masking the contribution of heterozygotes, leading to an underestimate of gene diversity (Lynch and Milligan 1994). When scoring the AFLP gels for presence or absence of bands, individuals showing no band at a locus were considered absent (null), and those with a band (of any intensity) were considered present. Irrespective of ploidy, it was assumed that if there was no band present, the individual was homozygous recessive, whereas if there was a band, the individual was assumed to have at least one copy of the dominant allele. For a diploid, the presence

of a band could indicate either a heterozygote (one null and one dominant allele) or homozygous dominant (two copies of the dominant allele). For an octoploid, the presence of a band could indicate anywhere from one to eight copies of the dominant allele.

Some researchers have suggested that AFLP gels can be reliably scored based on band intensity to designate individual genotypes for each locus (Jansen *et al.* 2001). However, the program used to analyze gels in the present study, SAGA-MX, does not enable differential scoring of band intensity (i.e. cannot output quantified band intensity). While there are programs available that rate band intensity, they are extremely expensive, such as AFLP-Quantar (Keygene Products, Wageningen, The Netherlands), and often require using an entire PCR and gel-imaging system such as with Genescan (Applied Biosystems, Foster City, CA, USA). To score individuals for band intensity it must be planned at the beginning of the research to standardize and regulate the procedure to compare between gels. To informatively score by intensity in salal, more specific information would need to be known about chromosomal arrangements and behaviour of the chromosomes in the populations used in this study. It was decided that the most appropriate analysis would be to assume that salal is acting like a diploid, and analyze the data accordingly, knowing that the genetic diversity observed would represent a minimum of the total genetic diversity present in the populations.

**3.2.5. Data Analysis**. AFLP gels were scored using SAGA-MX for presence or absence of bands, which ranged from 64 - 660 bp. Based on frequency of the dominant (present) alleles, loci with frequencies >0.95 and <0.05 were trimmed to reduce the statistical error and variance introduced by these loci (Kohli and Kohn 1998, Hardy 2003); this approach is more conservative than the 3/N pruning recommended by Lynch and Milligan (1994). Individuals with missing data were not included in the analyses as

they biased pairwise comparisons. Average gene diversities and heterozygosities were calculated across all loci from the square root of the frequency of the null genotype ($q^2 = N_{null}/N_{total}$), assuming a dominant, diploid system (Hendrick 2000). Fingerprints were compared within and among populations using Arlequin Version 2.001 (Schneider *et al.* 2000) to determine the number of pairwise mismatches between individuals. The observed mismatch distribution was also compared to expected distributions based on a step-wise expansion model using a parametric bootstrap approach in Arlequin.

Differentiation among salal populations was determined from principal components analysis (PCA) in SAS (SAS Statistical System Version 8.0) and AMOVA analysis (Excoffier *et al.* 1992) using Arlequin. A matrix of population pairwise genetic distances ($F_{ST}$) and number of mismatched loci between populations was also obtained. The pairwise $F_{ST}$ values were calculated from gene frequencies corrected for sample size (Lynch and Milligan 1994) based on the method of Reynolds *et al.* (1983). The pairwise $F_{ST}$ values were used to create an unweighted pair group method with arithmetic mean (UPGMA) dendrogram with 100 bootstrapped replicates of the data in PHYLIP Version 3.57c (Felsenstein 1995). A mantel test was performed in Arlequin to determine if there was a correlation between population pairwise matrices of genetic and geographic distances where the null hypothesis is no association between the matrices.

To study fine-scale population structure of the Shawnigan Lake (SL) population, SPAGeDi (Version 1.1, Hardy and Vekemans 2002) was used as it does not assume that populations are in H-W equilibrium (Hardy 2003). It does require previous knowledge of the inbreeding coefficient, although since it was unknown, the analysis was run using several different inbreeding coefficients. To test if genetic relatedness between individuals was correlated with spatial distance, the kinship estimator was

used, defined as $F_{ij} = Q_{ij} - \bar{Q} / 1 - \bar{Q}$ where $Q_{ij}$ is the probability of identity in state (IIS) between random genes *i* and *j* within a population and Q is the probability of IIS between random genes within a reference population (Hardy 2003). Hardy (2003) reported that similar averages and standard deviations were obtained when this model was compared to co-dominant markers. Different population sizes did not affect the results, although both types of markers showed downward bias of kinship estimates and higher standard deviations when populations were small (30 individuals). The SL population had 111 individuals once samples with missing data were removed so the results were not biased due to sample size.

## 3.3 RESULTS

The four primer pairs used to generate AFLP fingerprints resulted in a total of 230 scorable loci. Some individuals had missing data (i.e. did not amplify for one or more primer pairs), so when these individuals were removed from the analysis, total sample size was reduced from 361 to 273 individuals and resulted in two of the 230 loci being monomorphic for the dominant allele. Combined over all populations the loci were 99.1% polymorphic (Table 3.1). The average percentage of polymorphic loci within populations was 89.7% where the Shawnigan Lake population had the lowest diversity (87.0% polymorphic loci) compared to Sayward and Deak's Peak, where 92.0% of the loci were polymorphic (Table 3.1). Once loci with >0.95 and <0.05 were removed, it resulted in a total of 171 loci being used for population-level analyses and 143 loci used to for population structure analysis of SL (Table 3.2).

Table 3.1. Sample size, number of monomorphic loci, and % polymorphic loci in each population based on 230 loci, where the dominant allele refers to the presence of a band and the recessive allele refers to the absence of a band.

| Population | Sample size[1] | No. monomorphic loci | | % Polymorphic loci |
|---|---|---|---|---|
| | | No. dominant | No. recessive | |
| SL | 111 | 30 | 0 | 87.0 |
| ML | 56 | 21 | 2 | 90.0 |
| SA | 35 | 15 | 3 | 92.1 |
| PH | 39 | 24 | 5 | 87.4 |
| DP | 31 | 9 | 9 | 92.1 |
| Total | 273 | 2 | 0 | 99.1 |

[1] Individuals with missing data were excluded.

Table 3.2. Number of loci obtained from each AFLP primer pair for all populations and number of loci used in among-population analysis and within SL analysis, after pruning loci with very high or low frequencies[1].

| Primer pair | Total No. Loci | No. Loci used for all pops | No. loci for SL |
|---|---|---|---|
| ACC/CGT | 71 | 53 | 49 |
| ACC/CCTT | 63 | 45 | 36 |
| ACA/CGT | 47 | 35 | 28 |
| AGG/CTC | 49 | 38 | 30 |
| Total | 230 | 171 | 143 |

[1] Loci with dominant frequencies >0.95 and <0.05 were not included in analyses.

**3.3.1. Genetic diversity**. Average gene diversity across all populations was calculated using the 171 loci. Due to the dominant nature of the markers and because salal has been assumed to be acting as a diploid, the frequency of the recessive locus can be estimated from the square root of the observed frequency of the null genotype in the populations. In this case, across all loci in all populations, the observed frequency of the recessive, null genotype ($q^2$) was 0.36, resulting in an average gene diversity of the null allele ($q$) of 0.56. Subtracting $q$ from 1 gives 0.44, the average frequency of the dominant allele ($p$) and an average heterozygosity of 0.49, calculated as $H=2pq$. For the SL population, the average gene diversity of the null allele based on 143 loci was 0.55, with an average heterozygosity of 0.50, similar to that obtained for the other populations, which ranged from 0.46 in DP to 0.50 in ML. The average heterozygosity within populations was 0.49, indicating that 49% of the individuals in the populations are heterozygous, with at least one copy of a null allele. No two individuals had identical fingerprints within or between populations and the percentage of polymorphic loci was very high. This is amplified by the nature of the dominant AFLP loci, as they underestimate the diversity in the populations. It is possible to obtain identical fingerprints by removing variable loci; however, this may further misrepresent the level of diversity in the populations.

The number of pairwise differences between individuals within each population, ranged from 47 (SL) to 61 (DP) mismatches between individuals (Table 3.3). In some cases, pairs differed by only a few loci, and could therefore be considered as belonging to the same clone. For example, in SL, six individuals differed by ≤5 mismatches. The $p$-values for the mismatch distributions from each population were not significant, so the null hypothesis of fewer differences observed than expected, could not be rejected, suggesting that the number of differences observed is less than expected from a

randomly mating population and provides support for some level of clonality or selfing in the populations.

Table 3.3. Number of pairwise mismatches between individuals within each population, showing the observed variance, minimum and maximum number of mismatches between pairs, and the *p*-value obtained from comparing observed and expected mismatch distributions.

| Population | No. Pairs[1] | Ave. No. Mismatches | Minimum No. mismatches | Maximum No. mismatches | Observed Variance | *P*-value[2] |
|---|---|---|---|---|---|---|
| SL | 6105 | 47.183 | 1 | 85 | 174.490 | 0.67 |
| ML | 1540 | 51.627 | 10 | 92 | 190.655 | 0.87 |
| PH | 741 | 55.968 | 12 | 93 | 198.588 | 0.76 |
| SA | 630 | 62.298 | 25 | 96 | 122.703 | 0.96 |
| DP | 465 | 61.338 | 23 | 87 | 166.129 | 0.54 |

[1] Number of pairs determined by n(n-1)/2, where n is the population size.
[2] *P*-value is for the null hypothesis where the expected mismatch distribution, calculated using a step-wise expansion model, is greater than or equal to the observed distribution based on a parametric bootstrap approach (Schneider *et al.* 2000). Significant *p*-values (≤0.05) would reject the null hypothesis and indicate that more differences were observed than expected from the simulated distribution.

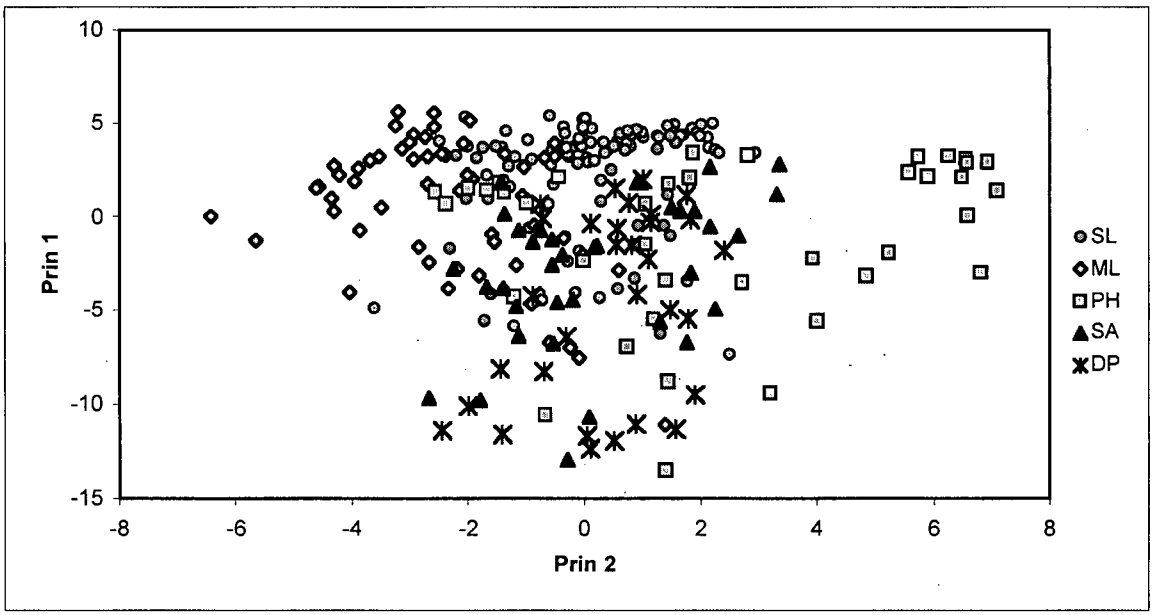


Figure 3.2. Ordination of the first two principal components of all five salal populations, explaining 14.6% of the variation.

**3.3.2. Differentiation among populations.** To assess the level of differentiation between salal populations, principal components analysis (PCA) was performed and the first two principal components, explaining 15% of the variation, were plotted (Figure 3.2). PCA resulted in 57 principle components with eigenvalues greater than one, explaining 76% of the variation, suggesting many loci are required to represent the variation observed in these populations. The ordination reveals differentiation of some individuals from SL, ML, and PH; however, other individuals and populations SA and DP, in particular, were not separated based on these two principle components and suggests that either there is very little differentiation among these populations, or that the differences are not explained by these principal components (Figure 3.2). The AMOVA resulted in an $F_{ST}$ of 0.0957 where 90.43% of the variation was partitioned within the populations and 9.57% of the variation was explained by differences among populations (Table 3.4). Using Wright's (1943) island model, gene flow can be estimated from $Nm=1/4[(1/F_{ST}) - 1]$ (Slatkin 1987), suggesting that there are on average 2.36 migrants per generation between populations.

The matrix of population pairwise $F_{ST}$ values in Table 3.5 suggest that SA and DP are the least differentiated populations ($F_{ST}$=0.0532), although it should be cautioned that these two populations also had the smallest sample sizes. SL and ML are also very similar ($F_{ST}$=0.0620) compared to SL and DP, which had the highest $F_{ST}$ of 0.1239 (Table 3.5). UPGMA analysis with the population pairwise $F_{ST}$ values resulted in a dendrogram showing the genetic distances (Figure 3.3). SL and ML formed one cluster while PH, SA, and DP formed a second cluster, in which SA and DP shared the most similarities (Figure 3.3).

Table 3.4. Analysis of molecular variance (AMOVA) for five salal populations resulted in an $F_{ST}$ of 0.0957. All tests were highly significant ($p<0.0001$) based on 1023 permutations[1].

| Source | d.f. | Sum of Squares | Variance Components | % of Variation |
|---|---|---|---|---|
| Among Populations | 4 | 673.025 | 2.800 | 9.57 |
| Within Populations | 268 | 7088.484 | 26.450 | 90.43 |
| Total | 272 | 7761.509 | 29.250 | |

[1] A second AMOVA was done removing SL from the analysis to see if it biased the $F_{ST}$ due to different sample sizes. The $F_{ST}$ that resulted was 0.0858, similar to that when all populations were included.

Table 3.5. Matrix of population pairwise genetic distances ($F_{ST}$) values for salal populations corrected for sample size.

| Population | SL | ML | SA | PH | DP |
|---|---|---|---|---|---|
| SL | -- | | | | |
| ML | 0.0620 | -- | | | |
| SA | 0.0713 | 0.0779 | -- | | |
| PH | 0.0839 | 0.1100 | 0.0712 | -- | |
| DP | 0.1239 | 0.1157 | 0.0532 | 0.0915 | -- |

A Mantel test comparing the matrix of population pairwise $F_{ST}$ values and pairwise spatial distances among populations was not significant suggesting the populations are not differentiated based on geographic distance, although this could also reflect the reduced power of the Mantel test with only five populations. The populations show low differentiation based on the AMOVA and PCA; however qualitative comparisons of the pairwise $F_{ST}$ values and the bootstrap values of the UPGMA dendrogram suggest there may be some isolation by distance (Table 3.5, Figure 3.3). For example, SL is most genetically similar to ML, which is the closest population geographically, and most genetically differentiated from DP, which is the most isolated from SL (interior island compared to coastal mainland populations). It

also suggests that populations are separated latitudinally, as SA is more differentiated from PH than it is from DP.

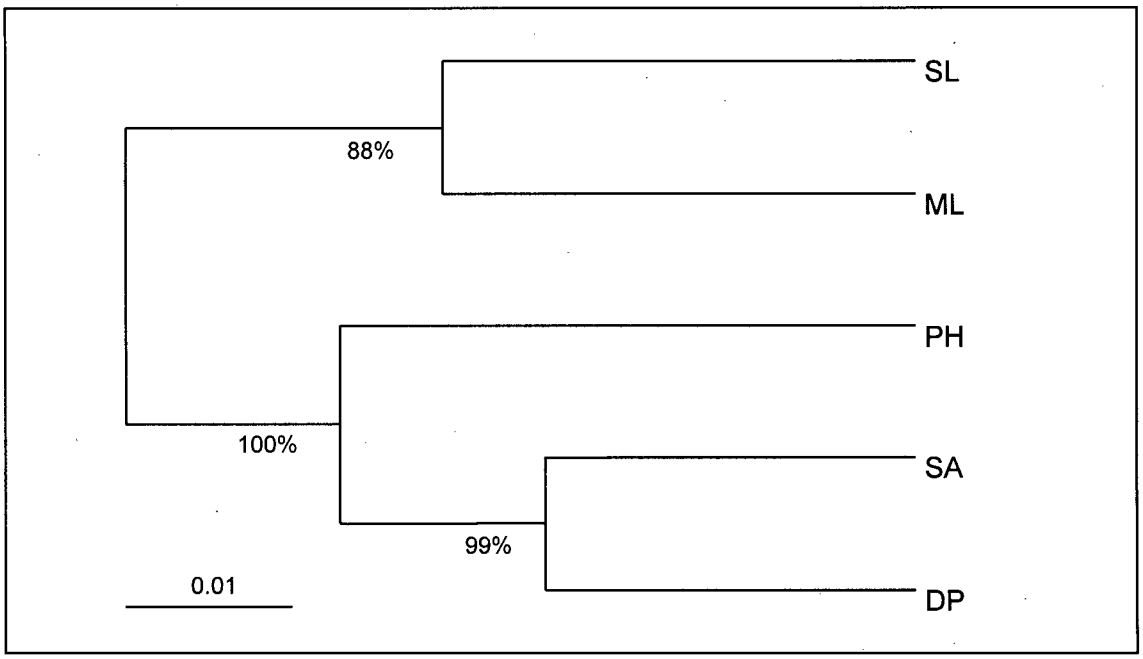


Figure 3.3. UPGMA dendrogram of salal populations based on pairwise $F_{ST}$ genetic distances. The percentage of 100 bootstrapped replicates are indicated at each branch point.

**3.3.3. Fine-scale structure within the Shawnigan Lake population**. The SL population was sampled more intensively than the other populations in order to analyze population structure within the population. Based on percentage of polymorphic loci, SL was not as diverse as the other populations where 30 of the 230 loci were monomorphic (Table 3.1). Trimming of loci for the SL population reduced the original 230 loci to 143 (Table 3.2), and of the 87 loci removed, 82 had frequencies greater than 0.95. SPAGeDi was used to determine the kinship coefficient and spatial distance between pairs of individuals and showed that as spatial distance increased, genetic similarity decreased (Figure 3.4). An increase in kinship coefficients was observed as spatial distance increased from 5 m to 10m, which could be due to fragmentation of clones

followed by local competition among different clones. The average distance at this interval was 8.5 m, suggesting that diagonal neighbours from the sampling grid are closely related. The analysis suggests that individuals within 10 m are closely related and possibly clonal, compared to individuals greater than 10 m apart (Figure 3.4).

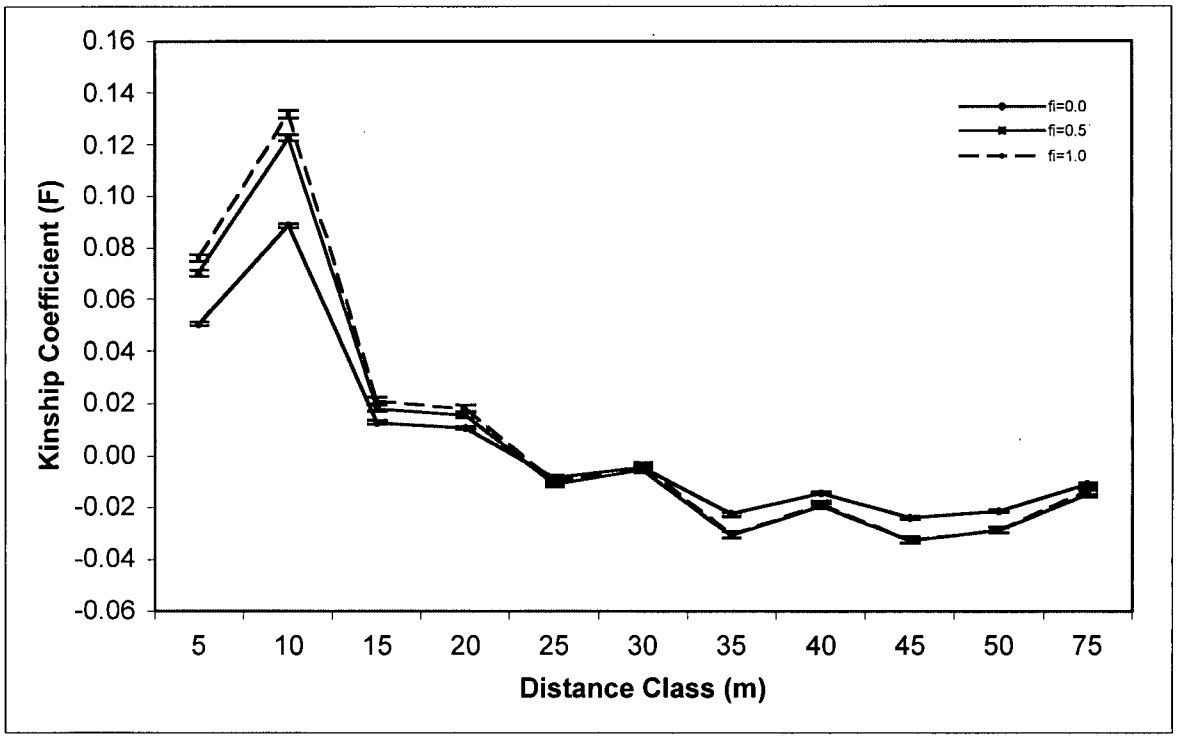


Figure 3.4. Relationship between genetic similarity (kinship coefficients, Hardy 2003) and spatial distance classes for salal individuals sampled from SL. The analysis was repeated with a range of inbreeding coefficients ($f_i$) from 0 to 1. Error bars are average jackknifed standard errors based on measures of actual variance over all loci for each inbreeding coefficient (Ritland 1996).

To visualize the level of relatedness between individuals, pairwise kinship coefficients were partitioned into groups (Figure 3.5). Some individuals shared very high kinship coefficients, but most were from 0.1 to –0.1, suggesting that most were no more related to each other than to the whole population. The closely related individuals could be from the same clone; however, as most of the pairwise comparisons were low,

it suggests there may be more random mating in this population than expected from a clonal population where many individuals would have high kinship correlations (i.e. greater than 0.95).

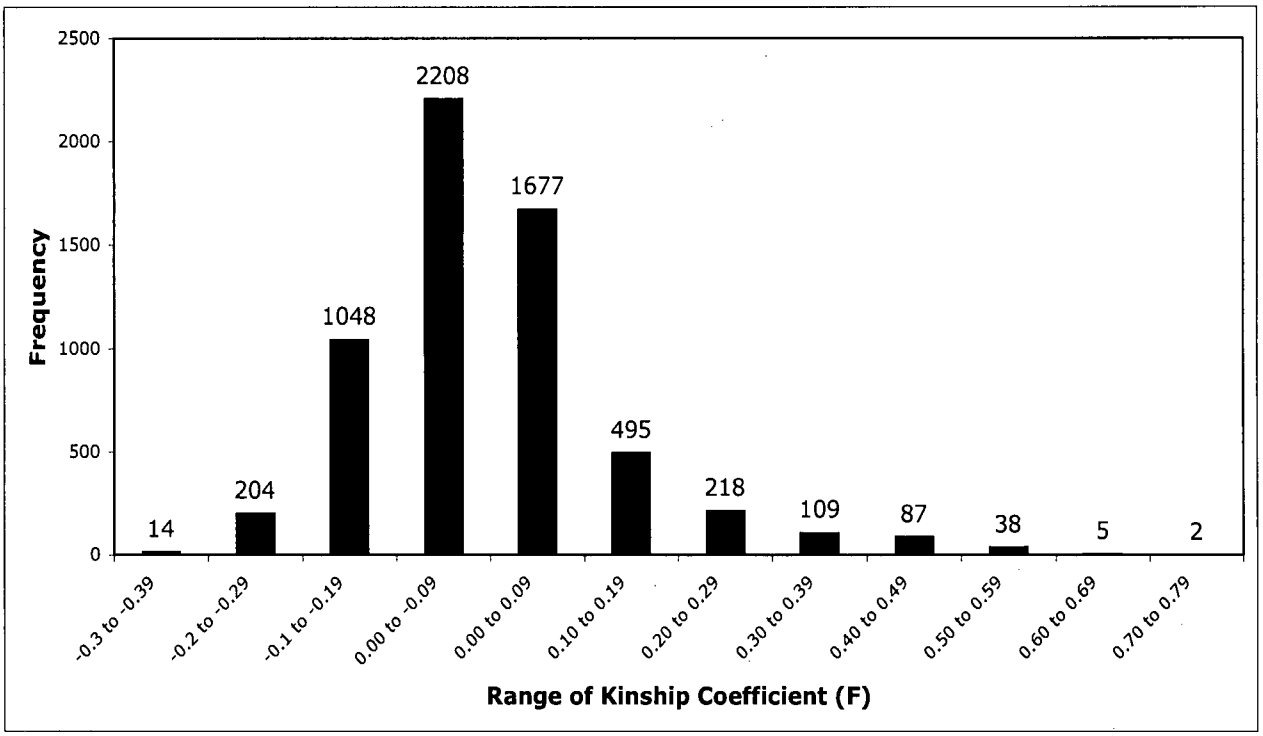


Figure 3.5. Frequency distribution of 6105 pairwise kinship coefficients between salal individuals from SL (n=111). Labels above each bar indicate the number of pairs within that kinship coefficient class.

## 3.4 DISCUSSION

Extremely high genetic diversity was observed within and among all five salal populations in this study as determined from the percentage of polymorphic loci. The average within population percent polymorphic loci was 89.7%, and the total percent polymorphic across all populations was 99.1%. As well, the average heterozygosity across all populations was 0.49. These results are similar to those found on studies with other ericaceous species. Using intersimple sequence repeat (ISSR) markers on *Gaultheria fragrantissima* Wall, Deshpande *et al.* (2001) revealed 83.7% polymorphism and Escaravage *et al.* (1998) found AFLP loci used to analyze *Rhododendron ferrugineum* L. were 88% polymorphic. Albert *et al.* (2003) found levels of genetic diversity in three clonal *Vaccinium* species equivalent to those observed in non-clonal *Vaccinium* species. While it is probable that the high ploidy in salal resulted in the higher levels of polymorphism than would be observed in the diploid species, these other studies suggest that the level of genetic diversity even in diploid Ericaceae species is very high, and are consistent with the results obtained for salal.

**3.4.1. Differentiation among populations.** Principal component analysis (PCA) and AMOVA showed little population differentiation among the salal populations used in this study. An overall $F_{ST}$ of 0.0957 was observed, which is comparable to the level of population differentiation observed for other long-lived woody perennial species (0.084, Hamrick *et al.* 1992), including other ericaceous species. For example, *Leiophyllum buxifolium* Elliot was found to have low population differentiation based on isozyme analysis (Strand and Wyatt 1991) and a study on *Leucopogon obtectus* Benth. using AFLPs found that 10.3% of the observed variation was partitioned between populations (Zawko *et al.* 2001). Low population differentiation in salal could be explained by high gene flow mixing the gene pools among populations. Assuming the

island model, an estimated 2.36 migrants would be necessary to maintain the level of differentiation observed. This level of migration could easily be achieved given the pollen and seed dispersal mechanisms in salal, especially among the populations used in this study, which were all from a relatively localized geographic region.

From population pairwise $F_{ST}$ values, SA and DP appear to be the most related populations (smallest $F_{ST}$) and there is some evidence of isolation by distance from the dendrogram (Figure 3.3). For example, SL and ML were geographically close to each other and had a correspondingly low $F_{ST}$. In contrast, the SA population was the most genetically similar to DP, which is on the mainland, compared to PH, which is the closest population geographically on Vancouver Island. This relationship could be explained by wind patterns, effectively shortening the distance between mainland and island populations, or by a founder effect, where SA was founded by genetically different individuals (perhaps from mainland populations given its high similarity to the DP populations). There was also some evidence of north-south partitioning of the populations based on the observed genetic differentiation, where populations were separated on a north/south axis, which has been observed for many plant and animal species of the Pacific Northwest (Soltis *et al.* 1997).

Low population differentiation was also observed by Dorworth *et al.* (2001) in a study on phenotypic variation among salal provenances. They proposed that genetically different individuals established geographically close populations or that birds may disperse seeds over wide geographic distances (Dorworth *et al.* 2001). High levels of gene flow could explain the low levels of differentiation observed, although if this was the case, highly related individuals should have been observed among populations. Alternatively, the sampling may not have been truly representative of clones in the natural populations. Low differentiation could also be due to the AFLP

markers, where perhaps different loci or a different marker system would have revealed more differentiation among populations. Another possible explanation is related to the geographic distribution of salal, which is limited to the Pacific Coast from northern California to southern Alaska. The populations used for this study are from a relatively small area in the center of the species range, which could explain the high diversity observed within populations and the low differentiation between populations. If the populations were further apart, for example from the edges of the range, it is likely they would be more differentiated.

**3.4.2. Fine-scale structure in Shawnigan lake**. A relationship between genetic similarity and spatial distance was observed within SL, where as spatial distance increased, genetic similarity decreased. This relationship was also observed using AFLP markers in *Rhododendron ferrugineum* populations (Escaravage *et al.* 1998). Salal was predicted to have a predominantly clonal population structure based on previous studies of reproductive and growth strategies of salal; however, the level of genetic similarity between individuals in this study does not seem to indicate a primarily clonal population. For example, no two individuals with identical fingerprints were detected. While some individuals had high kinship coefficients and differed by only a few loci, which could be explained by somatic mutations (for example in disconnected ramets of a clone), or even by scoring error, this was true for only a small fraction of the population. Therefore, only some of the individuals sampled from the population are likely from the same clone. When genetic similarity was compared to spatial distance, higher kinship coefficients were observed between individuals 10 m apart than those 5 m apart, which could be explained by local competition among clones, or could be reflecting a guerilla growth form resulting in widely spaced, intermingling ramets. These results indicate that clonal (or closely related) individuals were found within 10 m of

each other, which could indicate a limit to clone size, or possibly a switch from clonal to sexual reproduction. The result is more genetic differentiation between individuals than expected for a clonal population suggesting more random mating in the population than previously thought. Since the level of diversity is underestimated due to the dominant AFLP markers, the level of relatedness or clonality inferred from this study would be an overestimate. The most highly related individuals are likely found at a smaller, more local spatial scale than 10 m, for example within the 5 m blocks sampled. This is supported by Huffman *et al.* (1994) who found that the largest salal clone in their study was 29 $m^2$.

If more informative markers did show a reduced relationship between genetic similarity and distance, it would not necessarily mean that the population is not clonal. For example, with the ericaceous shrub *Elliottia racemosa*, Godt and Hamrick (1999) found that while the populations were highly clonal, the distribution was not related to spatial distance within the population, and they proposed that separating and dying off of ramets made room for the growth of other clones, leading to a population of widely dispersed stems of the same clone. If this was true with salal, one would still expect to find many individuals with high genetic similarities within populations (regardless of spatial distance), which was not observed in this study.

**3.4.3. Ploidy level and chromosome behavior**. The results from this study are an underestimation of genetic diversity largely because the ploidy level and segregation of chromosomes in these particular samples is not known. Since salal has been reported to be an autopolyploid (Callan 1941), it is feasible that it could be acting like a diploid, able to form homologous pairs of chromosomes and undergo normal meiosis. Other ericaceous polyploids have also been observed to behave as diploids, such as *Vaccinium corymbosum* L. (Krebs and Hancock 1991).

Some researchers have suggested that by observing the intensity of bands produced from AFLP, the number of genotypes can be predicted where, assuming all reaction conditions are identical, the number of dominant alleles present in an individual would be correlated to band intensity (for example, Jansen *et al.* 2001). By observing locus 459 (Figure 3.6), the band intensity of many of the individuals is approximately half that of the individuals with the darkest bands. This suggests that the moderate intensity refers to an heterozygous individual and the darkest intensity to homozygous dominant individual. The three band intensities (absent, light, and dark) could correspond to the three genotypes expected from a diploid and support that salal may be acting like a diploid for these loci. If it is octoploid, the moderate band intensity could represent four null and four dominant copies. Alternatively, there may be two functional genome copies (that result in the observed three genotypes) and six non-functional copies.

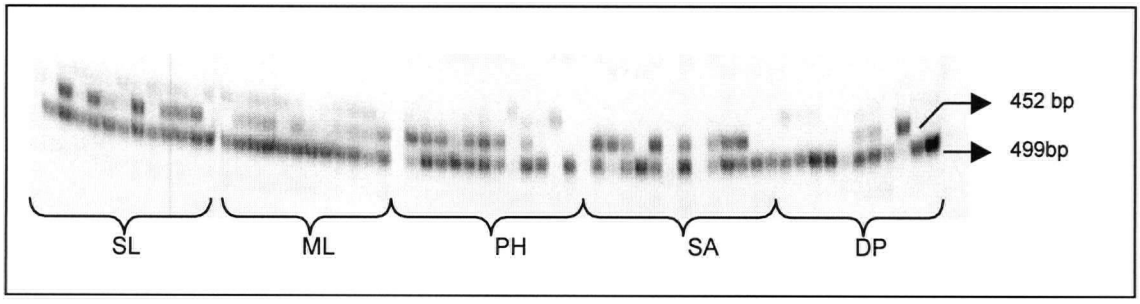


Figure 3.6. A salal AFLP gel showing samples from each population at two loci, 452bp and 449bp (50-750 bp molecular size marker not shown), which could be interpreted as diploid as there are three predominant genotypes observed: AA (dark intensity), Aa (moderate to light intensity), and aa (null, no band intensity). Some variation between intensities is due to experimental error, as the samples have not been normalized to account for variation between lanes.

Using AFLP fingerprints to indicate ploidy level and allele number can also be complicated depending on how the restriction enzyme cut sites are maintained within the genome copies. If there are eight copies of a locus in salal, over time, mutation,

crossing over (of homologous chromosome pairs), and chromosomal rearrangements will create variation among genome copies. With enough mutation, the location of restriction enzyme cut sites may change, varying the size of the fragment produced from digestion. Since AFLP loci are distinguished by size, loci separated by only a few base pairs may be variants of the same locus. If mutation and recombination are the major forces creating variation between the copies in neutral markers, the different variants may be observable on AFLP gels. For example, the ACC/CGT primer pair yielded a strong locus 279 bp followed by a series of what appear to be stutter bands, over a range of 40 bp from the locus (Figure 3.7). It is interesting to consider that these stutter bands could represent different alleles, rather than PCR artefacts. The stutter bands were not scored for inclusion in the above analyses because they were not present for all samples or clearly interpretable. However, it would be interesting to test if these bands are simple variants of the same locus, differentiated by mutation and recombination, by sequencing the fragments to determine the level of homology between the loci.

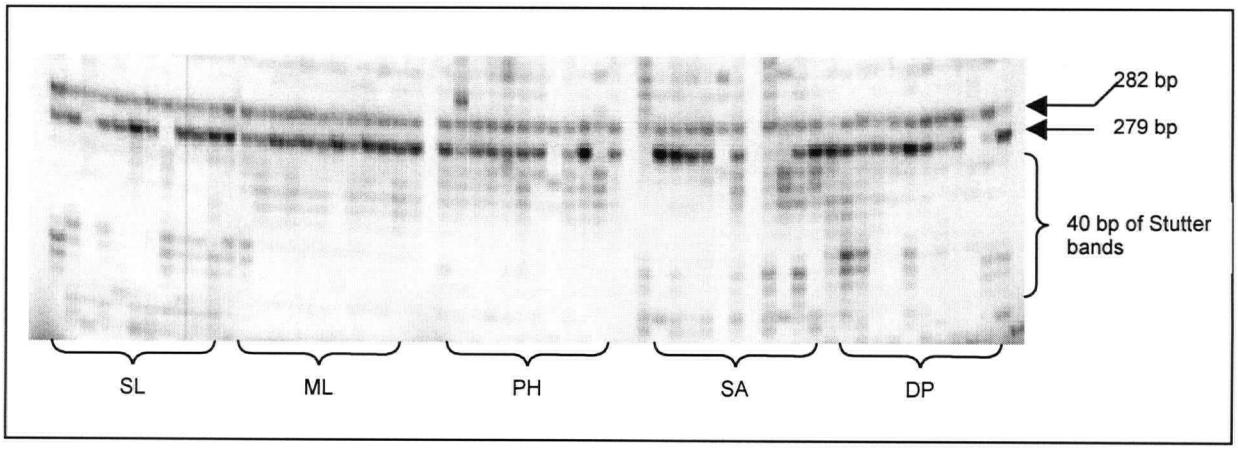


Figure 3.7. Section of a salal AFLP gel using primer pair ACC/CGT showing a 40 bp range of stutter bands, which may be sequence variants of the locus resulting from polyploidy (50-750 bp molecular size marker not shown).

**3.4.4. Recommendations for future research**. The results obtained from this study show that salal is very diverse; however they are somewhat limited due to the data available from the AFLP markers. If more was known about the mating system and cytology of salal, a more appropriate marker system could have been chosen to gain more detailed information about population structure. This type of information is important, since studies of polyploid species have shown that most of them have formed recurrently, and undergo significant chromosomal rearrangements as well as divergent evolution among the duplicated genomes (Soltis and Soltis 2000).

Flow cytometry could be used to approximate the amount of DNA in individuals from different populations to determine if the same ploidy level is observed throughout these populations, as was done for blueberry (Costich *et al.* 1993). Observing meiosis in cells from each population would have helped determine if salal behaves as a diploid in terms of chromosome segregation and could reflect the level of variation that has occurred between duplicated genomes within these populations (i.e. if there were more pairs of chromosomes than multivalent associations of chromosomes, Stebbins 1971). The level of homology between chromosomes could indicate time since polyploid formation, which would greatly affect the populations because when polyploidy initially arises, it is thought that populations experience a bottleneck, where reproduction is primarily asexual and then over time the level of sexual recombination increases (Stebbins 1971).

A more definitive method to determine whether salal behaves as a diploid would be to determine the number of functional gene copies. This could be assessed by studying mRNA expression and sequencing cDNA libraries. The level of sequence homology between functional genes and the number of gene copies expressed could provide insight into how salal 'uses' its polyploidy, and the potential it may have to

evolve pathogen resistance, key to developing a successful biocontrol agent. Crosses could also be done to assess segregation of loci in the offspring. Segregation ratios could indicate whether there is preferential pairing of chromosomes, if salal behaves as a diploid, and the origin of the ploidy (auto- or allo-polyploid, as in Barcaccia *et al.* 2003).

A codominant marker system would likely have been more informative than AFLP for this species; however, those markers also have both advantages and disadvantages. For example, with microsatellites, considerable research must be invested to develop primers. Alternatively, isozymes are readily available and have been used in many other studies to analyze polyploidy, but show lower levels of diversity. However, as previously mentioned, preliminary isozyme analysis with salal revealed complex banding patterns which were difficult to interpret. Other studies of isozymes in the Ericaceae have found that they are highly variable, for example, 90% of the loci were polymorphic for cultivated diploid blueberry species (Bruederle *et al.* 1991), which would be even more complicated with an octoploid species. With any marker system it seems that some sequencing may be necessary to confirm the observations in the electrophoretic markers.

## 3.5 CONCLUSION

The AFLP loci used in this study represent a minimum level of diversity due to their dominant nature; however, they show that within populations salal is very diverse with low genetic differentiation among populations, corresponding with other studies of woody perennial species (Hamrick *et al.* 1992). The level of genetic differentiation between populations was not consistently related to geographic distance, although this could be due to high gene flow, wind patterns among populations, or the relatively small distances between populations. Within the population from Shawnigan Lake, genetic similarity was related to spatial distance, indicating that there may be a limit to clone size or possibly a switch from predominantly vegetative growth to sexual reproduction at a distance of 10 m. These results suggest that for further studies on salal, samples should be collected >10 m apart to obtain different individuals. While a clonal population structure was not readily observed in this study, it may be that salal is only clonal at a very local scale at these study sites.

## CHAPTER 4. Discussion of efficacy and potential risks of using *Valdensinia heterodoxa* as a biocontrol for salal.

### 4.1 RISK ANALYSIS

The objective of understanding the plant and pathogen populations in a biocontrol system is to predict the effects of releasing the agent into the environment. With biocontrol of native plants using mycoherbicides, the outcome and effect on the ecosystem should therefore be rigorously analyzed. When considering risks (defined as the probability of a hazard or harmful effect) one must consider all the organisms which may be affected, including nontarget organisms that may be impacted by a change in the environment (Cook *et al.* 1996). Recombination, sources of variation, gene flow within and among populations (and even between species), and selection can all potentially affect the population dynamics of both the host and natural pathogen populations.

**4.1.1. Plant-Pathogenic Fungal Populations**. Plant-pathogenic fungi vary considerably in the level of diversity and mating systems, although many are predominantly clonal and produce haploid asexual spores with the incorporation of a (rare) diploid sexual phase somewhere in their life cycle. Migration and colonization are often limited by spore dispersal, which is dependent on abiotic factors such as wind and rain, or by animal and insect vectors (Te Beest *et al.* 1992), all of which apply to *V. heterodoxa*.

Predominantly clonal populations typical of *V. heterodoxa* are expected to have low effective population size, reduced variation, and accumulation of deleterious mutations resulting in mean reduced fitness (Kohn 1995). Recombination resulting from sexual reproduction, however infrequent, is sufficient to maintain genetic diversity in these populations (Taylor *et al.* 1999). Gene flow can also create diversity within

populations by spreading new genes and combinations of gene complexes while selection and drift within each population will amplify differences among populations (Slatkin 1987). Particularly when studying plant-pathogen systems, gene flow is key in cycles of extinction and recolonization, dependent on the source and number of founder individuals and random mixing between individuals from residual populations (Slatkin 1987, McDermott and MacDonald 1993). The patchy distribution in *V. heterodoxa* makes this a likely scenario. It is possible for virulence genes to be passed via gene flow among pathogen populations when used as a biocontrol. The strength of selection will determine if a virulence gene or complex of genes will be maintained at high frequencies within populations despite the presence of gene flow (McDermott and McDonald 1993).

The release of large amounts of a biocontrol strain could disrupt the existing population structure of the pathogen by introducing virulence alleles. If the natural pathogen populations are not very diverse, they may not have the ability to adapt or compete with the high concentrations of the biocontrol strain until the target plant population is substantially reduced. By colonizing all of the available target hosts, the biocontrol strain effectively suppresses the natural strains of the pathogen, either sending them toward extirpation or 'forcing' them onto nontarget hosts (Cook *et al.* 1996).

The level of gene flow within and among populations can also alter the effectiveness of the biocontrol and its interactions with natural populations. Virulence may persist beyond the epidemic caused by releasing the mycoherbicide (McDermott and McDonald 1993). High gene flow could spread the biocontrol strain across many populations, maintaining it at a low concentration. Low gene flow would restrict the biocontrol strain to the populations where it was applied. This may help control the

spread of the biocontrol, but it could potentially be more damaging to organisms in the populations where the biocontrol is applied.

The persistence and abundance of the biocontrol strain or frequency of the virulence alleles (if recombination has occurred) is largely dependent on natural selection. If the biocontrol strain is reduced to a low concentration after the initial epidemic, selection may be less likely to increase its frequency. Without such selection, the virulence genes would be lost from the population over time due to the predominate clonal reproduction. However, if the virulence alleles are maintained at moderate frequencies in these populations, selection could act on these alleles, eventually bringing them to fixation. Even if this does happen, the interactions between host and pathogen may necessitate the evolution of other virulence loci as the plant population evolves, increasing resistance to the virulence introduced by the biocontrol strain.

**4.1.2. Other microorganisms**. The release of a biocontrol could also affect other microorganisms in the ecosystem by inhibiting their growth and persistence on the host plant and decreasing their ability to compete. While many of the risks that apply to the natural populations of the mycoherbicide also apply to other microorganisms in the surrounding environment, other specific risks apply to associated nontarget microorganisms. These may include other pathogens, or they may be beneficial organisms that aid the plant, for example endophytes that provide protection from insect pests. The entire microflora of the target plant and its surrounding could be affected (Cook *et al.* 1996, Myers 2001). Population size of other microorganisms may be limited by host depletion. This has been a concern with the release of *Bacillus thuringiensis* (Bt) preparations in forests, which kills its gypsy moth target host but also kills other lepidopteran larvae, thus reducing the available food source for birds (Cook *et al.* 1996). Selection pressure caused by the high concentration of mycoherbicide may

increase the likelihood of horizontal virulence gene flow (the stable transfer of genetic material between species). Microorganisms such as bacteria have several different mechanisms for horizontal gene flow: conjugation, transformation, and transduction, and while the mechanisms in fungi are largely unknown, there is evidence that it occurs with the transfer of plasmids, introns, and transposable elements (Rosewich and Kistler 2000). If virulence is transferred to non-related microorganisms, including other plant and possible animal pathogens, overall levels of disease in the ecosystem would increase by altering the pathogen dynamics or synergistic interactions with other pathogens, including the mycoherbicide (Cook *et al.* 1996).

**4.1.3. Host plant populations**. The risks to the target plants are often not of great concern in control strategies. However, where the host plant is native to the area, it becomes important to consider what will happen to the residual populations particularly since the goal of the mycoherbicide is not to eradicate the entire host populations, but rather to reduce them (Wall *et al.* 1992). One major concern is that continued application of highly virulent pathogen strains will select for resistant plant strains as population bottlenecks after each application of the biocontrol result in increased frequency of resistant residual plants, depending on the amount of gene flow and migration among founder populations (Slatkin 1987, McDermott and McDonald 1993). As the host recolonizes an area, it will do so with these resistant strains, making it more difficult to control, necessitating the development of more virulent natural strains, which could be found by surveying natural populations for disease in putatively these resistant plants, or by genetically engineering the existing mycoherbicide for increased pathogenicity, which is highly contentious (Greaves *et al.* 1989). The target plant populations may be induced from the stress caused by the epidemic, to shift from predominantly vegetative growth to increased sexual reproduction. This would then

result in more recombination and increase the probability of the development of new resistance phenotypes in the plant on which selection could act (Barrett 1982).

The results from this study suggest that salal has very high genetic diversity and low interpopulation differentiation, which has several consequences for biocontrol. Firstly, it may be difficult to find one strain of *V. heterodoxa* that is effective on salal such that a cocktail of several different strains may need to be developed, effective over a wide range of salal, otherwise, the mycoherbicide may have limited effect. Secondly, high diversity and polyploidy indicate salal could quickly reduce its susceptibility to a biocontrol strain, especially if there is more sexual reproduction and recombination than previously thought and if the additional gene copies resulting from increased ploidy level are a source of variation (Soltis and Soltis 2000). Finally, depending on the goal of the biocontrol, the low genetic differentiation among populations would make the biocontrol more predictable across populations. However, if the goal was to apply the biocontrol to a more localized area, then low differentiation may be a disadvantage as high gene flow would spread the biocontrol strain to other populations. The ideal situation would be to have differentiated populations with very low gene flow so the biocontrol strain would be restricted to the target area, and potentially easier to control and monitor.

**4.1.4. Non-target plants**. The biocontrol strain could also affect nontarget plant populations. One of the most controversial issues surrounding the release of a biocontrol agent is the shift of the pathogen to nontarget hosts (Cook *et al.* 1996, Myers 2001). Increased levels of the pathogen may persist in the environment, due to direct application or secondary infections of the mycoherbicide surviving beyond the initial epidemic, combined with reduced availability of the target plant from the successful primary attack by the mycoherbicide. The threat of shifting to nontarget hosts is of particular concern when there are known, economically important alternative hosts

nearby. For example, a mycoherbicide of *Chondrostereum purpureum* was developed for control of *Prunus serotina* in the Netherlands (Teng and Yang 1993) and weedy hardwood species in Canada (Hintz et al. 2001) and prior to release, a "safe distance" from commercial *Prunus* orchards had to be established in order to prevent spread to the crop trees. *V. heterodoxa*, has many hosts other than salal, including *Vaccinium* species, some of which, such as blueberries, are of considerable economic value in British Columbia.

## 4.2. COMPARISON OF GENETIC VARIATION IN *V. HETERODOXA* AND SALAL.

The results from this study have shown that the potential biocontrol and target host have vastly differing levels of diversity and population structure that in some ways differ from ideal characteristics of candidate species. UPGMA dendrograms based on population pairwise genetic distances (Reynolds *et al.* 1983) were obtained from gene frequencies corrected for sample size (Lynch and Milligan 1994) using Phylip Version 3.57c (Felsenstein 1995) and trees viewed in Treeview (Page 1996). The dendrograms show that the same clusters were observed in *V. heterodoxa* and salal among populations and as expected, there is greater differentiation among *V. heterodoxa* populations than salal populations (Figure 4.1). In both species, Port Hardy (PH) and Deak's Peak (DP) were more similar to each other than to Mesachie Lake (ML), indicating, as did the Mantel tests, that factors other than geographic distance differentiate these populations, further emphasizing the patterns of population differentiation observed in Chapters 2 and 3. The two matrices of population pairwise genetic distances were highly correlated (r=0.996), suggesting a strong relationship between *V. heterodoxa* and salal.

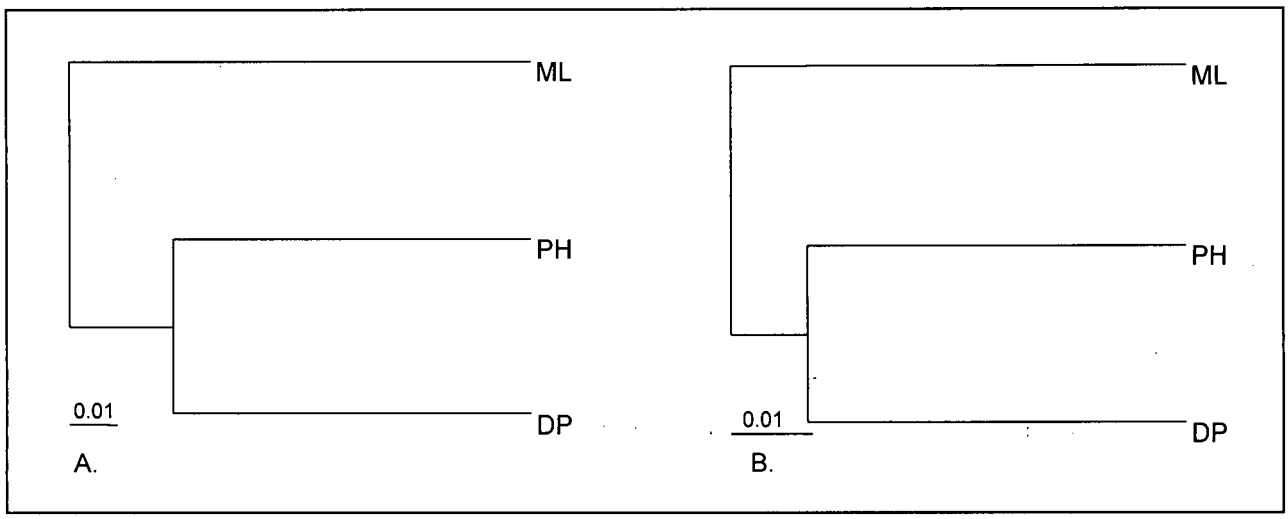


Figure 4.1. UPGMA dendrograms of genetic distances among A) *V. heterodoxa* and B) salal populations where *V. heterodoxa* and salal were sampled in tandem. All branches supported 100% by 100 bootstrapped replicates.

To further test the relationship between *V. heterodoxa* and salal, pairwise comparisons of alleles were made between species for samples collected from the same salal stem using a program compiled by K. Ritland (Forest Sciences Department, UBC). The program calculated normalized correlations between pairs of loci from each species across all individuals using the formula, $r_{ij} = \sum_i \sum_j \frac{(P_{is} - \overline{P}_{is})(P_{jv} - \overline{P}_{jv})}{\sqrt{\overline{P}_{is}(1-\overline{P}_{is})\overline{P}_{jv}(1-\overline{P}_{jv})}}$, where $P_{is}$ is the allele frequency for salal at locus $i$, $P_{jv}$ is the allele frequency for *V. heterodoxa* at locus $j$, and $\overline{P}$ is the within population frequency. All polymorphic loci were used in the analysis, 65 from *V. heterodoxa* and 228 from salal, resulting in 14,820 total pairwise comparisons. These multiple comparisons make significance testing difficult as any one pair is only a small fraction of the total; however, by comparing the observed distribution of correlations to a random distribution, those correlations that lie within the ±0.25 tails of the distribution can be considered significant.

This analysis was done to test for any associations between the species. For example, if a particular *V. heterodoxa* race commonly infects a certain salal variety, it would result in a high correlation of bands across individuals for each pair of loci. Figure 4.2 compares the distribution of observed correlations for pairs of alleles from *V. heterodoxa* and salal collected from the same stem to a random distribution (pairwise comparisons between *V. heterodoxa* and randomly selected salal loci) where pairwise comparisons were restricted to within each population. The observed distribution of correlations between pairs is wider and includes very high correlations (-1.0 and 1.0) compared to the random distribution (Figure 4.2). Associations between the species suggest that there may be different pathogenicity groups of *V. heterodoxa* and different varieties of salal in these populations that vary in their level of susceptibility and resistance to *V. heterodoxa*. This could limit the distribution of *V. heterodoxa* and explain the patches observed in the natural populations used for this study.

Some of the associations observed may be due to error, such as artifacts of the AFLP technique or contamination of the samples. However, contamination was likely minimal since pure cultures of *V. heterodoxa* were used and only salal leaves free of visible lesions were sampled. While it is possible that secondary pathogens associated with *V. heterodoxa*, such as viruses or bacteria not readily observed were present, it is unlikely that this would have a strong enough effect on banding patterns to create the significant correlations observed between the species. To test the biological relevance of these correlations, it would be interesting to conduct pathogenicity testing, where if the above relationship is true, certain *V. heterodoxa* isolates should be able to infect some salal plants more readily than others. Conversely, with known associations between pathogenicity and susceptibility of *V. heterodoxa* and salal, respectively, AFLP

fingerprints could be obtained and analyzed to see if the corresponding pattern of correlations is observed.

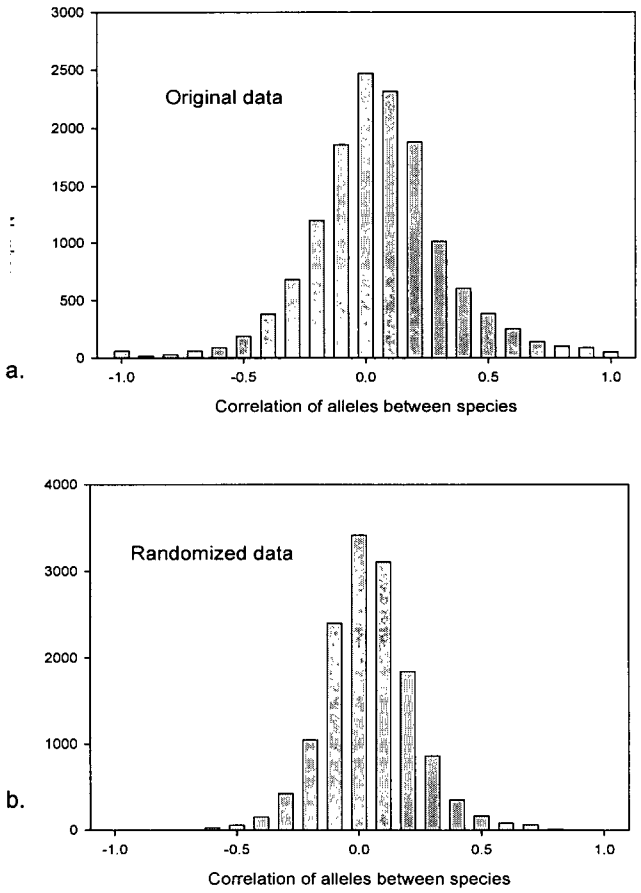


Figure 4.2. Distribution of correlations between pairs of *V. heterodoxa* and salal alleles showing the number of pairs at each correlation for a) samples collected from the same salal stem and b) randomly selected pairs.

The observed associations between *V. heterodoxa* and salal alleles suggest that there may be different genotypes of salal with varying levels of susceptibility to *V. heterodoxa* in these populations. If these associations reflect biological differences in susceptibility between salal genotypes than there should be a difference between infected and healthy salal plants, which would explain the patchy distribution of *V. heterodoxa* in these populations. In this case, comparisons of genetic distance between groups of infected and healthy salal plants should show more excessive differentiation

than comparisons between groups of infected and infected or healthy and healthy plants. Recall that in populations where both salal and *V. heterodoxa* were collected, healthy salal samples from between patches of infected salal were also collected (Figure 2.2). The samples collected from these populations (ML, PH, SA, and DP) can be divided into two groups, infected and healthy. Pairwise genetic distances (Reynolds *et al.* 1983) between infected and healthy salal groups were calculated and a dendrogram was created using Phylip (Figure 4.3). It was found that the healthy and infected groups were clearly differentiated from each other as the infected groups from each population all formed one cluster. The dendrogram also shows that the greatest differences are between healthy and infected groups and that infected groups are more similar to each other than to healthy groups. This suggests there are likely some commonalities among all infected salal groups that results in their greater susceptibility to infection by *V. heterodoxa*. Interestingly, the healthy group from SA was the closest to the infected groups, suggesting that perhaps it is not as differentiated as the other healthy groups. This could indicate that it may over time become infected with *V. heterodoxa* although it could also be a result of the shorter distances between healthy and infected plants within the SA population such that the plants are not as genetically differentiated.

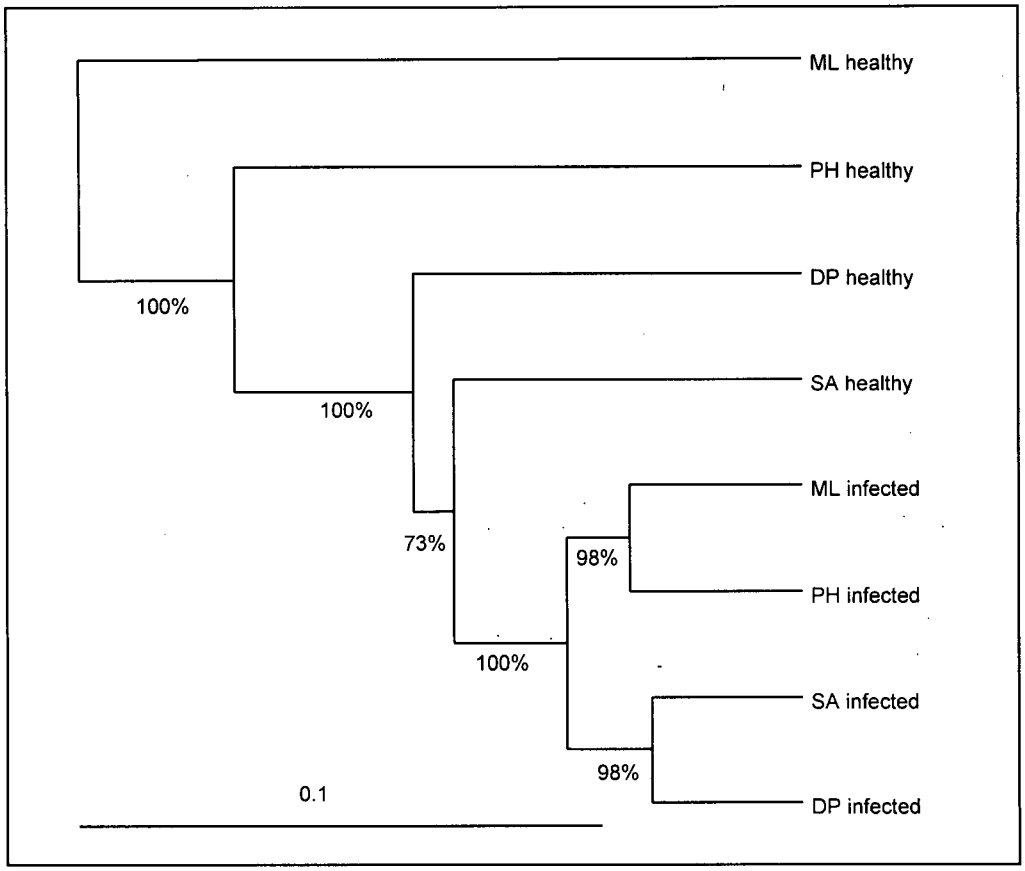


Figure 4.3. UPGMA dendrogram of healthy and infected groups of salal samples, based on pairwise genetic distances. The percentage of 100 bootstrapped replicates are indicated at each branch point.

## 4.3 CONCLUSION

Overall, this study found that *V. heterodoxa* has very low diversity and moderate population differentiation compared to the high diversity and low population differentiation more expected in an ideal biocontrol agent. As a result, *V. heterodoxa* may not be an ideal candidate for biocontrol in terms of the potential impact on the natural populations of the fungus based on the amount and distribution of genetic diversity. In salal, high diversity was observed where low diversity would be ideal, which could make developing a biocontrol extremely challenging. It may also partially explain why salal has been so difficult to control with traditional methods. The level of

population differentiation desired in the target population may change depending on the goal of the biocontrol, but if the intention was to control salal over a wide geographic range, than the low population differentiation may desirable as one effective biocontrol strain could be developed for the entire range.

The release of a mycoherbicide into its native habitat to control an indigenous weed has complex implications for both the target and pathogen populations, as well as for nontarget species and their ecosystem dynamics. Altering a species' distribution, particularly with respect to resistance and virulence in the plant and pathogen, has serious conservation and evolutionary implications, amplified when the species are native to the area. Native species are particularly important as they provide insight into local evolutionary patterns and indicate which traits are optimal for an environment.

In order to quantitatively assess the risks of releasing a mycoherbicide into the environment, strict guidelines must be followed to ensure that rigorous greenhouse and field testing are done prior to registration including susceptibility testing of as many nontarget organisms as possible. Stringent monitoring and management strategies specific to the biocontrol system will determine the long-term effects of releasing the mycoherbicide as well as controlling the application of the mycoherbicide so that it is only applied to target species and areas (Cook *et al.* 1996, Myers 2001).

Genetic variation, recombination, gene flow, and natural selection were the primary evolutionary factors shaping the structure of these plant-pathogen systems. Analyzing the risks of releasing a mycoherbicide from a population genetics perspective reinforces the importance of investigating the population structure of the natural plant and pathogen populations in order to predict the effects a particular mycoherbicide may have and to provide a baseline for retrospective comparisons to determine the actual effects of releasing the mycoherbicide into the environment.

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## Appendix 1

Table 1. Loci, allele frequencies, and gene diversity for within-population analyses.

| Mesachie Lake (n=60) | | | Port Hardy (n=36) | | | Deak's Peak (n=52) | | |
|---|---|---|---|---|---|---|---|---|
| Name | p[a] | $H_e$[b] | Name | p | $H_e$ | Name | p | $H_e$ |
| ACCAC597 | 0.567 | 0.499 | ACCAC597 | 0.639 | 0.475 | ACCAC597 | 0.769 | 0.362 |
| ACCAC490 | 0.900 | 0.183 | ACCAC490 | 0.583 | 0.500 | ACCAC222 | 0.769 | 0.362 |
| ACCAC205 | 0.250 | 0.381 | ACCAC210 | 0.333 | 0.457 | ACCAC210 | 0.096 | 0.177 |
| ACCAC203 | 0.383 | 0.481 | ACCAC193 | 0.306 | 0.437 | ACCAC203 | 0.250 | 0.382 |
| ACCAC191 | 0.067 | 0.127 | ACCAC191 | 0.389 | 0.489 | ACCAC191 | 0.365 | 0.473 |
| ACCAC143 | 0.433 | 0.499 | ACCAC172 | 0.556 | 0.508 | ACCAC172 | 0.154 | 0.266 |
| ACCAC130 | 0.150 | 0.259 | ACCAC143 | 0.167 | 0.286 | ACCAC143 | 0.192 | 0.317 |
| ACCAC94 | 0.450 | 0.503 | ACCAC94 | 0.028 | 0.056 | ACCAC76 | 0.654 | 0.462 |
| ACCAC76 | 0.717 | 0.413 | ACCAG603 | 0.111 | 0.203 | ACCAG603 | 0.115 | 0.208 |
| ACCAG231 | 0.200 | 0.325 | ACCAG599 | 0.222 | 0.356 | ACCAG361 | 0.192 | 0.317 |
| ACCAG203 | 0.800 | 0.325 | ACCAG361 | 0.528 | 0.513 | ACCAG231 | 0.192 | 0.317 |
| ACCAG157 | 0.067 | 0.127 | ACCAG203 | 0.778 | 0.356 | ACCAG203 | 0.789 | 0.340 |
| ACCAG137 | 0.800 | 0.325 | ACCAG198 | 0.167 | 0.286 | ACCAG160 | 0.558 | 0.503 |
| ACCAG134 | 0.167 | 0.283 | ACCAG152 | 0.083 | 0.157 | ACCAG157 | 0.269 | 0.401 |
| ACCAG131 | 0.200 | 0.325 | ACCAG131 | 0.444 | 0.508 | ACCAG137 | 0.789 | 0.340 |
| ACCAG130 | 0.033 | 0.066 | ACCAG106 | 0.417 | 0.500 | ACCAG131 | 0.289 | 0.419 |
| ACCAG106 | 0.300 | 0.427 | ACCAG87 | 0.444 | 0.508 | ACCAG130 | 0.308 | 0.434 |
| ACCAG87 | 0.233 | 0.364 | AGCAC673 | 0.250 | 0.386 | ACCAG87 | 0.115 | 0.208 |
| AGCAC695 | 0.661 | 0.456 | AGCAC490 | 0.583 | 0.500 | AGCAC420 | 0.231 | 0.362 |
| AGCAC673 | 0.288 | 0.417 | AGCAC420 | 0.389 | 0.489 | AGCAC409 | 0.789 | 0.340 |
| AGCAC490 | 0.898 | 0.186 | AGCAC409 | 0.833 | 0.286 | AGCAC391 | 0.192 | 0.317 |
| AGCAC409 | 0.797 | 0.330 | AGCAC380 | 0.417 | 0.500 | AGCAC380 | 0.077 | 0.145 |
| AGCAC331 | 0.915 | 0.158 | AGCAC331 | 0.611 | 0.489 | AGCAC371 | 0.173 | 0.292 |
| AGCAC327 | 0.085 | 0.158 | AGCAC327 | 0.417 | 0.500 | AGCAC331 | 0.827 | 0.292 |
| AGCAC230 | 0.119 | 0.213 | AGCAC230 | 0.556 | 0.508 | AGCAC327 | 0.173 | 0.292 |
| AGCAC228 | 0.085 | 0.158 | AGCAC228 | 0.556 | 0.508 | AGCAC202 | 0.250 | 0.382 |
| AGCAC204 | 0.441 | 0.502 | AGCAC194 | 0.722 | 0.413 | AGCAC194 | 0.192 | 0.317 |
| AGCAC194 | 0.220 | 0.350 | AGCAC188 | 0.833 | 0.286 | AGCAC188 | 0.808 | 0.317 |
| AGCAC188 | 0.780 | 0.350 | AGCAC186 | 0.194 | 0.322 | AGCAC186 | 0.192 | 0.317 |
| AGCAC186 | 0.237 | 0.368 | AGCAC169 | 0.639 | 0.475 | AGCAC143 | 0.173 | 0.292 |
| AGCAC143 | 0.322 | 0.444 | AGCAC143 | 0.167 | 0.286 | AGCAC135 | 0.077 | 0.145 |
| AGCAC94 | 0.424 | 0.497 | AGCAC135 | 0.222 | 0.356 | AGCAC94 | 0.173 | 0.292 |
| AGCAC93 | 0.237 | 0.368 | AGCAC88 | 0.250 | 0.386 | AGCAC93 | 0.231 | 0.362 |
| AGCAC88 | 0.797 | 0.330 | AGCAG364 | 0.500 | 0.514 | AGCAC88 | 0.231 | 0.362 |
| AGCAG157 | 0.050 | 0.097 | AGCAG358 | 0.361 | 0.475 | AGCAG253 | 0.462 | 0.507 |
| AGCAG129 | 0.817 | 0.305 | AGCAG219 | 0.250 | 0.386 | AGCAG219 | 0.039 | 0.075 |
| AGCAG125 | 0.050 | 0.097 | AGCAG170 | 0.500 | 0.514 | AGCAG194 | 0.231 | 0.362 |
| AGCAG120 | 0.300 | 0.427 | AGCAG129 | 0.806 | 0.322 | AGCAG170 | 0.423 | 0.498 |
| AGCAG106 | 0.333 | 0.452 | AGCAG125 | 0.250 | 0.386 | AGCAG157 | 0.231 | 0.362 |
| | | | AGCAG106 | 0.500 | 0.514 | AGCAG129 | 0.808 | 0.317 |
| | | | | | | AGCAG120 | 0.500 | 0.510 |
| | | | | | | AGCAG106 | 0.308 | 0.434 |
| No. Loci | Ave. p | Ave. $H_e$ | No. Loci | Ave. p | Ave. $H_e$ | No. Loci | Ave. p | Ave. $H_e$ |
| 39 | 0.399 | 0.314 ± 0.160 | 40 | 0.425 | 0.410 ± 0.208 | 42 | 0.349 | 0.338 ± 0.171 |

[a] Frequency of the dominant allele, p at locus i, given by $p_i=x_i/n_{pop}$ where x is the number of individuals with a band and $n_{pop}$ is the total number of individuals in the population.

[b] $H_e$ is gene diversity corrected for sample size. Ave. $H_e$ is average gene diversity: the probability of obtaining different alleles at randomly chosen homologous loci, shown with standard deviation.

Table 2. Average frequencies of alleles used in population-level analyses of all *V. heterodoxa* populations.

| Locus | ML | PH | DP | Overall |
|---|---|---|---|---|
| ACCAC222 | 1.000 | 1.000 | 0.769 | 0.919 |
| ACCAC210 | 0.000 | 0.333 | 0.096 | 0.115 |
| ACCAC205 | 0.250 | 0.000 | 0.000 | 0.101 |
| ACCAC172 | 0.017 | 0.556 | 0.154 | 0.196 |
| ACCAC143 | 0.433 | 0.167 | 0.192 | 0.284 |
| ACCAC94 | 0.450 | 0.028 | 0.000 | 0.189 |
| ACCAC76 | 0.717 | 0.667 | 0.654 | 0.682 |
| ACCAG361 | 0.017 | 0.528 | 0.192 | 0.203 |
| ACCAG198 | 0.350 | 0.167 | 0.000 | 0.182 |
| ACCAG157 | 0.067 | 0.111 | 0.269 | 0.149 |
| ACCAG152 | 0.383 | 0.083 | 0.000 | 0.176 |
| ACCAG87 | 0.233 | 0.444 | 0.115 | 0.243 |
| AGCAC391 | 0.000 | 0.000 | 0.192 | 0.068 |
| AGCAC380 | 0.017 | 0.417 | 0.077 | 0.136 |
| AGCAC331 | 0.915 | 0.611 | 0.827 | 0.810 |
| AGCAC327 | 0.085 | 0.417 | 0.173 | 0.197 |
| AGCAC230 | 0.119 | 0.556 | 0.192 | 0.252 |
| AGCAC228 | 0.085 | 0.556 | 0.269 | 0.265 |
| AGCAC204 | 0.441 | 0.000 | 0.000 | 0.177 |
| AGCAC202 | 0.186 | 0.000 | 0.250 | 0.163 |
| AGCAC188 | 0.780 | 0.833 | 0.808 | 0.803 |
| AGCAC186 | 0.237 | 0.194 | 0.192 | 0.211 |
| AGCAC143 | 0.322 | 0.167 | 0.173 | 0.231 |
| AGCAC135 | 0.000 | 0.222 | 0.077 | 0.082 |
| AGCAC94 | 0.424 | 0.000 | 0.173 | 0.231 |
| AGCAC93 | 0.237 | 0.000 | 0.231 | 0.177 |
| AGCAC88 | 0.797 | 0.250 | 0.231 | 0.463 |
| AGCAG364 | 0.083 | 0.500 | 0.250 | 0.243 |
| AGCAG219 | 0.000 | 0.250 | 0.039 | 0.074 |
| AGCAG170 | 0.000 | 0.500 | 0.423 | 0.270 |
| AGCAG157 | 0.050 | 0.000 | 0.231 | 0.101 |
| AGCAG129 | 0.817 | 0.806 | 0.808 | 0.811 |
| AGCAG81 | 0.150 | 0.028 | 0.019 | 0.074 |
| Ave. Gene diversity | 0.231 | 0.288 | 0.263 | 0.293 |
| SD | 0.121 | 0.150 | 0.137 | 0.150 |