CONSERVATION GENETICS OF WHITEBARK PINE (*Pinus albicaulis* Engelm.) IN BRITISH COLUMBIA

by

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ABSTRACT

Pinus albicaulis Engelm. is a keystone subalpine species found throughout mountainous regions of western North America. Population genetic investigations in British Columbia using isozymes (17 populations, 12 loci) extracted from bud tissue revealed that the species has high levels of observed and expected heterozygosity compared to other pine species (0.213 and 0.262, respectively). Isozyme analysis (two populations, ten loci) using maternal gametopyte tissue and embryos extracted from seed elucidated that biparental inbreeding, and possibly selfing, is common (mean multilocus outcrossing rate = 0.73, mean single-locus outcrossing rate = 0.69). There is moderate population substructuring (F_{ST} = 0.061), typified by the clumped distribution of trees, influencing gene flow, although seed distribution by Clark's nutcracker appears to be the overriding factor influencing genetic patterns. There were few rare alleles found and genetic distances between populations were small (Nei's 1978 distance ranged from 0.006 to 0.134 and Cavalli-Sforza and Edwards' (1967) chord distance from 0.086 to 0.297). Genetic distances were weakly related to physical distances between populations (Mantel test, p = 0.036). Observed heterozygosity was significantly negatively correlated with longitude (R^2 = 0.295) and latitude (R^2 = 0.357). Population genetic parameters were consistent with other studies suggesting northerly postglacial recolonization from refugia in the Washington and Oregon Cascades and several more northern refugia in the Rockies, including the possiblity of a refugium near Roger's Pass, BC.

Nearly all populations were observed to have *Cronartium ribicola* Fisch. (white pine blister rust) infections, mortality of trees of all ages was often present (due to various causes), and regeneration was often sparse or absent. A conservation strategy was developed based on the results of these investigations, concurrent with the priorities and recommendations of other agencies involved with whitebark pine conservation. Priorities included continuing surveys of natural stands in order to identify and monitor putatively resistant trees, collecting seed from all

available seed sources and especially these selected individuals, establishing common garden tests to assess adaptive variation and screen for disease resistance, establishing field trials in natural habitats with a variety of hazard ratings for blister rust, developing appropriate seed and scion transfer guidelines, and maintaining a cooperative exchange in terms of materials and research with other jurisdictions involved in whitebark pine conservation. Future research may involve isolation of any specific resistance mechanisms, genetic transformation or cross-breeding of susceptible individuals, and bulk propagation of resistant individuals or families via rooting cuttings or somatic embryogenesis. In the longer term, breeding strategies involving controlled crosses of putatively restitant parents in order to produce hardy and disease resistant planting stock for a variety of hazard-rated sites should be instituted. Due to the extremely long generation time of this species, it is critical that conservation measures begin immediately.

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CHAPTER 1 – INTRODUCTION AND OBJECTIVES

"A tree is a tree. Now how many more do you need to look at?"
- Ronald Reagan, 1965

1.1 INTRODUCTION

1.1.1 WHITEBARK PINE: AUTECOLOGY OF A KEYSTONE SPECIES

Whitebark pine (*Pinus albicaulis* Engelm.) is a high-elevation conifer, typically found from the subalpine to timberline (Achuff 1989; Arno and Hoff 1989; Callaway 1998; Douglas and Bliss 1977). It ranges from central British Columbia and Alberta south to the Sierra Nevadas, from 55°N to 37°N, along the Cascade and Coast ranges and the Rocky Mountains. The species is subdivided into eastern and western populations (Ogilvie 1990), separated at the closest point (in southern British Columbia) by 100 kilometres (Arno and Hoff 1989, 1990; McCaughey and Schmidt 1990). It survives on ridgetops and exposed talus slopes, enduring extreme abiotic conditions (Perkins and Swetnam 1996) such as wind dessication, high ultraviolet exposure, freezing temperatures and a very short growing season (Arno and Hoff 1989, 1990; Campbell 1998; McCaughey and Schmidt 1990).

Whitebark pine is the only member of the stone pines (subgenus Strobus, section Strobi, subsection Cembrae) (Critchfield 1986; Price *et al.* 1998) in North America (Bruederle *et al.* 1998; Goncharenko *et al.* 1992; Krutovskii *et al.* 1995), although the phylogeny and taxonomy of this group is still unresolved (Bruederle *et al.* 1998, 2001; Krutovskii *et al.* 1995; Liston *et al.* 1996; Politov and Krutovskii 2001, unpublished data; Price *et al.* 1998). The geographic isolation of whitebark pine from the other stone pines and ambiguous results of previous studies using various markers and characteristics led to some contention regarding its alliance with the other stone pines. Recently, several studies (Krutovskii *et al.* 1995; Liston *et al.* 1996; Price *et al.* 1998) have found support for the monophyly of the stone pines based on cpDNA sequences.

This has provided support for recognition of this debated taxonomic group originally defined by morphological characteristics (Little and Critchfield 1969; Critchfield 1986; Axelrod 1986).

This subsection includes several haploxylon five-needled pines found throughout Eurasia and Northern Europe which feature heavy, wingless seeds (Arno and Hoff 1989; Critchfield 1986), indehiscent cones (Arno and Hoff 1990; Krutovskii *et al.* 1995; Tomback 1986) and a mutualistic association with birds of the *Nucifragia* genus (Arno and Hoff 1990; Bruederle *et al.* 1998; Jorgensen and Hamrick 1997; Krutovskii *et al.* 1995; Tomback 1982; Tomback and Linhart 1990), nutcrackers which facilitate seed dispersal (Callaway 1998; Lanner 1982; Stuart-Smith 1998). *P. albicaulis* has coevolved with the Clark's nutcracker (*N. columbiana* Wilson) (Critchfield 1986; Tomback 1982) to the point where the tree species is completely reliant on the nutcracker for dispersing its seeds, which also provides ideal conditions for germination and establishment (Hutchins and Lanner 1982). It has been estimated that the nutcrackers consume approximately one third of the seeds they cache annually (Tomback 1982); the consumption rate is nearly 100% for small mammals which also cache the seeds (Arno and Hoff 1989).

Despite its narrow geographic range, *P. albicaulis* is a member of a variety of plant communities along its latitudinal gradient and grows primarily in association with subalpine larch (*Larix lyallii* Parl.), subalpine fir (*Abies lasiocarpa* [Hook.] Nutt.), Engelmann spruce (*Picea engelmannii* Parry ex Engelm.), limber pine (*Pinus flexilis* James) and lodgepole pine (*Pinus contorta* var. *latifolia* Dougl. ex Loud.) (Achuff 1989; Arno and Hoff 1989, 1990; Campbell 1998; McCaughey and Schmidt 1990; Ogilvie 1990; Perkins and Swetnam 1996). The large, heavy, high-fat and nutrient-rich seeds (Lanner 1982,1986; Lanner and Gilbert 1994; Tomback 1982; Tomback and Linhart 1990) serve as a key food source for a wide variety of animals (Arno and Hoff 1990; Keane and Arno 1993; Lanner and Gilbert 1994), including other birds, red squirrels (*Tamariscus hudsonicus*) (Arno and Hoff 1989), black bears (*Ursus americanus*) (Mattson and

Reinhart 1997), grizzly bears (*Ursos arctos horribilus*) (Mattson and Reinhart 1997; McCaughey and Schmidt 1990) and many small mammals (Arno and Hoff 1989; Kendall and Arno 1990; McCaughey 1994; McCaughey and Schmidt 1990). On average, per seed values for whitebark pine in the U.S. are: dry weight, 0.09g; 18 percent protein; 21 percent carbohydrate; 52 percent fat; as well as being high in many amino acids, fatty acids and minerals. Cone crop abundance has been linked to population cycles and behaviour of nutcrackers, squirrels and grizzly bears (Mattson and Reinhart 1997), as well as the animals with which they interact (Bruederle *et al.* 1998; Kendall and Arno 1990; Keane and Arno 1993; Tomback *et al.* 1995). For these reasons, it has been suggested that whitebark pine be regarded as a keystone or an umbrella species (Tomback *et al.* 2001; Campbell 1998; Stuart-Smith 1998), a species whose health and ecosystem status is integrally linked to, and an overall indicator of, the health and survival of other species and communities (Callaway 1998; Ledig 1988; Mattson and Reinhart 1997; Primack 1998). Currently, whitebark pine is considered threatened by several agencies in British Columbia since it is under direct pressure from a number of environmental and anthropogenic threats, although it is not formally listed as such under COSEWIC (Yanchuk and Lester 1996; Forest Health Committee of B.C. 1999; L. Pedersen, B.C. Chief Forester 1998, op. cit. Kieran 1998).

Since it already exists at the upper altitudinal periphery of its fundamental ecological niche, the potential for future global climate change may have a serious impact on the survival of not only whitebark pine but all of the biotic communities of which it is a component (Namkoong 1992). Based on predictions of doubled atmospheric carbon dioxide within the next century (Bradshaw and McNeilly 1991; Huntley 1991), some climate modelling projections forecast imminent warming of northern and high altitude areas by an annual mean of three to six degrees Celsius (Bradshaw and McNeilly 1991; Huntley 1991; Running and Nemani 1991; USEPA 2000; Watson *et al.* 1997). This is associated with unknown changes in moisture

regimes, although winter snowpack is likely to melt sooner than currently (IPCC 2001a,b; Franklin *et al.* 1991). Since the majority of moisture in whitebark pine habitat occurs as snow and much of the annual soil moisture is received as snowmelt throughout the warmer portion of the year (McCaughey and Schmidt 1990; Ogilvie 1990), global climate change will drastically alter the hydrology (McCaughey and Schmidt 1990; Perry *et al.* 1991; Running and Nemani 1991) and abiotic conditions of current whitebark pine habitat, including a significant change in the length of the growing season (Running and Nemani 1991; Watson *et al.* 1997).

Whitebark pine currently serves an important ecological role in the hydrology of montane and headwater systems by intercepting snow and serving as a moderator of snowmelt (Arno and Hoff 1989, 1990; Keane and Arno 1993; McCaughey 1994); a decrease in snowpack may lead to critical growing season moisture deficits and higher incidence and severity of fires in high elevation areas (Perry *et al.* 1991; Watson *et al.* 1997). Slope stability in whitebark pine habitat, typically steep, montane areas with shallow, rocky soils, will also be affected (Keane and Arno 1993) as these complex factors interact to alter the survival and establishment of living trees as well as the size and longevity of snags.

As future climate change alters the abiotic character of whitebark pine ecosystems, the trees themselves may no longer be optimally adapted to the sites they currently occupy (Franklin *et al.* 1991). They may then be even more susceptible to competition from other species with which they currently coexist at lower altitudes; above the timberline, they are the only trees present (Arno and Hoff 1990; Campbell 1998). Since generations are too long to evolve adaptive traits at a sufficient rate to keep pace with rapid climate change, the only means available to whitebark pine trees for long-term species survival would be to migrate (Delcourt and Delcourt 1998; Huntley 1991). This must occur via dispersal by Clark's nutcrackers. Typical seed dispersal distances of this bird have been gauged at one to three kilometres, although distances over twenty kilometres have been recorded (Tomback and Linhart 1990;

Vander Wall and Balda 1977), and dispersal direction is not related to prevailing winds (Tomback 2001). These distances may ensure that whitebark pine populations could adapt to a new regime of climatic zonation as a result of the observed rates of climatic change (McCaughey and Schmidt 2001).

It is unknown whether the birds would cache the seeds in such a manner as to extend the species range northward (Sedjo and Solomon 1988) at a rate approximating that of the location of suitable habitat, which is expected to move 150 to 550 km north, or 150 to 550 m upwards in elevation within the next century (Franklin *et al.* 1991; Rogers *et al.* 1999; USEPA 2000b; Watson *et al.* 1997). This is not an impossibility, however: since the most recent glaciation, the range of whitebark pine has been expanding northward to reoccupy its historic range throughout the mountainous areas of western North America (Jorgensen and Hamrick 1997; McCaughey and Schmidt 2001; Tomback 2001), and the only means by which this might occur, given the closed cones and wingless seeds is by nutcrackers (Baker 1990). The critical question is whether the current projections of the increased rate of climate change and corresponding ecosystem change can be overcome by the rate of northward migration of nutcrackers (Tomback and Linhart 1990).

Climate change notwithstanding, whitebark pine is under many other immediate threats throughout its range (Callaway 1998). White pine blister rust (*Cronartium ribicola*), an introduced fungal pathogen which infects many white pines, is a virulent disease of this species (Arno and Hoff 1990; Hoff *et al.* 1980,1994), causing extremely high mortality (Arno and Hoff 1989; Bruederle *et al.* 1998; McCaughey and Schmidt 1990), especially in Montana (Hoff and Hagle 1990; Keane and Arno 1993; McCaughey 1990; Tomback *et al.* 1995). Although mortality appears to be less severe in Canada, infection rates are still high and have no obvious constraints to their future expansion throughout the entire range of whitebark pine (Campbell 1998; Stuart-Smith 1998).

Research programs to locate and develop genetic resistance to this disease are under way in the United States (Arno and Hoff 1990; Hoff 1984,1986; Hoff and Hagle 1990; Hoff *et al.* 1994; Jorgensen and Hamrick 1997; Tomback *et al.* 1995), but no programs have been initiated in Canada to date. There are many other pathogens which cause injury and mortality in this species, including limber pine dwarf mistletoe (*Arceuthobium cyanocarpum*) (Arno and Hoff 1989; Hoff and Hagle 1990; Mathiasen and Hawksworth 1988), but none have had the severity of impact in BC that blister rust has had. The detrimental effects of blister rust are exacerbated by the longevity of whitebark pine: ages of 400 to 500 are not uncommon (Ogilvie 1990), and krummholtz specimens of over 1700 years have been found (Perkins and Swetnam 1996).

Insects such as the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Arno and Hoff 1990; Baker *et al.* 1971; Keane and Arno 1993; McCaughey and Schmidt 1990; Perkins and Swetnam 1996) and the ambrosia beetle *Ips pini* Say have also caused mortality in whitebark pine stands (Arno and Hoff 1989): however, their effects have been more serious in the United States than in Canada to date (Tomback *et al.* 1995). The severity of the impacts of both insects and pathogens has been attributed to fire suppression policies in North America (Arno and Hoff 1990).

Whitebark pine has evolved with a medium-intensity fire regime with a 50 to 350 year return pattern (Campbell 1998; McCaughey and Schmidt 1990), which would effectively kill competing trees and seeds in the substrate, enabling whitebark pine to play a role as both a pioneer and climax species, both as a true seral climax and a fire-maintained subclimax (Arno and Hoff 1989; Callaway 1998; Campbell 1998). Since the advent of fire suppression, successional changes have gone unchecked and competition-induced mortality is common (Arno and Hoff 1989; Tomback *et al.* 1995). Slow-growing whitebark pines are outcompeted by subalpine fir and Engelmann spruce, leaving age class gaps and weakening surviving trees (Campbell 1998). In addition, an abundance of old lodgepole pine stands, resulting from fire suppression,

are a significant factor causing mortality in coexisting whitebark pine via mountain pine beetle infection (Kendall and Arno 1990; McCaughey 1994).

Whitebark pine has developed a unique population genetic structure as a result of bird-mediated seed dispersal (Jorgensen and Hamrick 1997; Bruederle *et al.* 1998; Tani *et al.* 1998); adaptation to this symbiotic mechanism has been accompanied by selection for morphological and phenological adaptations which reflect the mutualistic association with nutcrackers. Unlike most coniferous species, stone pine cones lack the schlerenchyma in the female cones which cause them to open and release their seeds upon maturity (McCaughey 1994; McCaughey and Schmidt 1990). The cones are situated at branch tips in the top of the crown tree so they are difficult to locate from the ground but easily visible from above (Lanner 1982; Furnier *et al.* 1987). Clark's nutcrackers chisel the immature cone scales apart in July and August (McCaughey and Schmidt 1990; Tomback 1982), and collect up to 150 seeds at a time in a sublingual pouch, a unique adaptation of *Nucifragia* species (Arno and Hoff 1990; McCaughey 1994; Lanner 1982; Tomback 1982; Tomback and Linhart 1990). The seeds are then cached one to three centimetres deep solitarily or more often in groups of up to fifteen seeds (Bruederle *et al.* 1998; Tomback 1982,1986; Tomback *et al.* 1995), and a rock or cone is placed on top (Tomback and Linhart 1990). These groupings, combined with typical nutcracker behaviour of returning to the same caching area for several flights, results in a complex population genetic structure (Tomback 2001).

Typical dispersal distances range from several hundred metres to five kilometres (Arno and Hoff 1990), although distances of over 20 kilometres have been reported. Nutcrackers can collect 100,000 seeds annually (McCaughey 1994) and find up to 30,000 cached seeds each year (Tomback 1982), ensuring a year-round nutritive food supply. They can remember the locations of cached seeds for up to three years, and forgotten seeds typically germinate gradually over the course of up to three years (McCaughey 1994). Many of the embryos in the

mature cones are immature (Arno and Hoff 1989; Leadam 1986; McCaughey and Schmidt 1990; Pitel and Wang 1990), and seeds continue to mature after caching. The resulting delayed germination may be another adaptation reflecting the coevolution of the stone pines and nutcrackers: the trees have been subject to thousands of generations of selection for large seed size through bird preference, and the large seeds require one to three years to reach embryo maturity. The high seed weight may also reflect the optimal nutrition requirements for the embryo to survive in cold climates.

1.1.2 MATING SYSTEM

The mating system, or degrees of outcrossing (mating between unrelated individuals) and inbreeding (selfing and mating among relatives), a species typically exhibits is a critical factor both influencing and influenced by factors such as genetic structure, population density and distribution, and gene flow. The interrelatedness and sometimes opposing effects of these parameters makes it difficult to isolate the effects of genotype and environment, although clearly it is their interaction which results in the expressed mating system. Conifers (including most pine species) are generally highly outcrossing (Hamrick *et al.* 1992). The unique demographics, ecology and dispersal of stone pines may exert selection pressures on the genes controlling mating system parameters to such an extent that they may differ from other, wind-dispersed pines.

Rogers *et al.* (1999, p. 75) wrote, “whitebark pine is considered to have a largely outcrossing mating system, yet there is little local or empirical information to support the theory.” This view is also held by Krutovskii *et al.* (1995). However, based on the unique adaptations and population structure, it was suspected that it would likely feature a high degree of inbreeding (Tomback and Schuster 1994; Krutovskii *et al.* 1995). The isolation of populations and their low density (i.e., their sporadic distribution in subalpine and timberline areas) would likely contribute to inbreeding (Mitton 1992). This is supported by the hypothesis of kin selection which

facilitates survival and establishment of related genotypes while outcompeting or hindering other unrelated individuals. Traits such as root grafting, clumping and multiple stem formations all support some degree of increasing the fitness of relatives, perhaps at the expense of the interacting individuals (Tomback and Linhart 1990). The complex traits resulting from the coevolution of whitebark pine and the Clark's nutcracker, especially the adaptations concerning seed dispersal and establishment, appear to promote kin selection and increase the degree of mating among related individuals.

Krutovskii and his colleagues (1994, 1995) have documented varying levels of inbreeding among the Eurasian stone pines (and one single population of *P. albicaulis*); the results show substantially higher inbreeding in stone pines than in other taxonomic subdivisions of the genus *Pinus* with the exception of *P. maximartinezii* which exists as a single, isolated population of relatively small size in Mexico (Ledig *et al.* 1999) and other bird-dispersed pines, primarily the piñon pines. Human seed herbivory in the case of *P. maximartinezii*, and bird seed caching in the cases of piñon and whitebark pines (Richardson 2001), generate unique population structure and selection pressures, and would explain the relatively high inbreeding found based on their impact on dispersal and regeneration patterns.

1.1.3 GENETIC DIVERSITY

Several studies have examined genetic diversity of this species, but even the most comprehensive in terms of area covered, Jorgensen and Hamrick (1997), did not include any samples from British Columbia. One other study examined populations along the B.C.-Alberta border (Stuart-Smith 1998), but none have yet looked at genetic variation across the large and topographically complex province of B.C. Historical events, including the several glacial cycles in the last 100,000 years, have undoubtedly left a genetic signature on whitebark pine. Its long generation time, often 100 years, suggests in some cases that the effects are still evident (Richardson 2001). Glacial events during the Pleistocene era reduced many conifers' ranges in

North America and Eurasia and in some cases have led to genetic bottlenecks. These events contributed to different patterns of genetic structure and diversity in those areas. Typically, the heterozygosity of conifers decreases slightly with increasing latitude as a result of postglacial recolonization (Millar and Westfall 1992). Since conifers, and specifically whitebark pine, have such long generation times, these populations have often not yet returned to a state of genetic equilibrium with respect to drift, migration and selection.

Although whitebark pine exists in an ecologically peripheral habitat, global warming could potentially force species to migrate to more northerly latitudes as well as higher elevations. Since whitebark pine already exists at the the tops of many mountains, the only realistic option in terms of the overall species range is to migrate northward, in addition to upward elevational range expansion where physically possible.

Conifers generally have very high levels of heterozygosity, as measured both by isozymes and other molecular markers (Hamrick *et al.* 1992). There may be differences between observed (a direct count of the heterozygous individuals at each locus, H_o) and expected (calculated using allele frequencies, H_e) heterozygosity at the individual and population levels. A common index of the difference is the inbreeding coefficient F, where $F = 1-H_o/H_e$. This index estimates the relative excess or deficiency of heterozygotes in the actual population compared to Hardy-Weinberg equilibrium (HWE). Differences between H_o and H_e can arise from empirical causes such as localized anomalies in allele frequencies, errors, or deficiencies in the model used to calculate the frequencies, which typically oversimplifies real factors that interact in complex, often unquantified, ways.

While inbreeding typically reduces the observed heterozygosity, especially for selfing organisms, some primarily inbreeding species have fairly high expected heterozygosity, suggesting that many generations of inbreeding effectively purged deleterious alleles (many of which would have been recessives found at low frequencies in the population) and further

inbreeding resulted in no additional reduction of heterozygosity (Kirkpatrick and Jarne 2000). It is therefore possible to have somewhat contradictory results regarding genetic diversity and inbreeding at first glance. Heterozygosity, as measured by isozymes, has also been demonstrated to increase with age in conifers as individuals homozygous for deleterious or lethal alleles die during embryonic and juvenile life stages and are likely to be outcompeted (Bush and Smouse 1992).

Since genetic diversity and patterns in whitebark pine are dependent on Clark's nutcrackers, and to a lesser degree small mammals, there is a host of interesting evolutionary and ecological questions which may be posed. Will the nutcrackers be able to survive in more northerly environments? Will seeds result in established seedlings, or will they be outcompeted by other plants which may occupy the same ecological niche in the changing environment? Will the communities of other species which have evolved around whitebark pine be able to carry on their ecological roles in future climates? How will these communities change over time? These questions may determine the success of whitebark pine both as a species and as a keystone member of the timberline to subalpine community, both in the short and long term, with respect to natural and anthropogenic cycles of climate change.

1.1.4 CONSERVATION OF WHITEBARK PINE

Currently, B.C. has a Protected Areas Strategy under which conservation of natural resources, ecosystems and unique features are protected by law within a network of parks, wilderness areas and ecological reserves (Ecological Reserves Program 1993; Province of B.C. 1996). Although all ecosystem types are supposed to be represented in this system, high elevation ecosystems are over-represented in terms of area protected (Ecological Reserves Program 1993; Province of B.C. 1996). This is the natural habitat of whitebark pine, and large contiguous areas of current and potential future habitat are already under protection. In

Canada, there are vast tracts of wilderness which are seldom encountered by humans and not under immediate threat of develo25

pment (Achuff 1989; Yanchuk and Lester 1996). The preservation of landscape-level processes and dynamics has been deemed essential for the conservation of adequate levels of variation, as this approach takes into account the metapopulation structure and dynamics of most species and their associated communities (Delcourt and Delcourt 1998), and provides some long-term security in the event of future uncertainty (Erikkson *et al.* 1993; Noss 1990). In the United States, this is not the case: although much of the whitebark pine habitat is in extremely remote areas, much of it is under pressure of development: primarily road construction, resource extraction, heavy tourism and recreational ski areas (Cole and Landres 1996).

Since whitebark pine ecosystems are areas of heavy wildlife use, sensitive hydrology and a host of other non-timber values, including aesthetics and traditional values (Arno and Hoff 1989, 1990), preserving the habitat and the inherent genetic variation of whitebark pine in its natural habitat is likely to be a more difficult task in the United States, with its large human population and mixed-use wilderness areas. The current paradigm of delineating evolutionarily significant units, or ESUs (Moritz 1994), poses additional problems: should each region be considered an ESU? What about special adaptations such as blister rust resistance, other rare alleles, or interacting gene complexes (Williams *et al.* 1995), which serve as insurance against future change (Erikkson *et al.* 1993; Lande and Barrowclough 1987; Yanchuk and Lester 1996)? Identifying and legislating protection which conserves the inherent variability in the species will be difficult and costly (Hard 1995; Yanchuk and Lester 1996), but may be a necessary step in order to overcome the short-term threat of inbreeding to the health of the species (Erikkson *et al.* 1993; Millar and Westfall 1992; Namkoong 1992). *In-situ* conservation strategies must be designed to take into account the potential for future changes in areas such as land-use, public

opinion, genetic bottlenecks, natural catastrophes and climate change (Achuff 1989; Franklin *et al.* 1991; Ledig 1986; Millar and Westfall 1992; Namkoong 1992; Yanchuk and Lester 1996).

Developing comprehensive *ex-situ* collection of the genetic resources of the species is not likely to be feasible, given the expense of collecting in remote areas and preserving and cataloguing the material (McDonald and Hoff 2001; Millar and Westfall 1992; Yanchuk and Lester 1996). The remote nature of the populations and irregular nature of whitebark pine cone crops (Arno and Hoff 1989; McCaughey and Schmidt 1990; Weaver and Forcella 1986) make collecting seed a costly and uncertain proposition. Repeat visits to potential cone collecting sites are required each season as cones must be caged in early summer to prevent seed predation, which can otherwise lead to total loss. Large seed size, embryo immaturity and low germination percentage of whitebark pine, coupled with its susceptibility to fungal pathogens in storage which would further reduce the viability of stored seed, would also add to operational difficulties (McCaughey and Schmidt 1994; McCaughey and Tomback 2001). Various techniques to artificially enhance germination rates and embryo development have been attempted, with moderate to significant success (Leadam 1986; Pitel and Wang 1990). Regular viability testing, which is essential for *ex-situ* conservation in seed banks, is expensive, time-consuming and uses up valuable seed. Establishment of *ex-situ* collections in living genetic archives or clone banks is also very expensive.

1.2 THESIS OBJECTIVES

In light of the paucity of data regarding whitebark pine's mating system, and levels and patterns of genetic diversity in British Columbia, several objectives for this study were established:

1. To determine the mating system of whitebark pine;

2. to quantify the level and patterns of genetic diversity in whitebark pine in B.C. and to compare these results with those of related studies from other geographic areas; and

3. based on the results of the preceding objectives and existing frameworks, to propose a conservation strategy for whitebark pine.

It is hoped that the results of this study can be combined with other efforts currently underway to establish a feasible, fact-based management plan to mitigate the current decline of whitebark pine ecosystems in this region.

CHAPTER 2 – MATING SYSTEM

"People make the mistake of talking about 'natural laws.' There are no natural laws. There are only temporary habits of nature."

- Alfred North Whitehead, 1910

2.1 INTRODUCTION

The nature of a species' mating system is both a reflection and a result of the evolutionary forces influencing that species and the ecological niche it occupies. While there is certainly some degree of the "chicken and egg" argument regarding the influence mating systems have on other life history traits, the mating system (specifically, the relative degrees of selfing vs. outcrossing a species exhibits) can be influenced by factors such as density (Clegg 1980; Mitton *et al.* 1981), which could be altered by human intervention (Gooding 1998). Many models and techniques have been utilized for the analysis of mating systems; their accuracy varies with sampling design, availability of materials (in terms of seasonality, resource allocation and conservation requirements) and assumptions involved.

Assaying seeds using molecular markers is likely the most accurate way to determine the mating system of wind-pollinated species, and specifically conifers. Genetic information is available for both the mother via the seed megagametophyte, and embryo, and the genotype of the pollen parent can be inferred from differences between the two (Shaw *et al.* 1981). This procedure permits estimations of the degree of outcrossing, based on the degree of similarity between the embryo's two parents (Jarne and Charlesworth 1993). While conifers are typically outcrossing (Hamrick *et al.* 1991,1992), there are some clear exceptions (e.g., Ledig *et al.* 1999). Mixed mating models can be used to analyze mating systems and to accurately detect levels of inbreeding or selfing. Studies have shown that propogule dissemination, individual male or female fitness, and reproductive phenology all affect the results by altering factors influencing the rates of outcrossing and selfing (Clegg 1980; Hamrick and Allard 1972; Richards 2000; Shaw *et al.* 1981). Aborted and empty seeds may also reflect products of selfing and

since it is impossible to perform genetic analysis on them, these missing data would consequently lead to underestimation of selfing (Stettler and Bradshaw 1994).

Population genetics theory and empirical studies suggest that genetic bottlenecks, while causing an immediate reduction in heterozygosity, could also serve to purge the gene pool of recessive deleterious alleles, thereby facilitating a greater level of inbreeding (Kirkpatrick and Jarne 2000). The resultant increased inbreeding may not extend to complete selfing, however, since there would be no masking of deleterious or lethal alleles in later generations (Jarne and Charlesworth 1993) and would therefore be restricted to matings among relatives. Polyembryony in pines has also been found to act as an early selection agent against homozygotes and can impact the degree of selfing (Hedrick *et al.* 1999).

As a result of the population structure caused by related individuals growing in clumps, there is potential for a high degree of inbreeding in this species (Tomback and Schuster 1994; Bruederle *et al.* 1998). Pollen flow is more likely to occur between individuals within a clump, given the short reproductive window and physical proximity of the individuals. *Pinus* species do not possess SI (self-incompatibility) genes, which would effectively promote heterosis (Tomback and Linhart 1990; Politov and Krutovskii 1994), and help explain the high numbers of aborted and empty seeds which are found in cones (Stettler and Bradshaw 1994), although another explanation is a high genetic load.

Latta and Ritland (1994) have proposed that a stable mixed mating system is possible whereby strongly deleterious alleles are purged by selfing and mildly deleterious alleles, subject to weaker selection pressure, can be carried at a fairly constant genetic load. This model did not incorporate more complex permutations of mating such as biparental inbreeding, however, that may be more common in empirical situations. It is likely, however, under similar constraints, that the key results of the model would be similar but more gradual if mating among other classes of relatives was included.

Nothing about the mating system of whitebark pine is currently known. Estimates of relatedness within and among tree clumps have been calculated (Tomback and Schuster 1994; Furnier *et al.* 1987), but outcrossing rates have not. Mating systems analysis has been conducted for other stone pines (Krutovskii *et al.* 1995; Politov and Krutovskii 1994), and it is likely that whitebark pine has similar levels of consanguineous mating and selfing to those species, since they share demographic structural patterns as a result of nutcracker dispersal.

2.1.1 OBJECTIVES

Knowledge of the mating system of a species is important for formulating an effective management strategy. Obtaining this information will fill an important information gap for whitebark pine research. The objectives of this section are to:

1. Obtain quantitative estimates of single-locus and multilocus outcrossing rates of whitebark pine, and
2. Compare these data with the mating systems of other stone pines.

2.2 METHODS AND MATERIALS

2.2.1 FIELD COLLECTIONS

All the information contained herein is adapted from Meagher and Edwards (1997); see Figure 2.1. Ten to 20 cones per tree were collected from two populations on October 1, 1997. Sampling sites were located in E.C. Manning Provincial Park (with appropriate permits) in the meadows at the terminus of the Blackwall road (49°06'12"N, 120°45'40"W, 2000-2040m elev.) and around the ridges above the ski facilities at Mount Baldy (49°10'N, 119°15'W, 2100-2200m elev.). Trees sampled were at least five metres apart; cones were collected from multiple stems in a clump if a tree appeared to be multi-stemmed. Blister rust (*Cronartium ribicola*) incidence did not impact sampling decisions. If cones were partially damaged by birds, they were still collected if the majority of the cone appeared intact. Twenty-five trees were sampled at Manning, and 30 at Mount Baldy.

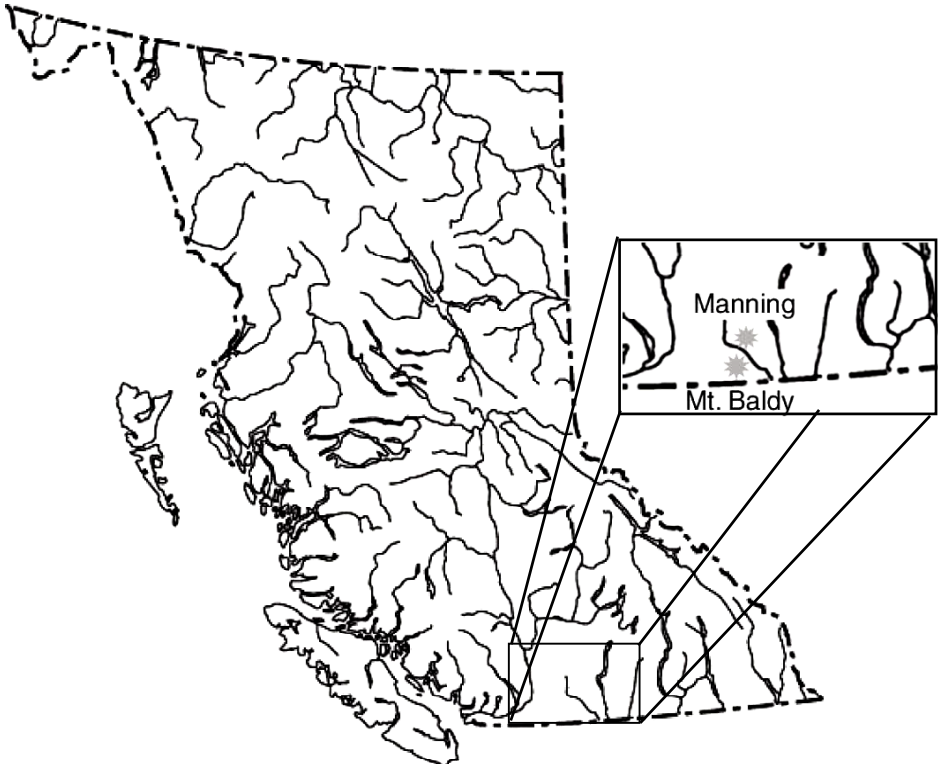

Figure 2.1. Location of sampling sites for mating systems study.

2.2.2 GENETIC ANALYSIS

2.2.2.1 Laboratory

Thirty filled seeds per tree for both populations were dissected to isolate the haploid megagametophyte and diploid embryo tissues. These samples were then subjected to isozyme analysis via starch gel electrophoresis during the summer of 1998. Five enzyme systems were assayed and ten scorable loci were detected (see Table 2.1) using slightly modified buffers detailed in Mitton *et al.* (1977).

Table 2.1. List of enzyme loci screened for mating systems analysis.

Enzyme	Locus	Enzyme name	E.C. number
Pgi	1,2	Phosphoglucose isomerase	5.3.1.9
Pgm		Phosphoglucomutase	2.7.5.1
6Pg	1,2	6-Phosphogluconic dehydrogenase	1.1.1.44
Idh		Isocitrate dehydrogenase	1.1.1.42
Mdh	1,2,3,4	Malate dehydrogenase	1.1.1.37

2.2.2.2 Analysis

Individual genotypic data were entered into a Microsoft Excel™ spreadsheet. These data were assessed for linkage disequilibrium utilizing a heterogeneity-G test, a modification of the χ^2 test (Sokal and Rohlf 1995) for each segregating pair of alleles. Linkage prevents Mendelian segregation and may obscure other genetic effects, so strongly linked loci should be excluded from mating systems analyses. Based on haploid genotypic data from the maternal and embryo tissue, an analysis was first conducted in order to assess the populations for linkage disequilibrium. Pairs of segregating alleles were compared and subjected to a heterogeneity-G test (Sokal and Rohlf 1995). The software application Popgene V.3.2 (Yeh *et al.* 1999) was utilized to determine linkage disequilibrium following Ohta's (1982) method by performing an analysis using components of variance. This approach was developed to elucidate effects of population structure and gene flow by calculating and then adjusting for linkage disequilibrium.

No consistent patterns of linkage disequilibrium were found in the loci examined, thus all loci were retained and the genotypic data were assessed for inbreeding levels and other genetic parameters using the program MLTR (Ritland 1989,1990). This program uses genotypic or allelic frequency data to calculate estimates of inbreeding at the family and population level via bootstrapping to a specified confidence level using a mixed mating model (i.e., both selfing and outcrossing are assumed to occur within the population). MLTR can estimate both selfing and biparental inbreeding, as well as other statistics correlating the relative proportions of inbreeding between parents and offspring, but utilizes the assumption that progeny are either products of selfing (t = 0) or complete outcrossing (t = 1) (Ritland 1990). If data from both parents and the offspring are not available, an inference technique using paternity exclusion is employed. A seed, or starting number is selected by the user, which is then the starting point for the bootstrapping estimates; a higher number of bootstrapped estimates will likely give a more accurate estimate of the parameters, assuming maternal fitness and potential paternal genotypes are equal among all parents, loci are not linked, and are selectively neutral (Clegg 1980; Gooding 1998).

Each family and population was assessed for single-locus and multilocus estimates of outcrossing, observed measures of all parameters, and 100 bootstrapped Newton-Raphson iterations were used to generate error estimates. Multilocus estimates of t (t_m) are much more informative than single-locus estimates (t_s), however, since they give an integrated estimate based on the total of all the information collected, and thus have far more degrees of freedom and statistical power, and they are more robust to violations of assumptions inherent in models which calculate t (Young *et al.* 2000). It is useful to estimate t_s since the difference between t_m and t_s can provide an estimate of the amount of biparental inbreeding: if no significant difference is found, then most inbreeding likely results from selfing, but if the difference is significantly higher than zero, then much of the inbreeding could be accounted for by mating among relatives (Gooding 1998). For any given data set (i.e., a family or population), the multilocus estimate of outcrossing will always be higher due to the robustness of the data to violations of assumptions and thereby would provide an estimate which would reflect more outcrossing than the single-locus estimate which would tend to be biased toward more selfing (Shaw *et al.* 1981).

2.3 RESULTS

Only heterozygous loci where it is possible to detect segregation can be used in linkage analysis, so *Mdh1*, which was monomorphic in both populations and *6Pg2*, which was monomorphic in one population, were not included in the analysis. Table 2.2 shows the results of the heterogeneity-G test in which each pair of segregating loci is tested for segregation distortion. No consistent trends were found and no pairs of alleles showed linkage when analyzed following Sokal and Rohlf (1995). All pairs of alleles thus appeared to segregate according to random Mendelian patterns. A summary of the numbers of trees which exhibited segregation distortion at each pair of loci is in Table 2.3.

The results of the heterogeneity-G test, presented in Table 2.3, reveal that no loci significantly deviated from expected random segregation patterns despite several families

showing isolated instances of disequilibrium in Table 2.2. Interestingly, when all loci were combined, the results of both the pooled and heterogeneity tests were highly significant (at α = 0.05 and 0.01, respectively), indicating that although no individual locus deviated from random segregation, the cumulative effect throughout both populations did show some systematic bias towards the common allele (1).

Table 2.2. Number of families with pairs of loci in linkage disequilibrium (Manning and Baldy populations combined, megagametophytes only)

	Pgi1	*Pgi2*	*Idh*	*Pgm*	*6Pg1*	*6Pg2*	*Mdh1*	*Mdh2*	*Mdh3*
Pgi2	2	-							
Idh	0	0	-						
Pgm	0	0	0	-					
6Pg1	1	2	2	0	-				
6Pg2	0	0	0	0	0	-			
Mdh1	0	0	0	0	0	0	-		
Mdh2	4	1	2	0	4	0	0	-	
Mdh3	0	1	0	0	0	0	0	0	-
Mdh4	5	3	4	0	1	0	0	2	0

Table 2.3. Log-likelihood G-test on segregation ratios of polymorphic loci for combined populations Manning and Baldy.

Locus	Alleles Detected	No. of Trees	Observed Ratio of Alleles	Pooled G (goodness of fit)	Heterogeneity G (test of independence)
Pgi1	1,2	15	225:205	0.930568	14.64466
Pgi2	1,2	9	138:132	0.133344	2.693182
Idh	1,3	2	30:30	0	0.266864
Pgm	1,3	17	258:248	0.197641	24.84116
6Pg1	1,3	1	16:14	0.133432	0
6Pg2	1,3	22	330:296	1.847554	25.32880
Mdh1	1	0	-	-	-
Mdh2	1,2	2	31:20	2.391295	0.018822
Mdh3	1,3	30	459:423	1.469796	25.47460
Mdh4	1,3	17	257:237	0.809937	21.44237
All			1605:3349	5.770842*	4636.929**

* Significant at α = 0.05
** Significant at α = 0.01

Furnier *et al.* (1986) detected slight linkage disequilibrium between several pairs of loci in whitebark pine; however, the lowest recombination rate (r) for any pairwise test was 0.35 (for *Adh:Pgi2*), which was not markedly lower than 0.50, the value representing completely unlinked loci (Falconer and Mackay 1996; Hartl and Clark 1997; Bruederle *et al.* 1998). None of the pairs of loci exhibiting linkage disequilibrium in that study were involved in similar patterns in this

study as different enzymes were investigated in both studies, and where the same loci were assessed, similar patterns were not observed. While no systematic bias was discovered among the ten loci analyzed, overall distributions of allele frequencies deviated significantly ($p < 0.05$) from the expected segregation under assumptions of random mating (χ^2 goodness of fit test). In the case of *6Pg1*, only one family was polymorphic, and only two families were polymorphic for *Idh* and *Mdh2*. The low number of heterozygous trees and total observations for these loci unfortunately decrease the statistical precision of the tests in these cases. The relatively small number of loci may have also obscured any extant linkage.

Table 2.4. Estimates of t at the population level. Single-locus (SL) and multilocus (ML) estimates are equivalent, except when all loci are combined. Standard errors of the mean in parentheses.

Locus	Mt. Baldy	Manning
Pgi1	0.762 (0.109)	0.493 (0.230)
Pgi2	0.888 (0.083)	0.777 (0.150)
Pgm	0.396 (0.282)	0.123 (0.057)
Idh	0.621(0.085)	0.709 (0.155)
Mdh1	0.000 (0.000)	0.000 (0.000)
Mdh2	0.646 (0.089)	0.758 (0.055)
Mdh3	1.319 (0.952)	0.614 (0.385)
Mdh4	0.897 (0.107)	0.759 (0.069)
6Pg1	0.913 (0.114)	0.684 (0.160)
6Pg2	0.000 (0.000)	0.294 (0.237)
Combined SL	0.735 (0.048)	0.650 (0.061)
Combined ML	0.736 (0.042)	0.722 (0.054)
t_m-t_s	0.001 (0.014)	0.068 (0.025)
r_t	0.082 (0.052)	0.074 (0.046)
r_n	0.208 (0.082)	0.148 (0.063)
No. of families	30	25
No. of observations	853	750

Single tree estimates of t varied from 0 to 1 for Mt. Baldy and Manning. Arithmetic means (± SE) were 0.550 (± 0.013) for the former and 0.519 (± 0.014) for the latter. Individual trees varied considerably in their estimated outcrossing rates by locus and there was wide variation among trees within populations. The single-locus and multilocus estimates of t for Mt. Baldy were nearly identical (0.735 and 0.736, respectively), while for Manning they were different (0.650 and 0.722, respectively), suggesting slight biparental inbreeding based on t_m-t_s in the latter population, while accounting for most inbreeding in the former by selfing. The difference

between single- and multilocus outcrossing rates was not statistically significant in either population at the 0.05 significance (α) level. t_m and t_s were not statistically different when comparing all families using a paired t-test at $\alpha = 0.05$. With respect to individual loci outcrossing rates, excluding fixed alleles (which by definition have t = 0), the minimum for Mt. Baldy was 0.396 for *Pgm* and the maximum 1.319 (which is effectively 1.000, since numbers greater than one are a statistical artifact of the estimation algorithm, and biologically impossible) for *Mdh3*. For Manning, the minimum value was 0.123 for *Pgm* and the maximum was 0.777 for *Pgi2*. Manning had generally lower t values, but Mt. Baldy had an additional fixed allele (*6Pg2*) which lowered the combined rate for the overall population. Excluding this locus, Baldy and Manning each had multilocus t values of 0.716 and 0.546, respectively.

All loci had differing estimates of the outcrossing rate (t) except for *Mdh1* which was fixed in both populations. In most cases, t for individual loci were within one standard error of each other, although this was not so for *6Pg2*, which had two alleles in Manning ($t = 0.294 \pm 0.237$) and was fixed at Mt. Baldy ($t = 0 \pm 0$). *6Pg1* had only one segregating family and *Idh* and *Mdh2* had only two, possibly leading to lower estimates at these loci. The standard error for *Mdh3* was quite large relative to the mean t value, since this was a highly heterozygous locus and there was considerable variation both among and within families.

The statistic r_t represents the correlation between parental and progeny values of t in a population (Ritland and Jain 1981; Ritland and El-Kassaby 1985; Ritland 1990), and this value was slightly higher for Baldy, although the results were not significantly different. r_p is the correlation of progeny, representing the chance that two randomly chosen progeny are full sibs (Ritland 1990). For Baldy, this value was 0.208, or almost 21%, and for Manning 0.148, or 15%. These values would double for the probabilities of randomly drawing half sibs, supporting a strongly structured population comprised of individuals with varying degrees of relatedness, but often sharing a parent or grandparent.

Displayed in graphical format, family (single-tree) outcrossing estimates for all loci combined reveal a bimodal distribution for both populations (Figures 2.2, 2.3). There was a very wide range of outcrossing rates for both populations, although there was a gap for the category of t = 0.90-0.99 in both populations. One family in each population appeared to have nearly complete selfing (t = 0.00-0.09), and one family in Manning and two at Mt. Baldy appeared to be completely outcrossing ($t \geq 1.00$), although the estimates for individual loci vary within those families.

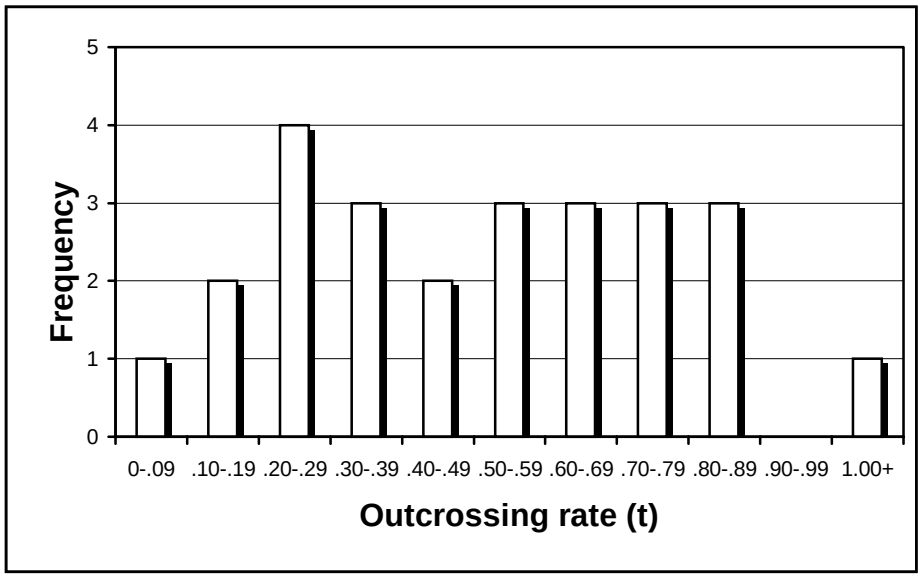

Figure 2.2. Frequency distribution of family outcrossing rates for all loci for Manning.

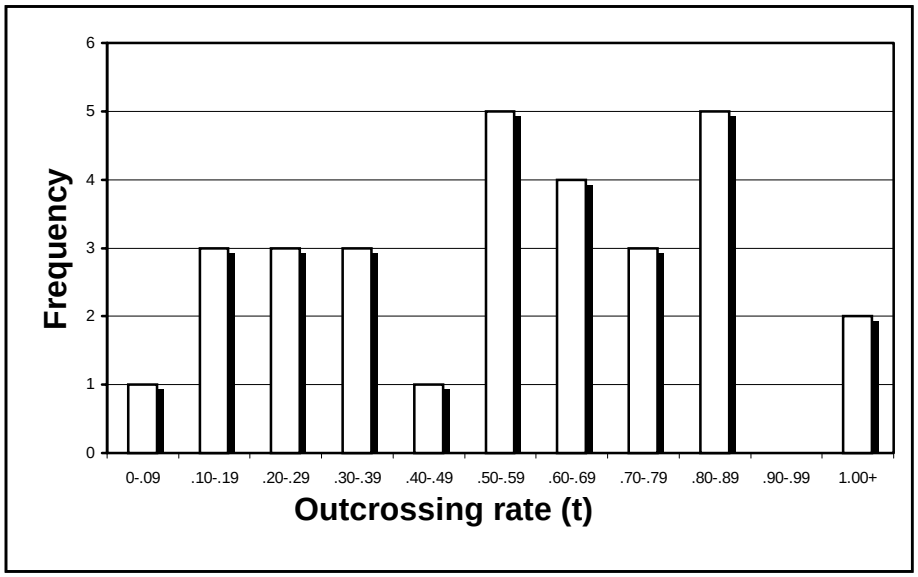

Figure 2.3. Frequency distribution of family outcrossing rates for all loci for Mt. Baldy.

2.4 DISCUSSION

2.4.1 MATING SYSTEM OF STONE PINES

Krutovskii and others (1994, 1995) have determined the mating systems of other stone pines (subsection Cembrae), but have not done so for whitebark pine. Table 2.5 includes outcrossing rates of the stone pines. Although data are lacking for the dwarf Eurasian *Pinus pumila*, outcrossing rates of stone pines are lower than many other pines, ranging from 0.686 (*P. cembra*) to 0.974 (*P. koraiensis*) for multilocus estimates and from 0.693 (*P. albicaulis*) to 0.936 (*P. koraiensis*) for single-locus estimates. While *P. koraiensis* was generally outcrossing, the other species all exhibited significant levels of inbreeding. This is not surprising given their similar habitat types and life history characteristics, all reliant on nutcrackers for seed dispersal.

Table 2.5. Outcrossing data for stone pines (subsection Cembrae) and other pines

Taxonomic group	Species	t_s (std. error)	t_m (std. error)
subsection Cembrae	*Pinus albicaulis*[1]	0.693 (0.055)	0.729 (0.048)
	P. cembra[2]	0.707 (0.045)	0.686 (0.025)
	P. koraiensis[2]	0.936 (0.051)	0.974 (0.058)
	P. pumila[2]	n/a	n/a
	P. sibirica[2]	0.862 (0.054)	0.894 (0.057)
subsection Ponderosae	*P. ponderosa*[3]	0.933 (0.052)	0.960 (0.030)
subsection Ponderosae	*P. jeffreyi*[4]	0.911 (0.081)	0.935 (0.021)
subsection Contortae	*P. contorta*[5]	0.974 (0.016)	0.926 (0.034)
subsection Strobi	*P. monticola*[6]	0.925 (0.056)	0.977 (0.023)
subsection Cembroides	*P. maximartinezii*[7]	0.816	0.761

[1]this study; [2]Krutovskii *et al.* 1995; [3]Mitton *et al.* 1981; [4]Furnier and Adams 1986; [5]Perry and Dancik 1985; [6]El-Kassaby *et al.* 1987; [7]Ledig *et al.* 1999; n/a data not available for this species

Murawski and others (1994) found that selective logging decreased the multilocus outcrossing rate of a tropical canopy tree by 18%; Gooding (1998) found a value of 0.642 for ponderosa pine in an area under pressure from harvesting and urban development, compared to the 0.960 found by Mitton and others (1981) in areas not subject to the same impacts. In light of the increased inbreeding caused by human impact, it is advisable that the effects of human intervention be carefully considered in whitebark pine ecosystems.

The documented history of coevolution between *Nucifragia* spp. and stone pines may have influenced the mating system of subsection *Cembrae* compared to pines with wind-dispersed

seeds. The caching of groups of related individuals together and their subsequent germination and synchronized phenology would probably lead to greater opportunity for self-pollination and mating among relatives (biparental inbreeding) than other pines. This would account for the relatively high inbreeding found in this study, but not for the wide range of outcrossing estimates for individual families. Other studies have documented similar levels of inbreeding among bird-dispersed pines in other taxonomic categories: ponderosa and maxipiñon pines.

The most likely explanation for the range and distribution of outcrossing coefficients found among families is that outcrossing rate is influenced by many genes which directly and indirectly affect the mating system, leading to a continuous, rather than a discrete, distribution. Genes affecting the mating system could impact factors such as male and female fecundity and fertility, pre- and postzygotic barriers to fertilization (especially in the case of inbred individuals), and reproductive phenology (e.g., timing and duration of gamete production and receptivity) (Jarne and Charlesworth 1993). The bimodal shape of the outcrossing rate distribution exhibited in both populations could be the result of diversifying selection acting differentially both on the loci and families. Some families clearly experience a very high level of inbreeding and possibly even selfing, while some appear to be primarily outcrossers. If selection, environment and their interaction had similar effects across families and loci, the curve would be normally distributed; instead, the individual trees generally appear to have differential responses resulting in some primarily outcrossing and others primarily inbreeding. The relatively low number of generations since glaciation would partially explain the persistence of families with intermediate outcrossing rates, since HWE has not yet been reached, both in terms of time and the inherent instability of a bimodal character distribution within populations. This distribution may reflect the recolonization of the species' range in the B.C. southern interior from two refugial populations, one primarily outcrossing and the other primarily selfing. During the several ice ages during the past 100,000 years, whitebark pine populations fluctuated and were relegated to genetically bottlenecked refugia during glacial maxima and expanding its range via founder effects in the

interglacial periods. The bottlenecked populations may have developed a greater tolerance to selfing, whereas more continuous populations retained higher outcrossing rates; as the refugial populations expanded and exchanged genetic material, the more gradual bimodal distribution could have developed.

Selection for different temporal and spatial levels of inbreeding has been found in other conifers, namely Scots pine (Hedrick *et al.* 1999), western white pine (El-Kassaby *et al.* 1994), lodgepole pine (Perry and Dancik 1985), ponderosa pine (Mitton *et al.* 1981) and Sitka spruce (El-Kassaby 1994). These differences have been found not only among families and provenances, but also among crown strata within individual trees (El-Kassaby 1994). Mating system differentiation must therefore operate at very fine scales and be exerted by a multitude of environmental and genetic factors. It is difficult to verify whether these differences are the direct result of selection since there are so many complex factors involved and it would take many years to conduct controlled tests in conifers to this effect.

Hedrick and others (1999) have suggested that populations have differential susceptibility to inbreeding depression. The number of lethal equivalents among individuals and populations would differ with many of the factors suggested in the preceding discussion. Populations may also have different intensities of selection acting on those factors, as well as direct selection against lethal equivalents. They postulated that polyembryony acted as a mechanism to effectively increase the tolerable genetic load in the event of inbreeding since the cost of producing offspring is lower since two or more proembryos are simultaneously produced, and in the event that one has a high genetic load, the remaining embryo(s) would likely still be successful. Kärkäinen and others (1999) using controlled pollination experiments, determined that individual *Pinus sylvestris* trees have differing levels of tolerance to inbreeding depression, and that although the early effects of selfing were that the vast majority of seeds were aborted, maternal genotype was the dominant factor determining fitness, measured by seed set. It is certainly possible that whitebark pine could manifest variation in selfing tolerance in a similar

fashion, both at the individual and population level, although in this study unfortunately it was not possible to determine whether aborted seeds were primarily the products of selfing. Given the genetic architecture of most whitebark pine populations in BC, the pollen pool available to most maternal parents likely consists of a high proportion of self and related pollen, and given the widespread occurrence of aborted, empty, and underdeveloped seed, it is likely that a large proportion of these types of seeds are the result of selfing.

One type of selection applicable in this instance and explored in the context of limber pine (*Pinus flexilis* James) by Schuster and Mitton (1991), is kin selection. This phenomenon occurs when it is advantageous, in terms of overall survival of genotypes, for related individuals to facilitate each others' reproduction and success at the potential temporary expense of the fitness of the individuals involved (Slatkin 1987). This would allow for a higher level of inbreeding, often associated with reduced individual fitness, to be compensated for by higher overall survival of related families (Jarne and Charlesworth 1993); models suggest that in some cases, mating among relatives may decrease the genetic load over time by purging deleterious alleles to a point where some inbreeding can be tolerated without further reducing the fitness of the population (Kirkpatrick and Jarne 2000). The common occurrence of root grafting and chemical transfer between individual related genotypes would also facilitate consanguineous mating by transferring photosynthates among grafted individuals for increased overall survival (Tomback and Linhart 1990.

For the Manning populations, the difference between the single- and multilocus outcrossing rates was 0.001, indicating that the inbred offspring were most likely the products of selfing. Figure 2.2 shows one family in the category of $t = 0\text{-}0.09$, which suggests that one family has consistently high levels of selfing. For Mt. Baldy, t_m-t_s was 0.068, suggesting that biparental inbreeding, as opposed to selfing, is the more common mechanism of inbreeding in this population, although one family in this population also showed nearly complete selfing (Figure 2.3). The correlated mating statistic, r_t, was under 10% for both populations, with a mean of

0.078, reflecting a 7.8% correlation between the outcrossing rates of parents and their offspring. r_p was relatively high (0.178 average for both populations) in whitebark pine; El-Kassaby and Jaquish (1996) reported a value of 0.082 for western larch (*Larix occidentalis* Nutt.).

2.4.3 SOURCES OF ERROR

While isozymes are an accepted and tested method of inferring mating system, some uncertainties remain. Statistical power obviously increases with the number of loci used (Hamrick and El-Kassaby 1987) and the sample size. The sample size in this study, ten loci and up to 30 samples per maternal parent (the cone collectors assumed each clump represented one parent, which may not be the case), is generally accepted as adequate (Sokal and Rohlf 1995), provided the loci show Mendelian inheritance, each parent has equivalent fitness compared to other parents in the gametic pool and all maternal parents have identical outcrossing rates, and the loci are selectively neutral (Mitton 1992). While the loci appear to follow Mendelian patterns of inheritance and other studies have not found strong effects of selection on the loci used in this study with respect to fitness of pine trees, Figures 2.2 and 2.3 reveal that each maternal parent has a different tendency towards outcrossing; these results are consistent with those El-Kassaby and others (1987) found in western white pine (*Pinus monticola*). The pollen pool contributions and relative receptivity of ovules were not tested in this study, but often these assumptions are violated in other species (Hedrick *et al.* 1999; Shaw *et al.* 1981). Outcrossing rates in western white pine have been found to vary from year to year (El-Kassaby *et al.* 1993), and this may also be the case for whitebark pine. The only way to verify this is to take samples from the same families for several years and assay the seeds for the same loci.

Although the use of multilocus outcrossing estimates is more robust to violations of the statistical assumptions than single-locus estimates, there may still be some inherent errors due to the effects of selection, genotype by environment interaction, and temporal variation in

outcrossing that was not measured in this study. In addition, high genetic loads associated with individuals which are the products of selfing may have resulted in early postzygotic barriers to embryo survival, causing an underestimation in the number of inbred individuals.

Adams (1992) suggested that for paternity analysis using haploid tissues, only 12 polymorphic isozyme loci are required for 90% confidence, and 13 loci using diploid tissues; for 99% confidence, 23 haploid markers would be required. If each stem in a clump that was collected from actually was a different genotype, then estimates of outcrossing were overestimated as the inclusion of different individuals would lead to inflated measures of genetic diversity. As detailed in Chapter 3, there are some subjective aspects to isozyme interpretation and analysis related to the laboratory conditions and analysis methods (Gillet 1993). The program MLTR, developed by K. Ritland, has been used extensively to estimate inbreeding parameters and is generally accepted as an effective tool. Occasionally, outcrossing coefficients (t) > 1.00 are calculated. While this is a statistical artifact of the calculation, a value greater than one is biologically impossible, although it could also be interpreted as a type of assortative mating for obligate outcrossers (Young *et al.* 2000).

2.4.4 COMPARISON WITH OTHER POPULATIONS

Due to the limited nature of this mating system study (only two geographically close populations were assessed), the results of this study may not be directly extrapolated to the entire species. While they do provide a good approximation for populations in the southern Coast Mountains, the scope of this analysis is likely too small to extrapolate much beyond that. One other possibility is that these two populations actually could be parts of the same metapopulation: they are close enough to exchange some genetic material. Studies of pollen flow (Latta and Mitton 1997) show that while the vast majority of propogules are disseminated close to the parent, many viable pollen grains can be transported considerable distances by prevailing winds. Seed dispersal could also account for this phenomenon since although

Clark's nutcrackers have been observed caching seeds 22 km from the seed source (Vander Wall and Balda 1977), black bears in the area also consume large numbers of seed and have very large home ranges, and could possibly disseminate unchewed seeds that pass intact through their digestive tracts over thousands of hectares via their droppings.

2.5 CONCLUSION

Whitebark pine has a fairly high level of inbreeding compared with other pines (mean multilocus outcrossing rate = 0.73), but these estimates are within the range of those of other bird-dispersed stone and piñon pines. For the two populations tested, each had a bimodal distribution of outcrossing rates among families; t was highly variable among families, from nearly zero to complete outcrossing. This distribution may reflect population genetic structure facilitated by bird seed caching, as well as differential individual tree responses to selection acting upon a complex group of genes impacting genetic load, reproductive fitness, and consequently, mating system.

CHAPTER 3 – GENETIC DIVERSITY IN BRITISH COLUMBIA

"Nature has good intentions, of course, but as Aristotle once said, she cannot carry them out."

- Oscar Wilde, 1891

3.1 INTRODUCTION

3.1.1 IMPORTANCE OF GENETIC DIVERSITY FOR WHITEBARK PINE

Maintaining the genetic diversity of whitebark pine is critical for the long-term survival of high elevation ecosystems of which it is a keystone species (Bradshaw and McNeilly 1991; Erikkson *et al.* 1993). Although whitebark pine communities typically have low timber value, they have extremely high values in other areas (Watson *et al.* 1997): watershed protection, slope stability, wildlife habitat, aesthetics (Arno and Hoff 1990), First Nations cultural heritage and biodiversity, to name a few. Natural ecosystems are regarded as a reservoir of genetic diversity which ensures future ecosystem stability (Boyle 1992; Millar and Westfall 1992). It is therefore essential that entire intact ecosystems be protected in order to preserve evolutional processes and linkages of interdependent species (Boyle 1992; Leopold 1933; Ledig 1986,1988; Millar and Westfall 1992). The extremely slow growth rate of whitebark pine lends extra weight to the consequences of the decisions that must be made now in terms of genetic conservation: any impact that management strategies have may take decades to appear, and centuries to remedy should they be the wrong ones (Brussard 1990; Bradshaw and NcNeilly 1991; Tomback *et al.* 2001; Cole and Landres 1996; Ledig 1986).

3.1.2 GENETIC DIVERSITY AND POPULATION STRUCTURE

As a consequence of nutcracker seed caching, whitebark (and limber) pine trees often grow in cohorts which contain related individuals (Linhart and Tomback 1985; Tomback and Schuster 1994; Bruederle *et al.* 1998). Trees may grow monopodially, but a multi-stemmed growth form is quite common (McCaughey 1994; McCaughey and Schmidt 1990; Ogilvie 1990; Tomback and Schuster 1994; Weaver and Forcella 1986). On exposed ridges, a krummholtz form of the tree exists (Ogilvie 1989; Tomback 1986), and it also reproduces vegetatively by layering (Arno

and Hoff 1989, 1990; McCaughey 1994; Rogers *et al.* 1999). Due to seed caching and abiotic influences on growth form, these multi-stemmed trees may be a single or several individuals (Linhart and Tomback 1985; Furnier *et al.* 1987; Weaver and Jacobs 1990). It is impossible to tell by observation since the trees are often grafted together; genotypic analysis is the only way to determine the identity of the individual stems within a multi-stemmed clump (Tomback and Linhart 1990).

Stands typically contain related individuals, from full-sibs to half-sibs (Brussard 1990; Furnier *et al.* 1987; Jorgensen and Hamrick 1997; Rogers *et al.* 1999; Schuster and Mitton 1991; Tomback and Linhart 1990; Chapter 2, this study), and there is no apparent pattern of relatedness among stands (Furnier *et al.* 1987; Bruederle *et al.* 1998; Rogers *et al.* 1999). This is likely due to many nutcrackers caching seeds randomly throughout their home ranges, where each seed cache is likely to contain some related individuals, although the placement of the caches themselves is essentially random (Tani *et al.* 1998; Tomback and Schuster 1994).

In many studies involving isozymes, populations coalesce into regional groups, reflecting an overall gradient of relatedness throughout the species' range (Bruederle *et al.* 1998; Jorgensen and Hamrick 1997; Yandell 1992). The western portion of the species range, found along the Rocky Mountains, displays only one third of the genetic variability of the eastern populations. This is probably a result of the recolonization of the species range northward by populations that survived in glacial refugia (Axelrod 1986; Baker 1990; Richardson 2001) that were more abundant in the eastern portion of the range. Founder effects (i.e., the founding of populations from a small number of individuals) due to subsequent recolonization via bird-mediated seed dispersal may have been one cause of the low level of population differentiation and high gene flow (i.e., low F_{ST} and high N_m) found in northern populations (Hard 1995; Jorgensen and Hamrick 1997).

Gene flow occurs between local populations via wind-pollination (Brussard 1990), but interpopulation pollen flow is limited between regional or distant local populations by factors such as wind dessication of pollen and phenological differences (Arno and Hoff 1989; Hamrick *et al.* 1992; Jorgensen and Hamrick 1997, personal observation). Most pollen drift occurs within populations (Brussard 1990) and seeds are typically dispersed randomly within several kilometres of the parents (Schuster *et al.* 1989). Gene flow patterns of whitebark pine thus generate a population structure that encompasses the majority of the population genetic variation among individuals (Bruederle *et al.* 1998; Yandell 1992), but low differentiation among populations (Gregorius and Baradat 1992; Hamrick *et al.* 1991,1992; Jorgensen and Hamrick 1997; Krutovskii *et al.* 1995; Schuster *et al.* 1989).

Other studies have found clear regional differentiation, and low differentiation among populations within regions (Jorgensen and Hamrick 1997; Yandell 1992; Stuart-Smith 1998): these characteristics may reflect the northward, radiative range expansion from refugial populations of the species following the most recent glaciation (Baker 1990; Ellstrand 1992). Founder effects resulting from nutcracker caching also influence the population genetic structure by creating a stepwise northward migration pattern where the mixture of genotypes is fairly heterogeneous among and within populations, but the mixture of genotypes within clumps reflects a high degree of relatedness (Jorgensen and Hamrick 1997; Latta and Mitton 1997; Tomback and Schuster 1994; Richardson 2001).

Many studies have shown in conifers that the percentage of heterozygotes increases significantly with age from embryos to mature individuals (e.g., Bush and Smouse 1992; Politov and Krutovskii 1994). Several studies have found that mature conifers in general, and whitebark pine in particular, have excess heterozygotes (Politov and Krutovskii 1994; Bruederle *et al.* 1998; Stuart-Smith 1998; Rogers *et al.* 1999; Koelewijn *et al.* 1999). This phenomenon could be interpreted as the result of overdominance: increased fitness of heterozygotes relative

to homozygotes, but this hypothesis has not been specifically tested. Selection against deleterious or lethal alleles which are more frequently expressed in homozygotes, especially those which are the products of selfing, is consequently manifested as selection against homozygotes, and especially inbred individuals (Gregorius and Baradat 1992; Krutovskii *et al.* 1995; Fu and Ritland 1994; Wang and Hill 1999; Morgan 2001). Both heterosis and genetic load, the former involving overdominance and the latter dominance, have been implicated in the cause of inbreeding depression in plants. While the ideal means of elucidating the root cause would involve multi-generation controlled crossing experiments and QTLs linked to deleterious alleles (Fu and Ritland 1996; Charlesworth and Charlesworth 1999), it is also possible to draw some conclusions from studies using isozymes based on Wright's inbreeding coefficient *F* (where $F = 1 - H_o/H_e$), which can assess pre-existing inbreeding levels (Ledig and others 1997, 2000).

The theory of kin selection could explain the common occurrence of grafting which occurs between roots or stems, indicating that the tissues are often compatible and allelopathic interactions seldom occur between related individuals (Tomback and Linhart 1990; Tomback and Schuster 1994; Weaver and Jacobs 1990); grafting has been noted in a variety of stress-tolerant conifers in ecologically severe conditions (Tomback and Linhart 1990). Another explanation which has been offered to support overdominance is that heterozygous individuals have increased fitness in extreme environments due to their inherently greater potential for adaptation and evolution (Lande and Barrowclough 1987).

Most of the genetic analysis performed on whitebark pine has been concluded using allozymes; extending this technique to BC populations facilitates comparisons among studies. For this purpose, isozymes are ideal markers: codominant, polymorphic, with clear alleles (Gregorius and Baradat 1992), they generate reproducible and reliable results, and are relatively inexpensive in terms of labour and equipment (Cruzan 1998). El-Kassaby (1991) and

others have calculated that the proportion of genic variation within an organism detectable by isozymes may be < 0.01%, and that allozyme diversity is generally not associated with adaptive traits (Berg and Hamrick 1997; Bush *et al.* 1987). The number of loci is limited due to the nature of protein expression (Parker *et al.* 1998), and in some instances their neutrality has been questioned (Bush and Smouse 1992; Markova *et al.* 2000). Since they are based on fundamentally different portions of the genome (coding vs. noncoding, respectively), results from isozymes and other molecular markers based on noncoding regions such as microsatellites are not directly comparable (Petkau *et al.* 1997). Traditional analytic measures developed for isozymes may not be applicable to microsatellites as their mutational mechanisms differ and their mutation rates differ by so much. Thus, it is difficult to make direct meaningful comparisons between DNA markers such as cpDNA (which has a unique mode of inheritance) or microsatellites and isozyme data.

3.1.3 OBJECTIVES

The results of a genetic analysis can reveal many types of information, depending on the initial objectives and sampling design. This study attempts to fill in existing information gaps by focusing on populations throughout B.C., encompassing the northernmost range limits of whitebark pine. Objectives of this study are:

1. to calculate basic genetic diversity statistics (expected and observed heterozygosity, alleles per locus, etc.) for populations encompassing the entire range of whitebark pine in B.C.;
2. to calculate Wright's *F*-statistics and compare them with the results from the mating systems analysis in Chapter 2;
3. to identify patterns of genetic diversity in B.C. whitebark pine; and
4. to compare the results of the above with those found in studies of whitebark pine from other geographic areas, and attempt to explain similarities or differences.

3.2 METHODS AND MATERIALS

3.2.1 FIELD COLLECTIONS

Between May and August 2000, bud samples were collected from 29 populations, including 26 populations from throughout the native range of whitebark pine around B.C., three from the Alberta Rockies and one in the Washington Cascades near B.C. Of these 29, 17 were successfully assayed in this study (See Figure 3.1 and Table 3.1 for populations analyzed in this study, see Appendix III for a list of all populations sampled.) One bud was collected per tree along with a sample of the previous year's needles from approximately 30 trees per population. Trees were sampled a minimum of ten metres apart, and only one stem was sampled per clump. Blister rust incidence and size did not influence sampling decisions, providing the tree was large enough to survive removal of the sample. Samples were then wrapped in aluminum foil, labelled and stored in a portable liquid nitrogen container until they were stored in a -80°C freezer.

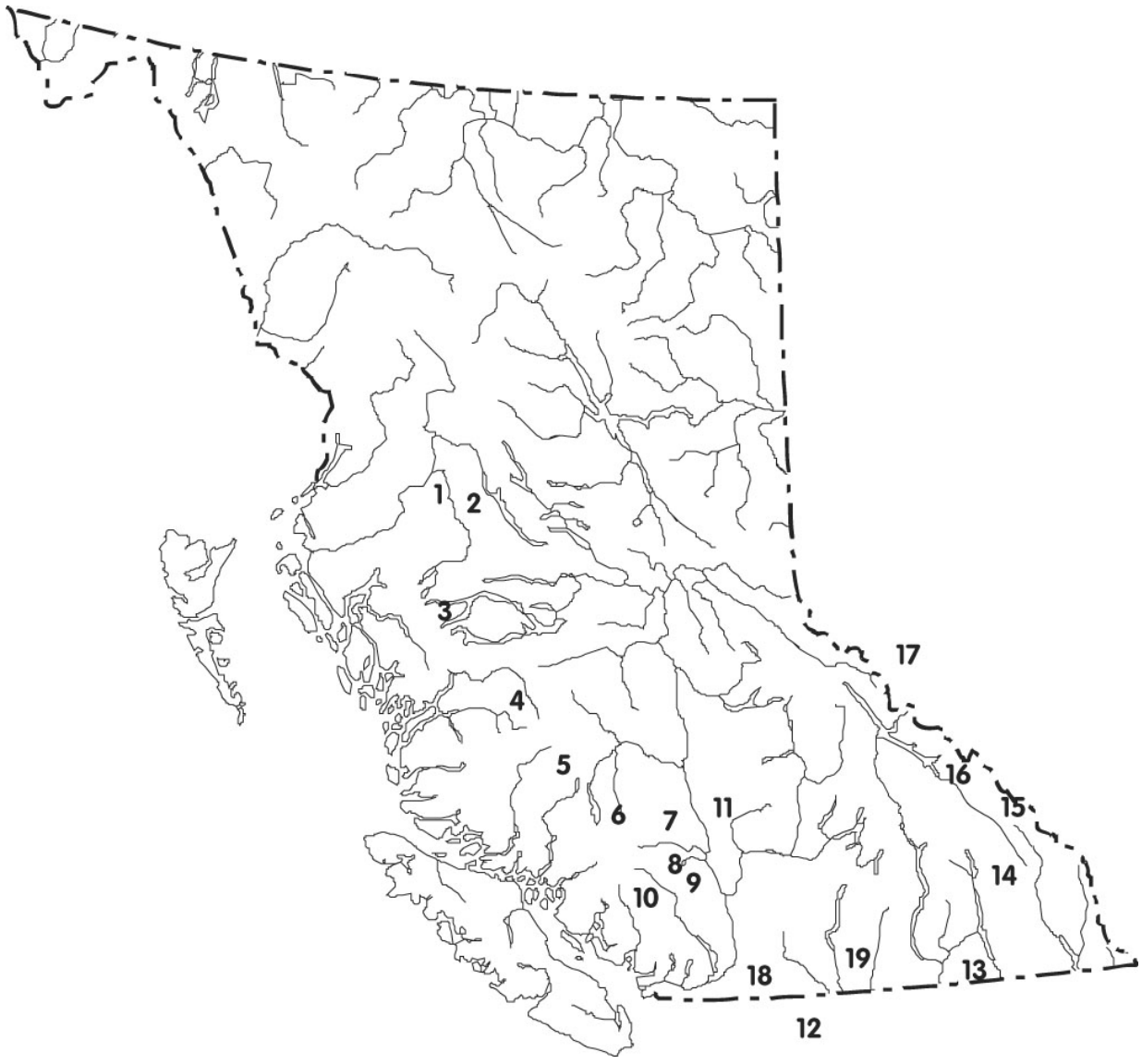

Figure 3.1. Genetic diversity sampling locations.

Table 3.1. List of sample locations summer 2000; PP = provincial park, NP = national park, Ck = creek, Mt = Mount; Mtn = mountain, Lk = lake, R = river.

Pop #	Location	Area	NTS 1:50,000 Mapsheet	Latitude (N) Longitude (W)	Elevation (m)
1	Hudson Bay Mtn	Smithers	Smithers 93L/14	54°56'25" 127°19'15"	1850
2	Higgins Creek	Babine Mtns PP	Driftwood Ck 93L/15	54°54'20" 126°46'55"	1600
3	Sweeney Lake	Houston	Newcombe Lk 93E/14	53°45'25" 127°12'35"	1630
4	Heckman Pass	Tweedsmuir PP	Tusulko R 93C/12	52°32'20" 125°48'40"	1600
5	Perkins Peak	Chilcotin	Tatla Lk 92N/15	51°50'45" 124°59'10"	1700
6	Tchaikazan R	Ts'yl-os PP	Tchaikazan R 92O/4	51°12'00" 123°39'30"	1600
7	Yalakom R	Lillooet	Big Bar 92O/1	51°04'50" 122°27'05"	1900
8	D'arcy	D'arcy	Birkenhead Lk 92J/10	50°31'15" 122°34'35"	1910
9	Van Horlick Ck	Lillooet	Duffy Lk 92J/8	50°16'20" 122°14'45"	2000
10	Whistler Mtn	Whistler	Whistler 92J/2	50°03'45" 122°56'00"	1700
11	Lime Lookout	Clinton	Clinton 92P/4	51°05'25" 121°39'55"	1980
12	Hart's Pass (Washington, U.S.A.)	Okanogan National Forest	USGS 1:24,000 Slate Peak N4837.5 W12037.5/7.5	48°42'30" 120°41'00"	2050
13	Kootenay Pass	Stagleap PP	Salmo 82F/3	49°05'10" 117°02'30"	1940
14	Jumbo Pass	Purcell Mts	Duncan Lk 82K/7	50°20'20" 116°38'00"	2060
15	Stanley Glacier	Kootenay NP	Mt Goodsir 82N/1	51°11'10" 116°04'40"	1850
16	Paget Peak	Yoho NP	Lk Louise 82N/8	51°26'50" 116°21'55"	2240
17	Mt Edith Cavell	Jasper NP	Amethyst Lks 83D/9	52°42'00" 118°03'30"	1750
18*	Blackwall Peak	Manning PP	Manning Park 92H/2	49°05'35" 120°45'35"	2000
19*	Mt Baldy	Grand Forks	Grand Forks 92I/4	49°10'20" 119°15'25"	2150

* Maternal megagametophyte tissue from seeds analyzed from these populations

3.2.2 GENETIC ANALYSIS

Samples were removed from the freezer, placed on ice and ground with two drops of grinding buffer developed by S. Barnes, modified slightly from Mitton (1977; see Appendix II for details). Three running buffer systems were used (Table 3.2). Buds were dissected to remove bud scales which may have contained secondary compounds that can interfere with isozyme analysis. If the current year's needles had grown past ~3mm, then they were removed and stored for future use and the remaining bud tissue was used. Samples were ground with a handheld Conair Zipwhip™ eggbeater on ice, then 1.5mm x 6mm chromatographic paper wicks were immediately inserted to absorb the supernatant.

Twelve per cent (w/v) starch gels with 5% (w/v) sucrose were utilized for electrophoresis. Gels were first run at half voltage for 30 minutes in a refrigerator at 4°C, after which the wicks were removed, a spacer inserted into the gel adjacent to the gel plate wall to enhance the

continuity of the matrix, and an icepack was added to the top of the gel. Then the voltage was increased to full power as the system ran for the duration in the refrigerator.

Table 3.2. List of loci scored for isozyme analysis.

Enzyme	Locus	Buffer system	Enzyme name	E.C. number
Aat (= *Got*)	1	2,3	Aspartate aminotransferase	2.6.1.1
Dia	1	1,3	Diaphorase	1.6.4.3
Est	3	1	Esterase	1.22.6.1
Gdh	1	2	Glutamate dehydrogenase	1.4.1.2
G6pdh	1	2	Glucose-6-phosphate dehydrogenase	1.1.1.49
Idh	1	3	Isocitrate dehydrogenase	1.1.1.42
Lap	1,2	2	Leucine aminotransferase	3.4.11.1
Mdh	1,2,3	1	Malate dehydrogenase	1.1.1.37
Pgm	1	1	Phosphoglucomutase	2.7.5.1
6Pgd	1	3	6-Phosphogluconate dehydrogenase	1.1.1.44
Pgi	2	3	Phosphoglucose isomerase	5.3.1.9
Sod	1	3	Superoxide dismutase	1.15.1.1
Prx	2,3	1,2	Peroxidase	1.11.1.7
Adh	1	1	Alcohol dehydrogenase	1.1.1.1
Sdkh	1,2	1	Shikimate dehydrogenase	1.1.1.25
Fdp	1	2	Fructose-1,6-diphosphatase	3.1.3.11

Table 3.3. Electrophoretic buffer systems. All buffer recipes are included in Appendix II.

System #	Buffer system	pH	Type	Run time	Reference
1	Morpholine	8.0	continuous	3.5 hrs	(Clayton and Tretiak 1972)
2	Lithium borate	8.3	discontinuous	6 hrs	(Ridgeway *et al.* 1970)
3	Tris citrate	7.0	continuous	3.5 hrs	(Stuart-Smith 1998)

Loci were scored as follows: the most common allele was scored as 1, and other alleles were designated sequentially as they appeared (see Appendix IV for clarification and zymograms). All gel slices were scored immediately, and then fixed with a 1:5:5 mixture of glacial acetic acid:water:methanol, wrapped in plastic and stored in dark, cold conditions for further verification.

3.3 ANALYSIS

Individual genotypic data were entered into a Microsoft Excel™ spreadsheet. Data were then formatted for use in BIOSYS-2 (Swofford *et al.* 1997) and Popgene V.3.2 (Yeh *et al.* 1999), where loci with significant amounts of missing data were excluded from the analysis to avoid skewing the results and to ensure that the programs would process the data optimally. These loci were *Prx-1, Est-1, Est-2, Mdh-4, Pgi-1, Dia, Adh, Pgm, Aat, G6pdh* and *Sod*.

For the two populations used in the mating systems study, maternal diploid genotypes were inferred using the megagametophyte haplotype data, assuming that under Mendelian segregation for two alleles at a locus, the likelihood of assuming the correct maternal genotype from the observed haplotypes was $1 – (1 – p)^n$, where n = the number of progeny scored, and p = the frequency of allele 1 (Hartl and Clark 1994). For 30 progeny, this would translate into a probability very close to unity. These genotypes were then used to conduct an analysis of the genetic diversity parameters for these populations with the same methods as mentioned above. All ten loci were used for these analyses.

In cases where populations have low genetic differentiation, such as whitebark pine, where nearly all of Nei's (1972) genetic identities are > 0.9 (Yandell 1992), Cavalli-Sforza and Edwards' (1967) chord distance can effectively highlight similarities and differences. Its converse, the arc distance, is a similar measure measured along a different multidimensional surface. Matrices of physical and genetic distances were compared using a Mantel test (Manly 1985) with the Mantel Nonparametric Test Calculator V.2.00© (Liedloff 1999). All pairwise combinations of geographic and genetic distances are simultaneously assessed using Monte Carlo simulations, and the relationship between the two is determined based on Type I error (α) limtations.

3.4 RESULTS

3.4.1 REMOVING LOCI

Although they were consistently present aross populations and had fairly high heterozygosities, the decision was made to remove esterase (*Est*) and peroxidase (*Prx*) loci for several reasons. First, they represent a series of complex products (Dukharev 1978) and are difficult to clearly score (Juo and Stotzky 1973; Lieu *et al.* 2001). Second, they can be produced by multiple metabolic pathways, and results from one locus to the next revealed with a single staining procedure may reflect differing physiological processes or environmental influences

(Copes 1978; Mayberry and Feret 1977). Third, *Prx* may be influenced at the constitutive level by the presence and severity of the *Cronartium ribicola* fungus in that not only may the tree's own metabolism respond by altering the level of peroxidases, but the fungus itself may also produce or secrete other peroxidase forms that would appear when tissues of the tree were analyzed (Adorada *et al.* 2000; Gay and Tuzun 2000a,b; Maa and Liao 2000; Tyagi *et al.* 2000). It is also possible that the fungal hyphae may secrete other enzymes that would be detectable by electrophoresis and may interfere with or confound the interpretation of the resulting bands, although this would be more likely when analyzing needle tissue, and depend on the degree of infection.

3.4.2 GENETIC DIVERSITY STATISTICS

The standard genetic diversity statistics and their estimates, such as expected and observed heterozygosity, number of alleles per locus, etc., can provide valuable information in characterizing the genetic architecture of populations. While various studies may employ differing sampling strategies or laboratory techniques, the standardized and accepted methods of analysis and interpretation for isozyme data facilitate the augmentation of existing data sets with new information, as well as enabling scientists to make comparisons between species and taxonomic groups.

There was one private allele found within the two populations analyzed for mating systems: *6Pg2-3* in Manning (see Appendix I for pollen and ovule allele frequencies). This may not actually reflect a true private allele, however; it may only indicate sampling error or be the result of small sample size of other populations since these data were generated from the inferred maternal genotypes of 55 trees. Distributions of alleles within loci tended to have one common allele with frequencies > 0.85. Berg and Hamrick (1997) advocate the use of no percentage criterion when calculating genetic diversity statistics such as alleles per locus (A) and percentage of polymorphic loci (P), in order to avoid artificially low heterozygosity estimates.

For populations where there may be many loci with very low frequencies of alleles other than the most common one, using restrictive criteria could also significantly alter the character of the population in terms of genetic description.

Comparison of the observed and expected heterozygosities of the mature parent trees for Manning reflects a homozygote deficiency, or heterozygote excess of 11%, in contrast to the selfing implied and resulting excess of homozygotes in seeds found in the analysis in chapter 2 (Table 3.4). Conversely, the opposite situation applied for Baldy: homozygote excess of 11% compared to expected values, concurring with the results in chapter 2 that most of the inbreeding in this population was due to biparental inbreeding. The heterozygote imbalances were not statistically different from zero (paired t-test, $\alpha = 0.05$). These two populations therefore do not statistically deviate from HWE.

Genetic variability statistics in the other 17 populations are also presented in Table 3.4. Yalakom, located in the Coast Mountains near the middle of the latitudinal distribution within B.C., had the lowest number of alleles per locus (A), averaging 1.6, and Edith, in the Rockies near the northern portion of the range, had A = 1.7. The highest allelic diversity was 2.6, found in Manning, and 2.5 in Baldy from the maternal seed genotypes; both populations are in the southernmost portion of the Coast Mountains, the former contiguous with the eastern portion of the range, and the latter an outlying population further to the east. Yalakom had the lowest proportion of polymorphic loci (P) at 50%, Edith was the next lowest at 58.3%. Paget in the northern Rockies and Perkins in the north Coast Mountains both had the highest value of P at 91.7%; Manning had 90%.

Observed heterozygosity averaged 0.213; Hudson, in the northern Coast Mountains had the lowest value (0.119) and the northern Washington population the highest (0.289). Other populations in the northwestern portion of the range also had low H_e, while those in the south were markedly higher. Expected heterozygosity averaged 0.257 across all populations, with a

minimum at Manning in the southern Coast Mountains (0.184); Perkins had the highest value (0.312). Populations in the Rocky Mountains did not exhibit clear trends with respect to heterozygosity. Over all 19 populations, H_e was greater than H_o in all but the populations at Tchaikazan, Washington and Manning, and the difference was statistically non-significant in Tchaikazan and Manning. In most cases, H_e was greater than H_o by at least 50%, and sometimes by a multiple of one or more. The strongest heterozygote deficiency occurred at the northwest extent of the species range, with the exception of the Sweeney population.

Table 3.4. Genetic diversity statistics for all populations. Standard errors of the mean in parentheses. N = mean sample number; A = mean alleles per locus, no criterion; P = percentage of polymorphic loci, no criterion; H_e = expected heterozygosity; H_o = observed heterozygosity. Manning and Baldy statistics based on ten loci.

Population	Pop #	N	A	P	H_o	H_e
Hudson	1	18.1 (0.6)	1.8 (0.2)	75.0	0.119 (0.051)	0.237 (0.057)
Higgins	2	17.8 (2.4)	1.9 (0.1)	83.3	0.169 (0.070)	0.298 (0.065)
Sweeney	3	19.9 (1.5)	1.8 (0.2)	66.7	0.192 (0.070)	0.210 (0.058)
Heckman	4	25.8 (2.0)	1.8 (0.2)	75.0	0.127 (0.048)	0.265 (0.060)
Perkins	5	23.8 (2.2)	2.0 (0.1)	91.7	0.205 (0.081)	0.312 (0.062)
Tchaikazan	6	23.6 (2.2)	1.8 (0.2)	75.0	0.264 (0.100)	0.262 (0.066)
Yalakom	7	23.3 (1.6)	1.6 (0.2)	50.0	0.147 (0.072)	0.194 (0.066)
D'arcy	8	23.8 (0.8)	1.8 (0.1)	75.0	0.277 (0.103)	0.309 (0.066)
Van Horlick	9	20.4 (1.7)	1.8 (0.2)	75.0	0.167 (0.054)	0.229 (0.052)
Whistler	10	29.1 (0.4)	1.9 (0.2)	75.0	0.229 (0.083)	0.301 (0.066)
Lime	11	30.0 (0.0)	2.0 (0.2)	83.3	0.253 (0.083)	0.300 (0.054)
USA	12	17.0 (0.0)	1.8 (0.2)	75.0	0.289 (0.112)	0.260 (0.067)
Kootenay	13	20.3 (1.3)	1.9 (0.1)	83.3	0.224 (0.081)	0.284 (0.064)
Jumbo	14	31.1 (1.5)	2.0 (0.2)	83.3	0.214 (0.078)	0.243 (0.059)
Stanley	15	29.3 (0.4)	2.1 (0.2)	83.3	0.284 (0.080)	0.291 (0.064)
Paget	16	27.9 (0.9)	2.2 (0.2)	91.7	0.274 (0.088)	0.262 (0.056)
Edith	17	16.3 (1.5)	1.7 (0.2)	58.3	0.180 (0.084)	0.204 (0.062)
Manning	18	750 (0.0)	2.6 (0.2)	90.0	0.184 (0.068)	0.204 (0.050)
Baldy	19	853 (0.0)	2.5 (0.2)	80.0	0.243 (0.065)	0.218 (0.057)
Mean		23.2* (1.3)	1.9 (0.058)	69.5 (2.4)	0.213 (0.012)	0.257 (0.009)

*Not including populations 18 and 19.

3.4.3 WRIGHT'S *F*-STATISTICS

Since the two populations included in the mating system study were analyzed using different tissues and a different (although partially overlapping) set of loci, as well as a haploid data set, the salient results will not be included with those from the other populations. The statistical power of the analysis for these populations is such that only a brief summary of the findings will be presented here. Further detail can be found in Appendix V.

For both Manning and Baldy, all of the mean fixation indices were not significantly different from zero (t-test, $\alpha = 0.05$). The proximity of the two populations to each other may explain the relatively similar results. It is in fact possible that they are representative of two sub-samples of one metapopulation, although the small sample sizes (25 trees for Manning, 30 for Baldy) would prohibit drawing any definite conclusions in this regard. F_{ST} between the two populations was

-0.024, but was not statistically different from zero. This slightly negative value implies low subpopulation differentiation, which is also supported by the allele frequencies in Table 3.4. Values for F_{IS} and F_{IT}, respectively, were -0.025 and 0.008, reflecting negligible effects of inbreeding with respect to heterozygosity of mature individuals in these two populations.

For the results presented in Table 3.5, all populations in the Selkirk, Purcell, Cariboo and Rocky Mountains are designated to be within the Rockies for analytical and interpretive purposes. With respect to the fixation index F_{IS}, the mean value for all populations of 0.345 shows a pronounced effect of inbreeding among individuals within populations. The high standard deviation reflects the high variability among loci. *Fdp* was not fixed, but had uncommon alleles at very low frequency, resulting in a high F_{IS} and F_{IT} (> 0.999 for both statistics). Nine loci had positive values, many with values > 0.5, suggesting a significant decrease in heterozygosity among inbred individuals within each subpopulation at these loci. Three loci had F_{IS} less than zero, although the value for *Skd2* was -0.017, very close to zero.

Table 3.5. Wright's *F*-statistics for 17 populations; standard errors of the mean in parentheses.

Allele	F_{IS}	F_{IT}	F_{ST}
Mdh1	0.538	0.540	0.004
Mdh2	0.085	0.091	0.006
Mdh3	0.349	0.369	0.031
Pgm	-0.395	-0.237	0.113
Skd1	0.697	0.704	0.023
Skd2	-0.017	-0.004	0.013
Fdp	1.000	1.000	0.071
Gdh	0.194	0.279	0.105
Lap1	0.936	0.940	0.077
Lap2	0.739	0.757	0.067
Idh	0.396	0.470	0.123
Pgi2	-0.379	-0.250	0.093
Mean	0.345 (0.129)	0.388 (0.118)	0.061 (0.012)

F_{IT} values were similar in magnitude and sign to F_{IS}, with an equally wide range of values. Overall, for all populations combined, inbred individuals express a 38.8% decrease in heterozygosity relative to expectation under panmixis.

Perhaps the most informative of these three measures, F_{ST} had an overall value of 0.061, which is within Wright's (1931) subjective category boundaries of 0.050 to 0.150 for populations with moderate levels of population differentiation. Values for loci ranged from 0.004 for *Mdh1* to 0.123 for *Idh*; all loci were therefore within the low to moderate range of population differentiation and there were no outstanding anomalies which affected the overall mean. The low standard deviation compared to the other *F*-statistics confirms this.

3.4.4 MEAN STATISTICS BY GEOGRAPHIC REGION

While the north/south divisions of population were somewhat arbitrary, due to the lack of definitive information on glacial refugia, general trends are still apparent (Table 3.6). Southern and northern populations had equal mean numbers of alleles per locus, and Coastal populations had a significantly lower number than other subdivisions. The Rockies had the highest proportion of polymorphic loci, and the Coast the lowest, although the value was very similar to the southern populations and the standard errors overlap between all subdivisions.

Expected heterozygosity was highest in the south and the Coast Mountains, and lowest in the Rockies. Observed heterozygosity was lowest in the north (0.202) and Coast Mountains (0.203), and substantially higher in the Rocky Mountain populations (0.235): a difference of 14% between east and west. Genetic variability was lowest in the Coast Mountains and highest in the Rockies, but the differences among groupings was not substantial; expected heterozygosity had slightly higher variability than observed. Wright's inbreeding coefficient F was very high (0.231) in the Coast Mountains which had the lowest H_o, and low (0.085) in the Rockies, which had the highest H_o. There was a heterozygote deficiency of 0.223 (22%) in the north and 0.151(15%) in the south.

Table 3.6. Genetic diversity statistics for subdivided population groupings; standard errors of the mean in parentheses; all abbreviations as in Table 3.4.

Group	Pop #	# of Pops	N	A	P	H_e	H_o
Coast Mtns	1-12	12	21.9 (1.5)	1.8 (0.03)	75.0 (2.9)	0.265 (0.012)	0.203 (0.017)
Rockies	13-17	5	25.0 (2.8)	2.0 (0.09)	80.0 (5.7)	0.257 (0.016)	0.235 (0.019)

North	1-6, 15-17	9	22.5 (1.6)	1.9 (0.06)	77.8 (3.7)	0.260 (0.013)	0.202 (0.020)
South	7-14	8	23.1 (2.3)	1.9 (0.05)	75.0 (3.9)	0.265 (0.014)	0.225 (0.018)

3.4.5 PHYSICAL VERSUS GENETIC DISTANCES

Physical and genetic distances often are not directly related. This may certainly be the case for whitebark pine in B.C.'s complex topography, which may show genetic patterns of relatedness along major drainages or mountain ranges, while physically adjacent mountain ranges may be closer. Bird seed dispersal may generate a stepwise dispersal pattern where adjacent populations are genetically closer to each other than to more distant populations, but other factors may obscure these patterns, if they do exist. While populations 1 (Hudson) and 2 (Higgins) appear very close to each other, they are located on opposite sides of a major drainage. Some populations, such as the northern Washington population, are physically closer to populations located in different mountain ranges than to those within the same range (Table 3.1).

Table 3.7. Physical most parsimonious distances between populations (km). Population numbers as in Table 3.1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2	25															
3	140	130														
4	265	270	145													
5	360	360	235	95												
6	450	445	340	135	115											
7	520	515	410	280	200	80										
8	560	555	445	305	220	110	55									
9	595	590	480	345	255	145	80	40								
10	595	590	470	330	240	145	110	55	60							
11	540	530	440	310	240	125	50	90	95	145						
12	815	815	710	565	550	370	295	260	220	240	290					
13	920	930	920	710	635	520	435	425	390	430	415	240				
14	845	825	760	645	580	475	395	405	375	430	350	315	160			
15	845	825	780	345	615	505	435	455	430	410	385	385	235	85		
16	795	775	730	630	575	475	405	430	410	465	355	400	265	105	50	
17	640	620	590	510	475	390	340	380	370	435	295	455	395	250	215	165

Nei's (1972) genetic distance was not highly informative, but is included here for comparisons with other studies (Table 3.8). Populations are too genetically similar to accurately

gauge relative genetic distances using this measure, exhibited by the fact that many pairs of populations have genetic distances of 0.000 and very few are > 0.1. This may reflect the close relationships among all populations following postglacial recolonization from few refugia, but more fine-scale relationships are difficult to detect using this method. Nei's (1978) unbiased distance, adjusted for population sample size, is slightly improved for this purpose. Using this measure, the populations most closely related to each other were 7 (Yalakom) and 9 (Van Horlick), which are separated by 80 kilometres; 12 (Washington) and 17 (Edith), separated by 455 km; and 16 (Paget) and 17, 165 km apart. Heckman (4) showed the greatest genetic separation from any other populations: genetic distances between 4 and 7, 8 (D'arcy) and 9 were highest, 0.132, 0.118 and 0.134, respectively, but all of these populations were within the Coast Mountains and not physically very distant.

Table 3.8. Nei's 1972 genetic distance for 17 populations (above diagonal) and unbiased (1978) genetic distance (below diagonal). Population numbers as in Table 3.1.

Pop	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*****	0.004	0.018	0.071	0.038	0.025	0.051	0.050	0.059	0.057	0.033	0.049	0.046	0.028	0.057	0.028	0.043
2	0.026	*****	0.023	0.072	0.032	0.037	0.066	0.052	0.065	0.069	0.043	0.051	0.055	0.039	0.053	0.030	0.040
3	0.026	0.044	*****	0.050	0.022	0.016	0.059	0.048	0.068	0.036	0.012	0.026	0.033	0.013	0.019	0.022	0.023
4	0.080	0.093	0.058	*****	0.043	0.067	0.122	0.109	0.126	0.092	0.077	0.081	0.080	0.093	0.070	0.082	0.096
5	0.048	0.056	0.032	0.053	*****	0.000	0.039	0.020	0.034	0.011	0.016	0.008	0.020	0.018	0.009	0.005	0.015
6	0.036	0.061	0.027	0.078	0.012	*****	0.012	0.031	0.012	0.002	0.014	0.005	0.013	0.000	0.014	0.000	0.010
7	0.061	0.090	0.068	0.132	0.050	0.025	*****	0.050	0.000	0.024	0.048	0.018	0.010	0.019	0.055	0.010	0.016
8	0.059	0.075	0.057	0.118	0.030	0.042	0.060	*****	0.047	0.023	0.027	0.019	0.029	0.026	0.032	0.022	0.023
9	0.068	0.088	0.077	0.134	0.045	0.024	0.006	0.056	*****	0.022	0.057	0.025	0.020	0.021	0.055	0.012	0.025
10	0.065	0.091	0.044	0.100	0.020	0.013	0.033	0.031	0.030	*****	0.015	0.007	0.015	0.009	0.020	0.011	0.020
11	0.041	0.064	0.019	0.084	0.025	0.024	0.057	0.036	0.066	0.023	*****	0.008	0.014	0.014	0.016	0.014	.0.12
12	0.059	0.074	0.035	0.090	0.018	0.017	0.029	0.029	0.035	0.016	0.016	*****	0.003	0.011	0.016	0.002	0.000
13	0.055	0.078	0.042	0.089	0.031	0.025	0.021	0.039	0.030	0.024	0.023	0.013	*****	0.014	0.017	0.008	0.003
14	0.035	0.059	0.019	0.100	0.027	0.010	0.027	0.033	0.028	0.016	0.020	0.019	0.022	*****	0.015	0.006	0.012
15	0.065	0.074	0.026	0.078	0.018	0.024	0.064	0.040	0.063	0.027	0.023	0.025	0.026	0.022	*****	0.018	0.021
16	0.035	0.051	0.029	0.089	0.014	0.006	0.019	0.030	0.020	0.018	0.021	0.010	0.016	0.012	0.024	*****	0.000
17	0.053	0.064	0.033	0.106	0.027	0.022	0.027	0.033	0.035	0.029	0.021	0.007	0.017	0.021	0.029	0.008	*****

The minimum Cavalli-Sforza and Edwards' (1967) chord distances were found between 6 (Tchaikazan) and 12 (Washington), which are located in the Coast Mountains 370 km apart; and 6 and 16 (Perkins), located in different mountain ranges (Table 3.9). The maximum chord

distance was between 4 (Heckman) and 8 (D'arcy), which concurs more or less with the results from Nei's distance statistics.

Table 3.9. Below diagonal: Cavalli-Sforza & Edwards (1967) arc distance; above diagonal: Cavalli-Sforza & Edwards (1967) chord distance.

Pop	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*****	0.132	0.182	0.227	0.184	0.171	0.178	0.236	0.175	0.211	0.197	0.206	0.187	0.177	0.220	0.162	0.223
2	0.133	*****	0.194	0.233	0.151	0.189	0.230	0.230	0.185	0.219	0.211	0.208	0.216	0.200	0.216	0.165	0.224
3	0.184	0.198	*****	0.176	0.159	0.124	0.202	0.222	0.195	0.159	0.151	0.143	0.169	0.140	0.172	0.140	0.159
4	0.231	0.237	0.179	*****	0.194	0.193	0.273	0.297	0.241	0.234	0.243	0.228	0.230	0.242	0.245	0.213	0.275
5	0.187	0.152	0.160	0.196	*****	0.111	0.195	0.158	0.144	0.113	0.148	0.127	0.154	0.145	0.152	0.102	0.172
6	0.172	0.192	0.125	0.196	0.111	*****	0.143	0.185	0.115	0.105	0.146	0.086	0.133	0.097	0.146	0.090	0.146
7	0.180	0.234	0.206	0.278	0.198	0.144	*****	0.215	0.106	0.177	0.208	0.153	0.153	0.145	0.213	0.146	0.158
8	0.240	0.233	0.225	0.311	0.159	0.187	0.218	*****	0.196	0.147	0.181	0.159	0.194	0.152	0.187	0.154	0.153
9	0.178	0.188	0.200	0.245	0.145	0.115	0.107	0.198	*****	0.142	0.195	0.143	0.148	0.138	0.194	0.115	0.169
10	0.213	0.222	0.160	0.239	0.114	0.106	0.179	0.148	0.143	*****	0.130	0.109	0.129	0.126	0.149	0.102	0.156
11	0.199	0.214	0.152	0.251	0.149	0.146	0.210	0.182	0.197	0.131	*****	0.131	0.111	0.124	0.153	0.133	0.158
12	0.208	0.211	0.144	0.234	0.127	0.087	0.154	0.161	0.144	0.109	0.132	*****	0.122	0.109	0.143	0.095	0.102
13	0.188	0.219	0.170	0.233	0.155	0.134	0.154	0.196	0.149	0.129	0.111	0.123	*****	0.116	0.132	0.114	0.157
14	0.178	0.203	0.141	0.247	0.146	0.097	0.146	0.153	0.138	0.126	0.124	0.110	0.116	*****	0.122	0.106	0.125
15	0.224	0.218	0.173	0.249	0.153	0.147	0.217	0.189	0.196	0.150	0.154	0.144	0.132	0.122	*****	0.125	0.171
16	0.164	0.167	0.141	0.217	0.102	0.090	0.147	0.154	0.116	0.102	0.133	0.095	0.115	0.106	0.126	*****	0.117
17	0.226	0.228	0.160	0.288	0.174	0.148	0.160	0.155	0.171	0.157	0.159	0.102	0.159	0.126	0.173	0.117	*****

Physical and chord distances were compared with a nonparametric Mantel test (Manly 1985; Jorgensen and Hamrick 1997) using the program Mantel V.2.00©. (Liedloff 1999). Results from 10,000 random permutations were significant at the $p = 0.05$ level adjusted for the number of pairwise tests, indicating that physical and genetic distances are correlated in this species in B.C. Using a standardized Mantel Z-score, the critical value was 1.645 for $\alpha = 0.05$ based on 136 pairwise comparisons; the test statistic, g (also termed the standard normal coefficient) was 1.872 resulting in a *p*-value of 0.0355. The Pearson correlation coefficient, r, for the two matrices was 0.24, indicating that 24% of the data in each matrix is explained by the other; conversely, 76% of the relationship between physical and chord distance could not be explained by either factor.

A dendrogram based on five iterations of the Wagner distance procedure using the Cavalli-Sforza and Edwards (1967) chord distance revealed in each iteration that there were only slight

correlations with spatial and genetic distances (final iteration shown in Figure 3.2). While some populations consistently grouped together, such as Hudson and Higgins, and Sweeney and Heckman, groupings of populations, especially among mountain ranges, were weak. Edith (in the east) and Lime (in the west), for example, are both found in clades which contain populations from the opposite, western and eastern portions of the species range, respectively. In the various iterations, the positions and branch lengths of most populations among clades was variable.

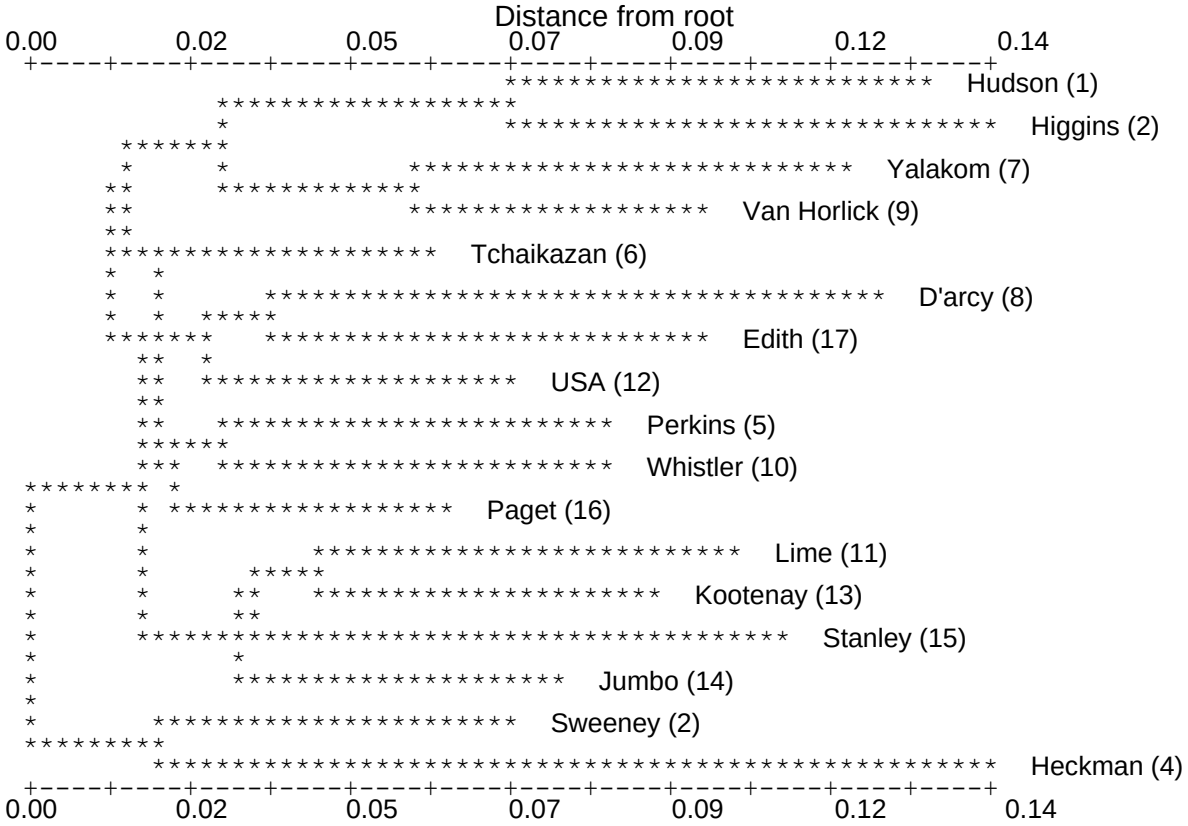

Figure 3.2. Wagner tree produced by rooting at midpoint of longest path (after optimization) by BIOSYS-2.

3.4.6 GEOGRAPHIC PATTERNS IN GENETIC DIVERSITY

There were surprisingly strong correlations between geographical variables and heterozygosity (Figures 3.3 to 3.8). Correlations with observed heterozygosity were statistically significant at a significance level of 0.05, and showed the strongest trends (for latitude: R^2 = 0.357, p = 0.011; for longitude: R^2 = 0.295, p = 0.024). Regressions on expected

heterozygosity were weak and not statistically significant (for latitude; $R^2 = 0.039$, $p = 0.450$; for longitude; $R^2 = 0.000$, $p = 0.992$). Genetic diversity generally increased towards the south and east.

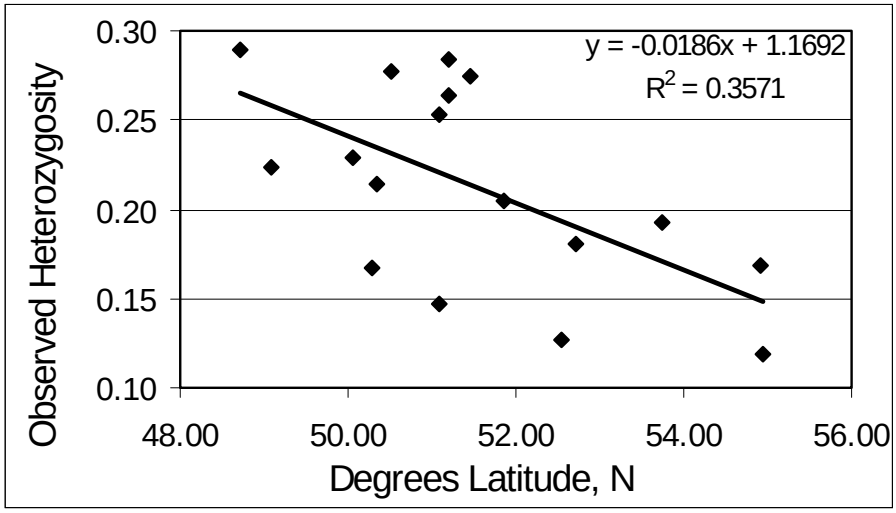

Figure 3.3. Regression of observed heterozygosity on latitude.

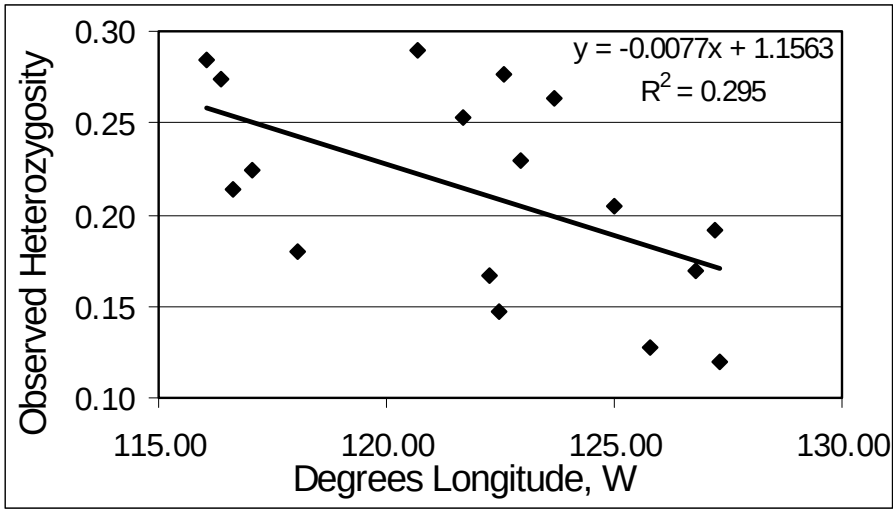

Figure 3.4. Regression of observed heterozygosity on longitude.

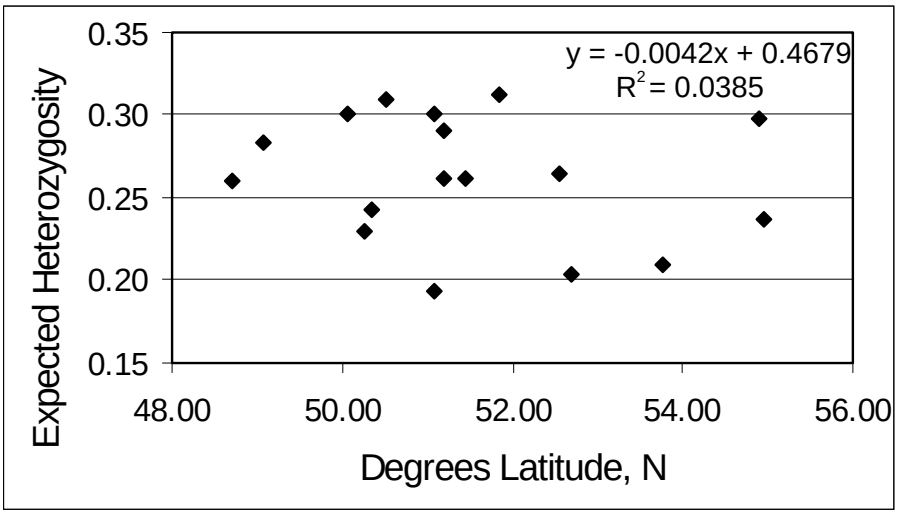

Figure 3.5. Regression of expected heterozygosity on latitude.

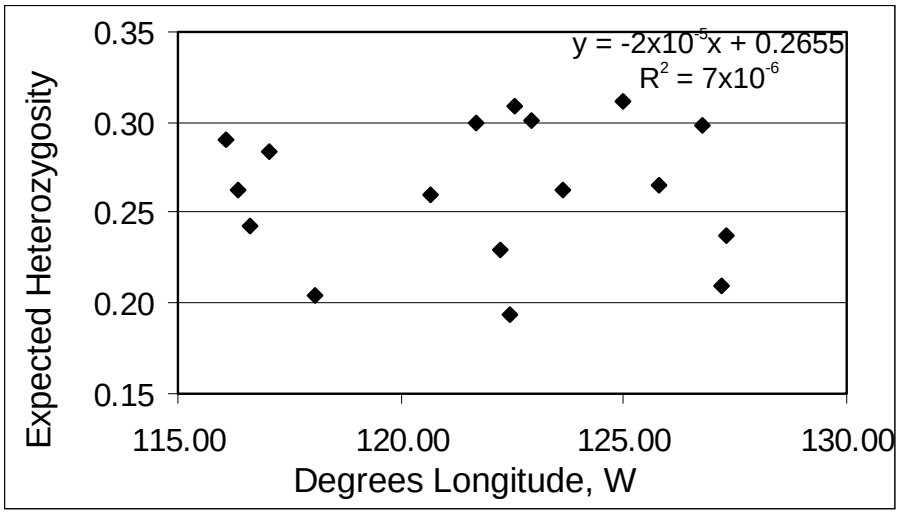

Figure 3.6. Regression of expected heterozygosity on longitude.

Correlations between F, Wright's index of heterozygote deficiency measured by $1-(H_o/H_e)$, also showed strong and significant patterns in relation to geographic variables (Figures 3.7 and 3.8). The regression coefficient (R^2) for F with latitude was 0.296 (p = 0.024) and with longitude 0.368 (p = 0.010). Heterozygote deficiency increased with increases in both longitude and latitude, indicating the strongest heterozygote deficiencies in the north and west (i.e., the Coast Mountains), and the tendency towards small heterozygote excesses in the south and eastern regions of B.C.

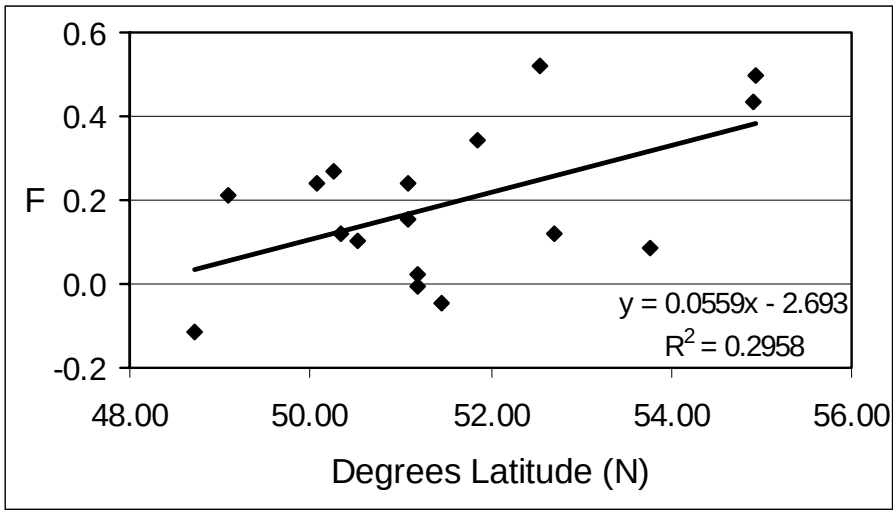

Figure 3.7. Regression of Wright's F on latitude.

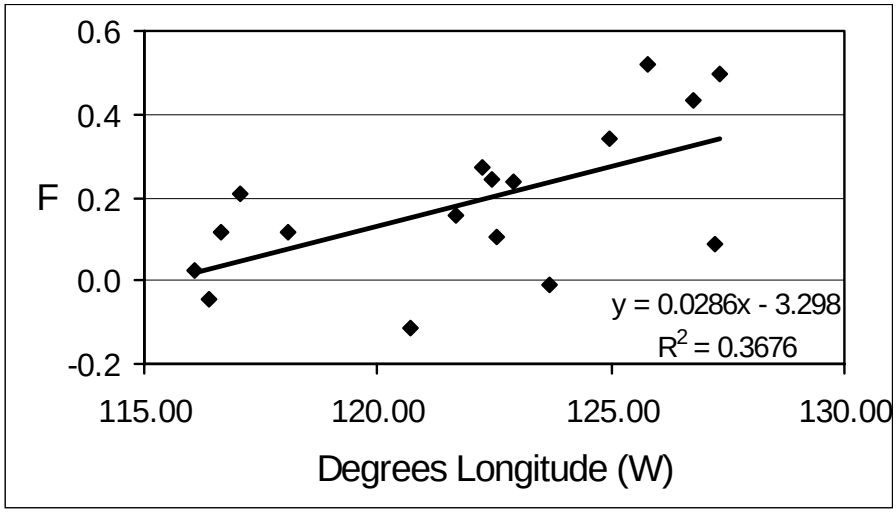

Figure 3.8. Regression of Wright's F on longitude.

3.5 DISCUSSION

3.5.1 PATTERNS OF GENETIC DIVERSITY

As Stuart-Smith (1998) pointed out, bird-mediated seed dispersal would likely obscure genetic patterns stemming from founder effects. This is due to the fact that birds have been documented to randomly cache seeds with respect to collection and deposition locations; i.e., a bird would be no more likely to cache seeds from the same stand near each other than near caches from other stands. This would lead to the integrated mosaic of genotypes that has been found by other researchers (e.g. Bruederle *et al.* 1998; Furnier *et al.* 1986; Miller and Westfall

1992; Rogers *et al.* 1999) in which individuals within clumps are likely to be related to each other, but genotypes between clumps show no isolation by distance relationships and tend to be randomly distributed.

The data from this study correlate well with the results of both Stuart-Smith (1998) and Yandell (1992), although again the standard deviations reflect the relatively small sample size and number of loci. Bruederle *et al.* (1998) and Jorgensen and Hamrick (1997) had lower estimates of H_e, in the order of 0.15 and 0.10, respectively for their studies, although the standard deviations overlap with ranges in this study. The geographic ranges of studies and laboratory conditions, including loci analyzed, may account for some of the differences (Conkle 1971).

Table 3.10. Summary of whitebark pine genetic data by study. Superscripts indicate criterion percentage.

Study	# of pops	Area	# of loci	Analysis level	A	%P	H_o	H_e or H_t	F_{IS}	F_{IT}	F_{ST} or G_{ST}	F
1	9	Yellowstone NP	19	Population	1.66^{no} 1.6^{95}	38.6	0.148	0.152	0.016	0.041	0.025	0.026
2	30	Entire range but BC	20	Population		25^{95}	0.088	0.092				0.043
				Species		85^{no}		*0.102*	0.267		*0.034*	
3	14	Great Basin	13	Population	1.6^{99}	48.8	0.191	0.204	0.060	0.143	0.088	0.064
4	29	BC/AB Rockies	16	Population	1.64	50.2	0.218	0.211				-0.033
				Species	2.06	56.3	0.218	*0.224*	-0.035	0.030	0.062	0.027
5	3	Mono Lk, CA	21	Hierarchical		5.6-19.1	0.033-0.089	0.026-0.087	~ -0.3	~0.3	~0.055	0.004-0.334
6	17	BC	12	Population	1.9	69.5^{no}	0.212	0.257	0.154	0.207	0.061	0.168

1 - Bruederle *et al.* 1998; 2 - Jorgensen and Hamrick 1997; 3 - Yandell 1992; 4 - Stuart-Smith 1998; 5 - Rogers *et al*. 1999; 6 - this study

The results of the province-wide isozyme analysis reflect heterozygosity statistics similar to those calculated by Stuart-Smith (1998) who performed an analysis based on populations along the B.C.-Alberta border (overlapping with the current study), as well as Yandell's (1992) study of Great Basin populations, none of which were assayed here. Results from this and the two aforementioned studies, however, have heterozygosities up to twice as high as those found in some other studies (Bruederle *et al.* 1998; Jorgensen and Hamrick 1997). Reasons for these discrepancies likely include the tissue sampled and the sampling season, whereby different loci

and levels of enzyme activity are expressed at different phenological stages, thus making expression somewhat influenced by sampling date. *Adh*, an enzyme involved in leaf abscission and dormancy, is one example of such a locus; *Prx*, involved in eliminating toxins and waste is another. Other studies have shown that there can be differences in the loci expressed among tissue types; while most other studies have used needle tissue, this study has used bud tissue that includes primordial needles, meristem tissue and possibly the following year's primordial reproductive tissues. Thus, isozyme studies examining different tissues or loci for the same organism could have differing results.

Studies involving northerly populations generally have higher overall heterozygosity, while those focusing on unglaciated and southerly areas have lower heterozygosity, in contrast to results shown in Figures 3.3 and 3.5. This may reflect differences in laboratory procedures, buffer systems, statistical methods and resolution of bands following staining, but it may also reflect actual differences. One way to resolve these discrepancies is to compare values for different studies where the same populations were sampled. Jorgensen and Hamrick (1997) sampled one population which was also sampled in this study, Mt. Edith Cavell. These results were quite different to Jorgensen and Hamrick (1997), yet similar to what Stuart-Smith (1998) found: both observed and expected heterozygosities were substantially higher in this study (0.204 and 0.180, respectively) than the former (0.080 and 0.088, respectively). The same differences were found between populations analyzed by Yandell (1992) and Jorgensen and Hamrick (1997): heterozygosity values were two to three times higher in Yandell's study. Stuart-Smith attributed the differences between Jorgensen and Hamricks' results and the other studies primarily to differences in laboratory technique and resolution of common alleles in electrophoresis; this possibility was supported by Hamrick (J. Hamrick, U. of Athens, GA, Depts. of Botany and Evolution, pers. comm. 2001). The use of the 95% criterion in the definition of statistical parameters was also implicated, and he demonstrated that the low 25% level of

polymorphism could be increased to 85% by employing a 99% criterion. Since the data in this study concur fairly closely with those of Stuart-Smith (1998), I am inclined to agree with his hypotheses, especially since similar buffer systems were used for this and his study.

Differences found in statistics and distribution for the same alleles among studies could be caused by selection acting on non-neutral loci linked to those analyzed in this study or drift caused by founder effects affecting allele frequencies. Latta and Mitton (1997) found very different dispersal patterns and selection regimes for different markers measured for limber pine, and several researchers emphasize the selective role that Clark's nutcracker plays for bird-dispersed species (Carsey and Tomback 1994; Vander Wall and Balda 1977; Schoettle and Rochelle 2000). The relatively recent glaciation and long generation time would also indicate that whitebark pine allele frequencies would not yet approach expected equilibrium values in many cases. Since each study analyzed different loci to calculate population estimates of genetic parameters, the inclusion in one study of several loci which depart from the typical pattern of allele distribution would skew the results from the same population.

An interesting relationship between expected and observed heterozygosity is revealed in this study. It is apparent from the geographic relationship between regional blister rust mortality (highest in the southern Rockies (Campbell 1998; Stuart-Smith 1998; pers. observ.) and Wright's F (Figures 3.7 and 3.8) that the areas where mortality from the disease is highest show a striking trend towards heterozygote excess, while those where the pathogen is either absent or has a less severe impact on the population tend towards heterozygote deficiency. This could possibly be interpreted as evidence supporting selection against homozygous genotypes, or conversely, that populations with a higher proportion of heterozygotes are able to withstand or tolerate the effects of white pine blister rust via the wider available range of biochemical mechanisms conferred by increased genetic diversity. A causal basis for this apparent correlation between heterozygosity and degree of blister rust infection would need to be verified

with a study specifically investigating the relationship between heterozygosity and resistance on an individual-tree basis.

Peripheral populations did not appear to show any striking genetic patterns, although there were higher heterozygosities in the south and east (Table 3.6). Lesica and Allendorf (1995) suggest that there may be some selective advantage in those populations in terms of future adaptations, since selection pressures differ in peripheral or extreme environments, but lower heterozygosity and a loss of rare or private alleles would be two consequences if they underwent genetic bottlenecks or were recently diverged (Nei *et al.* 1975). Certainly, both options are possible, although the high heterozygosity of nearly all populations would counter arguments for a genetic bottleneck. The general lack of private alleles may reflect repeated founder effects resulting from bird seed caching instead, and the high heterozygosity could result from multiple founding events in the same area, which has been documented (Tomback and Schuster 1994; Richardson 2001).

While their ranges do not generally overlap in Canada in terms of elevation, whitebark and limber pine share many similarities, especially morphologically. The two are generally indistinguishable in the absence of cones, which is the key differentiating characteristic in their taxonomies (Little and Critchfield 1969; Critchfield 1986). Both species also have corvids in common as their primary agents of seed dispersal (Tomback and Linhart 1990). While the cones of limber pine do open upon maturity, leaving a wider spectrum of opportunities for seed dispersal, birds remain a key factor influencing limber pine gene flow and population structure. Exact results from studies conducted on limber pine cannot be directly applied to whitebark pine, however general deductions regarding topics such as the role of birds influencing genetic neighbourhoods or gene flow using different measures and markers may be extrapolated when the circumstances are similar for both species.

Schoettle and Rochelle (2000) found strong selection effects on morphology and phenology of limber pine across a wide ecological amplitude. Schuster *et al.* (1989) found strong elevational differences for the limber pine, and that there was fairly high gene flow via seed. Latta and Mitton (1997) discovered strong differences in gene flow and selection pressures among male and female gametes using different molecular markers. The elevational clines apparent in limber pine may not apply directly to whitebark pine due to whitebark pine's more restricted nature in terms of elevation, but it is likely that the two species share some selection pressures and similarities in effective pollen and seed gene flow patterns that explain some of the large and small scale population differentiation.

The patterns of genetic diversity found in this study confirm reconstructed biogeographic patterns of postglacial recolonization (Richardson 2001), reflecting fairly recent founder effects. The higher levels of heterozygosity in the south and east imply that bird-mediated seed dispersal generally progressed northwesterly, and that there were either more refugia in the east, or that there were some refugia in the Rockies at more northerly latitudes, concurring with a hypothesized refugium east of Roger's Pass, just outside Glacier NP (B. Richardson, USDA For. Serv., pers. comm.).

When subjected to the Mantel test (Jorgensen and Hamrick 1997; Manly 1985) to gauge isolation by distance, a statistically significant correlation between physical and genetic distance was revealed. This does provide some support for the repopulation of habitat by founder effects from glacial refugia, reflecting a stepping-stone model. Very little, if any, cross-Cordilleran migration would have been likely: the closest distance between known whitebark pine provenances in the Coast and Rocky Mountain ranges (approximately 60 km) is still greater than the furthest recorded nutcracker caching distance (22 km, *cf.* Vander Wall and Balda 1977.

The complex life history factors affecting whitebark pine interact across many scales and exert different influences both at different life stages and ecological stages (Bruederle *et al.*

2001). These factors, which are all affected by selection, include dispersal of pollen and seeds, ecological micro and macro-scale effects, successional stage and life history characteristics of the species. Many trends would only be obvious using a highly specific and intensive sampling scheme across several spatial scales. Selection is generally stronger, although heritability is low for fitness and adaptive traits, which generally involve many genes (Merilä and Sheldon 1999). Acknowledging the subtle and complex interplay among adaptive traits, Vida (1994) suggested that there may be more adaptive traits than neutral, calling into question the broad applicability of allozyme markers. Further research on adaptive traits is clearly necessary in this species in order to understand the physiological aspects of intra- and interpopulation variation to develop effective conservation measures.

3.5.2 WRIGHT'S *F*-STATISTICS

The various *F*-statistics developed by Wright (1931,1951,1965) can be used to infer the prior history of inbreeding within populations as well as to gauge hierarchical levels of population differentiation. These statistics use the estimated reduction in heterozygosity among inbred individuals attributed to inbreeding as a gauge of the degree of relatedness among individuals within and among populations (Hartl and Clark 1997). Assumptions critical to these calculations include (1) that all loci follow Mendelian inheritance and (2) that populations are in Hardy-Weinberg equilibrium.

While other studies have demonstrated the general applicability of the former condition (Furnier *et al.* 1986), the latter is almost certainly not true: nearly all of the areas currently populated by whitebark pine in B.C. have been glaciated as recently as 8,000 years ago, and some even more recently. If the average generation time is approximately 80 years and some areas have been recolonized from a few glacial refugia near Roger's Pass as well as several others in the northern U.S., then approximately 100 generations have passed since the most recent glacial event. Most populations would likely have originated exhibiting severe founder

effects due to bird caching, and in such environmentally extreme habitats are subject to constant environmental selection pressure. In mountainous topography, extremely fine micro-scale differentiation could conceivably result due to environmental variability in terms of snowpack, temperature gradients, moisture availability and intra- and interspecies competition. This fine scale differentiation, if not swamped by local gene flow, could be quantified using systematic sampling. Recently, many populations have also been experiencing severe selection pressure from the introduced white pine blister rust pathogen. It is nearly impossible, even under the most liberal assumptions, to assume that any of these populations would be experiencing conditions approaching genetic equilibrium.

To sum up, many of the critical underlying assumptions of Wright's *F*-statistics and other calculations involving the preconditions of Hardy-Weinberg equilibrium (such as heterozygosity calculations) are violated to some degree by this species, particularly in the northern portion of its range. Assumptions made in estimating genetic parameters for unglaciated areas in the southern portion (the Great Basin, portions of the Sierra Nevada and Yellowstone NP, some other refugia) may be more accurate than the (glaciated) northern portion (Yandell 1992; Jorgensen and Hamrick 1997; Bruederle *et al.* 1998) due to the confounding effects of repeated bottlenecks and population size fluctuations. Despite assurances that these formulae are fairly robust with respect to violations of many of the assumptions required, it is unlikely that the degree of divergence can be overcome to provide highly accurate data. However, since these are the generally accepted universal modes of expressing and calculating genetic diversity statistics, and since other formulae are not generally used, I have employed the standard formulae, but with an added caveat.

The fact that 94% of population genetic variation was found among individuals within populations (F_{ST} = 0.061 for 12 loci) concurs with fairly high gene flow implied by the results of other studies (Stuart-Smith 1998; Jorgensen and Hamrick 1997; Yandell 1992), and is typical of

many conifers (Hamrick and others 1991,1992). Fixation indices (F_{IS}) were fairly high (> 0.3) for most loci assayed in this study. The most likely explanation for a positive F_{IS} using neutral markers (i.e., isozymes) is identity by descent, or that the individuals assessed for allele frequencies share a common ancestor; a high F_{IS} indicates a strong correlation between alleles within subpopulations relative to those found within that subpopulation under random mating (Wright 1965). This correlates fairly well with the level of inbreeding found in the mating systems analysis (Chapter 2, this study). F_{IT} values followed generally the same trends and variability as F_{IS}, showing that for each locus the effects of inbreeding among individuals within the total population were similar to those within subpopulations.

3.5.3 SOURCES OF ERROR

As Stuart-Smith (1998) pointed out, a Wahlund effect, where separate populations are combined and analyzed as a single population (Hartl and Clark 1997) may obscure population genetic patterns and account for higher than expected heterozygosity values. In whitebark pine, this may occur if a single population sample was taken from individuals representing two different populations. One case where this may occur is where ecological factors create a physical disjunction in the environment, causing two or more populations adjacent to each other to superficially appear as one population. This situation can occur where part of a population spans an area disturbed by fire or avalanche, creating a successional disjunction, or where one population is spread over an area large enough to be characterized by different slope aspects, soil or geomorphological types. This also could result if the local environment caused a disjunction in reproduction by affecting phenology, e.g., elevation affecting the dates at which physiological threshhold values of climatic variables are reached. Given the longevity of the species, a Wahlund effect cannot be ruled out in some cases, although the majority of populations were sampled along a single slope face within a fairly narrow elevational range (< 100 m) and appeared to form a single continuous population.

One other possible source of error was scoring. While many alleles were clearly detectable and distinguishable from each other, there were some cases where the distinctions were less obvious. Attempts were made to mitigate this problem by including samples from several different populations on the same gel to facilitate comparison, and preparing several runs using the same buffer mixture and keeping them frozen until used. Double runs were often conducted so that many samples were processed using the exact same grinding and running buffer systems and stains. All gels were fixed and then rescored following the completion of all initial runs to ensure consistency within and among populations with respect to scoring methodology and interpretation. El-Kassaby (1991) suggests a sample size of 40-60 for isozymes to accurately measure patterns of genetic diversity where allele frequencies approach 0.5; the lack of sufficient sample size may have obscured some of the relationships in this study although most common allele frequencies (p) in the majority of populations were > 0.8.

Populations with very high proportions of trees of infected by blister rust often had very weak staining or did not produce scorable results, and those loci and populations were dropped from the analysis. Consequently, there may be some inherent bias in the results of this study in that some genotypes or alleles may have been underrepresented as a result of this. Since it appears likely that blister rust would have some metabolic effect on the enzyme expression of an infected tree or *vice versa*, trees more susceptible to blister rust may have resulted in some systematic enzyme signature or allelic expression (which may or may not have been detectable by electrophoretic technique). One possible result of this interaction is the possibility of selection on an electrophoretically detectable locus. The results shown both in this study in Figures 3.7 and 3.8, and by Stuart-Smith (1998) who found an extremely strong correlation between F_{IS} and white pine blister rust, do lend support to this hypothesis, although isozyme loci are generally considered neutral. It is therefore possible that some of the populations which were heavily infected with blister rust may have been less heterozygous, or more monomorphic,

or expressed specific alleles which were not detectable during the course of this study. This would account for some of the high heterozygosity values calculated for the other populations which showed only minimal to moderate blister rust infection.

3.6 CONCLUSIONS

For whitebark pine populations encompassing the species' range throughout BC, observed heterozygosity averaged 0.213, and expected 0.257. Populations and individual loci were highly variable, and the majority (94%) of genetic variability occurred among individuals. Populations were moderately differentiated (F_{ST} = 0.061); there was a statistically significant but weak isolation by distance effect, and populations within major mountain ranges were more genetically similar than among mountain ranges. Observed heterozygosity was highest in the south and east, but trends were weak for expected heterozygosity. Effects of glaciation, founder effects caused by avian dispersal and high levels of inbreeding would all contribute to the observed patterns and selection pressures involved in maintaining them.

CHAPTER 4 – A CONSERVATION STRATEGY FOR WHITEBARK PINE IN BRITISH COLUMBIA

"We're getting environmental Band-aids when we need intensive care."
- Mike Harcourt, 1989

4.1 INTRODUCTION

4.1.1 JUSTIFICATION FOR CONSERVATION

There are as many reasons to conserve or preserve as there are opinions; there are similarly as many arguments to the contrary. Randall (1986) lists several reasons in favour of conservation which may apply in this instance: inherent existence value of a species, anthropocentric altruism, and the role a species plays within an ecosystem, including generating oxygen and providing a carbon sink (Oldfield 1984; Salwasser 1990; Ledig *et al.* 1998). Other arguments put forward include concepts such as species rights, potential future importance, economic value, the value of the knowledge gained from a species, preserving a "natural order", a reverence for life for its own sake, a nature-centured empathy, and a theistic model (Callicott 1986; Lovejoy 1986; Leopold 1933; Ledig 1988). Clearly, all of these share some subjective and even emotional component, making them practically impossible to rank or quantify. In the modern context, decisions are often reduced to a fiscal scale with stakeholders or interest groups presenting their relative rankings to influence conservation decisions (Falk 1991). The key flaw in this methodology is that many, or sometimes most, of the values related to conservation arguments have no definable monetary value or price, and there is often no way to place such a value on abstract or preexisting ecological or subjective functions (Oldfield 1984; Hanemann 1986; Lande 1999).

Whitebark pine is a species of little immediate financial value when harvested; it would be costly to create infrastructure to facilitate harvesting and generally of poor form. The real value of this species is evident when whitebark pine trees are left intact within their indigenous ecosystems. Production of wildlife food, aesthetics, soil anchoring, a foundation for high-

elevation succession, keystone of subalpine biodiversity, meltwater channelization, insect and fungal habitat and microclimate modification at the timberline are all functions which whitebark pine has in its natural setting (Arno and Hoff 1990).

In British Columbia, f whitebark pine ecosystems are currently under less threat (re: levels of blister rust infection) than in southern Alberta or the Intermountain regions of the United States (Campbell and Antos 2000; Keane *et al.* 1990; Wilson and Stuart-Smith 2000; Tomback *et al.* 2001). Most of the land in B.C. suitable for current and future habitat is owned by the Crown, i.e., the Province. While a large area of high-elevation ecosystems is already preserved in a contiguous, large network of national and provincial parks, some is also allocated under both short- and long-term tenured licences for resource extraction such as logging and mining. A tiny component is privately owned, primarily for outdoor recreational purposes.

Whitebark pine has recently received unprecedented attention from the provincial government due to its threatened status (Yanchuk and Lester 1996; Yanchuk 2001), and even the province's chief forester has recognized its precarious predicament (Pederson 1998, op. cit. Kieran 1998). The Rocky Mountain National Parks are in the midst of formulating a conservation and ecosystem restoration strategy (Wilson and Stuart-Smith 2000; Rob Walker, Chief Ecologist, Parks Canada, Rocky Mountain National Parks, pers. comm., 2000). Based on knowledge gained from ecological studies (Campbell 1998; Campbell and Antos 2000; Stuart-Smith 1998), provincial protected areas policy (Sawicki 2000) and the current genetic study, a feasible conservation strategy for the province can now be created with a sound scientific basis which adheres to *a priori* goals such as protecting biodiversity through maintaining all components of fully functioning ecosystems (BCMoELP 2000).

4.1.2 CLIMATE CHANGE

The current buzzwords "global climate change" imply catastrophic changes but actually provide little information. Global climate has historically been in a constant state of flux; the

current rate of change, however, has led concerned citizens and scientists to press for more information and action (Jackson and Overpeck 2000). Agencies such as the United States Environmental Protection Agency (USEPA), the United Nations-sanctioned International Panel on Climate Change (IPCC) and many other regional, national and international organizations have been attempting to clarify and quantify the nature, scope and scale of this dramatic change.

Most of the scientific endeavours to define the magnitude and potential effects of climate change on the biosphere and specific regions of it involve General Circulation Models (GCMs), which attempt to model the potential magnitude and impact of global carbon allocation on the biosphere according to several scenarios. While these models are steadily involving more complex variables and able to produce more specific and varied effects, they still leave huge gaps in our knowledge. Such models only provide hypothetical scenarios and many of the critical input variables and feedback mechanisms are still unknown or too complex to model (Shafer *et al.* 2001). No matter which body or study is consulted, the final projections still emphasize the tremendous uncertainty associated with the estimates (USEPA 2001b; IPCC 2001a,b). These levels of uncertainty are so large that many estimates involving even 80% confidence intervals still encompass both positive and negative temperature change scenarios.

Attempting to project the future climate of areas that currently and in future could support whitebark pine ecosystems is even more difficult. Hydrological regimes are difficult to model (including estimates of snow accumulation and melting patterns) and complex topography 'throw a wrench' into the most sophisticated modelling systems. Satellite data has helped ameliorate some of these problems, but very little empirical climate data is available from high elevation sites, where there is a paucity of recording stations (Prentice *et al*. 2000). While the impact of climate change is widely expected to be greater in magnitude in the northern latitudes and higher elevations, the nature of the change is poorly understood. Planning for future

ecological management of whitebark pine is hence made even more complicated, with a generation time approaching the century mark, and drastic changes expected to be evident within 50 years, thus decisions made now are even more critical (Peters II 1986).

Most projections for montane and cordilleran regions of the Pacific Northwest estimate a mean temperature increase of two to six degrees Celsius within the next century (USEPA 2000, 2001a; Watson *et al.* 1997; IPCC 2001a). Mean summer temperatures are expected to remain the same or increase, while summer drought is likely to increase as snowpack in cordilleran areas melts faster, creating more water stress and increasing the likelihood of associated disease and insect susceptibility. This may result in a longer growing season of up to three weeks in the subalpine and alpine in terms of temperature, but this may be limited by moisture availability and increased frequency of extreme events, such as early and late growing season frosts (Easterling *et al.* 2000). Peterson *et al.* (1990) have already found that within the last 150 years, and especially within the last 30 or so, tree radial growth at the timberline of whitebark pine and other species has drastically increased, possibly as a result of a longer growing season due to warmer temperatures. Wildfires are generally predicted to increase in frequency and severity as a result of climate change (Perry *et al.* 1991; Keane *et al.* 1999; Keane and and Arno 1993; USEPA 2000; IPCC 2001a,b). The effects on precipitation regime vary with the predictive model used, but generally summers in mountain habitats will be drier, although winters are expected to be warmer, resulting in decreased mortality of seasonal pathogens and insects from lighter winter kills (Ayres and Lombardero 2000; Simberloff 2000).

Species' ranges are expected to shift generally northward and upward, causing an increase in the timberline and shifting species ranges north (Watson *et al.* 1997, IPCC 2001a,b; Peters II 1986; Shafer *et al.* 2001; Davis and Shaw 2001). The rate of climatic change, however, is not necessarily expected to be matched by the availability of suitable substrates or seedbeds. Species may therefore not be able to migrate at the rate the climate shifts (Davis and Shaw

2001; Tomback *et al.* 2001); moreover, earlier life stages are less resilient to extreme climate events than mature trees. Such off-site populations will be suffering from maladaptation or competition from other species better adapted to the new environmental conditions, especially "weedy" species (Simberloff 2000). Most ecological communities as they currently exist will likely not retain their character in the next century (Huntley 1991). Micro- and macrofauna associated with many of the plant species will also experience similar migrational difficulties as their hosts or habitat and food sources may be maladapted to their current habitat as climatic conditions change (Jackson and Overpeck 2000; Ledig and Kitzmiller 1992).

While historical shifts both in species composition and elevation of timberline and the historical range of whitebark pine have been dated using palynology and other techniques (e.g., Luckman and Kearney 1986; Kearney and Luckman 1983), the future remains uncertain. For whitebark pine, it is uncertain whether the Clark's nutcracker will shift its range at a rate consistent with climatic change. Birds have been observed caching seeds in sites outside of whitebark pine's current range (Carsey and Tomback 1994), and the potential for seed dispersal to ideal sites is certainly enhanced with a mutualist, long-distance seed disperser. It is difficult to predict whether animal behaviour will shift in a similar manner to other ecological factors (Peters II 1986). As the ecological character of current whitebark pine habitat alters, however, it is likely that other species may be able to outcompete it: lodgepole pine, subalpine fir and Engelmann and white spruce (*Picea glauca* (Moench.) Voss) being the prime candidates in B.C. On sites where fires may be far more frequent and severe, subalpine larch (*Larix lyallii* Parl.) may also increase its range and density.

Some research has been conducted specifically regarding whitebark pine ecosystems by Keane and Arno (1993) in Montana's Glacier National Park. Besides the startling prediction that there will be no glaciers left in this U.S. national monument in 70 years, the nature and location of ecosystems containing whitebark pine were projected to shift upwards and northwards, with

severe summer droughts and consequent increases in catastrophic wildfires accompanying these changes (Keane *et al.* 1999; Watson *et al.* 1997; IPCC 2001a). Shafer and others (2001) have also suggested that species currently found on the windward (western) side of North American mountain ranges may also shift to the leeward (eastern) side. If fire suppression programs currently in place are discontinued, the potential for whitebark pine to survive in these areas may be enhanced as populations of the alternate herbaceous blister rust host would be periodically diminished in the area. If current fire suppression regimes continue, they will drastically increase in expense (and likely decrease in success), and whitebark pine ecosystems will rapidly be replaced by more closed-canopy subalpine fir-Engelmann spruce ecosystems. Subalpine meadows will also become more scarce as they will be subject to constant tree recruitment without moderately frequent fires to maintain the position of the timberline (Keane and Arno 1993; Keane *et al.* 1990; Kendall and Keane 2001). Observations substantiating this phenomenon have been made over much of the southern portion of the range of whitebark pine aleady (Murray *et al.* 2000). Eighty to one hundred percent mortality is expected for whitebark pine stands in southeast B.C. (Campbell 1998; Campbell and Antos 2000; Kendall and Keane 2001), although there were no evident correlations with either weather or climate (Campbell 1998; Kendall and Keane 2001).

4.1.3 GENE CONSERVATION

An effective gene conservation strategy is the key to conserving adaptive variation (Aitken 2000). The potential for evolution is quantified in large part by genetic diversity, which is roughly analogous to intra- and interpopulation measures of heterozygosity. Since trees are sessile and can only migrate during the seed dispersal stage (although portions of the genome may also be dispersed during pollination, this does not provide an opportunity for range expansion), the long generation time of whitebark pine makes the option of *in situ* adaptation to climate change a critical component of any conservation strategy for the species. By targeting

highly diverse populations as well as genetically unique ones for special status or active management, the most efficient use of resources can be achieved.

Many unique alleles are only detectable in DNA sequences, or DNA markers based on these sequences, since many mutations are functionally neutral or serve to inactivate a protein. Many mutations also will only appear at the most fundamental level of DNA since several different sequences may produce the same RNA or protein based on genetic redundancy of codons. Many mutations are either detrimental to, or have no effect on, an individual's fitness in the current climate or in certain environments and are found at infinitesmally low frequencies (Holsinger *et al* 1991; Caughley 1994). Some researchers have suggested that if resources or time are limiting, it may be most prudent to capture the majority of common alleles instead, based on the distribution of rare alleles and the diminishing returns inherent in expending resources to capture them (Falk 1991; Brown and Briggs 1991), especially given the neutral (i.e., non-adaptive) nature of allozyme variation (El-Kassaby 1982; Karhu *et al.* 1996).

Determining the genetic composition of individuals can be done at many levels of resolution (Dekker-Robertson *et al.* 2001; Bruederle 1998); however, the finer the resolution, the more expensive the technique. Heterozygosity and fitness are not always linked (Frankham 1995), although heterozygosity may provide some genetic insurance against future environmental change (Huennke 1991; Vida 1994). The most cost-effective technique would be to visually survey individuals and populations and gauge the myriad complex relationships between and among genes, individuals and the environment by the physical and phenological adaptations they display in their native habitat. This will not provide much specific information about the actual genetic composition, and none at all about genes or alleles which are not expressed under those conditions. With respect to the critical conservation issue of locating individuals which may be resistant or highly tolerant of white pine blister rust, however, visual assessments

would actually provide such genetic information as well as capturing the most important genetic adaptations for the present as well as the future (Ledig 1988).

While ideally every individual and genotype of every species could be conserved in order to preserve the optimal adaptive potential for future generations, this is impossible and unnecessary in reality. A target effective population size (N_e) can be used as a surrogate for the proportion of genetic variability captured in a population, and estimates can be approximated from heterozygosity statistics in the ratio of roughly H_e being proportional to the inverse of $2N_e$. Precise calculations of N_e are difficult in any case for a hermaphroditic organism with overlapping generations, a mixed mating system, unknown exact population size, variable risk and susceptibility to mortality in different areas, long-distance multi-directional vector-mediated seed dispersal, wind pollination, and subdivided population structure (Cain *et al.* 2000; Nunney 1999; Nomura 1999; Hartl and Clark 1994). An exact estimate would involve extremely complicated models and formulae which are still being developed and tested (Cain *et al.* 2000; Case and Taper 2000). The stepping-stone population structure of whitebark pine results in relatedness among nearby populations and would result in spatially differentiated requirements for N_e, depending on the relatedness of adjacent populations. Yanchuk (2001), following recent suggestions that a traditionally suggested size of 500 may not be sufficient (e.g., Lynch 1996) has suggested a target size of 1000 for N_e, roughly corresponding to a census adult population size of 5000 for B.C. conifer species. The inbreeding found in this study may necessitate a slightly higher census population, since Yanchuk was assuming an inbreeding coefficient of < 0.1, so a census population on the order of 6000 may be more appropriate for *in situ* conservation purposes. A better actual number would consider the frequency of any disease resistance genes or genotypes in each conservation area, since a key goal of *in situ* conservation for whitebark pine would be to capture a threshhold percentage (one to five percent has been suggested by Hoff *et al.* 2001) of those genes.

While the results from Chapter 2 indicated that whitebark pine exhibits substantial inbreeding relative to other pines, although within a range typical for stone pines, results from Chapter 3 revealed a fairly high level of heterozygosity. Most of the genetic variation was found among individuals within populations, and there were geographical trends with both heterozygosity and heterozygote deficiency. Based on these observations, effective population sizes for whitebark pine conservation may not be strongly impacted by current levels of inbreeding. This may be due in part to the high interpopulation gene flow, thus a conservation strategy should incorporate more than one population from each general area in order to maintain gene flow and minimize potential inbreeding. Although heterozygosity was lowest in the north, it may not be necessary to compensate for the lower N_e in this region by conserving a greater number of individuals relative to the south since the populations in the south are at a far greater risk of immediate mortality from white pine blister rust. As will be explained in the subsequent section, conservation efforts in the south should not be geared towards a gross number as may be appropriate in the north; the pressing problem of blister rust engenders a conservation strategy targeted towards specific individuals or families, and will require more aggressive management.

Since future environmental conditions are not known, the best strategy may be to conserve as wide a spectrum as possible of genotypes (Frankham 1995; Ledig 1986,1988; Ledig and Kitzmiller 1992). The most cost-effective conservation strategy would undoubtedly be to provide for *in situ* reserves containing as many individuals as possible which would allow for migration to habitat suitable in the future (Peters II 1986; Aitken 2000; Ledig 1986). Making comprehensive *ex situ* germplasm collections, seed orchards or seed collections would likely be too expensive due to the extremely long period of time required for propogule production and costs of seed collection, processing and storage. Some ancillary *ex situ* or *inter situ* collections may be instituted as a complementary measure for areas where the creation of natural reserve areas is either not feasible or the local populations are under immediate threat of extirpation

(Yanchuk 2001). The most effective option would probably be cryogenic seed or germplasm storage in those cases, although the effects of long-term seed storage on germplasm viability remain largely unknown for this species and its seeds often deteriorate rapidly in conventional types of storage (Ledig 1988; Bonner 1990; D. Kolotelo 2000, BCMoF Seed and Cone Officer, pers. comm.).

4.1.4 WHITE PINE BLISTER RUST

In terms of species conservation, it is probably most effective to target those populations most at risk for special conservation efforts, thereby providing trickle-down benefits to other ecosystem components (Namkoong 1992). While B.C. is fortunate to have fairly large, contiguous regions occupied by whitebark pine ecosystems, populations in the south appear to be especially susceptible to blister rust and mountian pine beetle attacks. Intensive surveys currently being conducted by the B.C. Ministry of Forests (Zeglen 1999, 2000) are attempting to locate individuals throughout the province which appear to be resistant or tolerant of blister rust. Identified trees will be subject to further screening in the future; however, it is critical to institute screening programs combined with site hazard assessment in order to correctly assess the resistance of the stock and determine suitable outplanting sites (Kendall and Keane 2001; McDonald and Hoff 2001).

Other five-needle pines affected by this disease have been extensively studied. A single-gene mechanism for resistance has been found in both western white pine (*Pinus monticola* Dougl. ex D. Don) and sugar pine (*Pinus lambertiana* Dougl.) (Devey *et al.* 1995), but different genes for each species. Adaptive traits such as the multigenic bark reaction resistance to white pine blister rust are likely to be more robust in the long term than a single-gene trait. The complexities of a host-pathogen system are also not always fully understood, and recently virulent strains of the blister rust have been found in areas where there was strong selection due to a high frequency of the single-gene resistance, which the pathogen can now overcome in

both sugar and limber pine in some regions. The stability and efficacy of the multigene tolerance or bark reaction mechanism has a trade-off in that there will likely be some growth impact on the tree caused by the disease (Hoff 1984; Kojwang 1994). These contradictory effects on fitness conferred by the multigenic disease resistance mechanism may be an artifact of the variation inherent in the complex of genes acting to cause this reaction. Should a screening process for a naturally-occurring resistance gene be developed, it would be ideal for whitebark pine conservation since individuals can be screened in a non-destructive manner to identify resistant genotypes.

4.2 WHITEBARK PINE CONSERVATON STRATEGY FOR BRITISH COLUMBIA

4.2.1 INTRODUCTION

While a conservation strategy for such a widespread species which is a component of so many different ecological communities cannot focus around a single species or geographical area (Lovejoy 1986), this plan is intended as one link within an integrated framework including the Rocky Mountain National Parks Strategy (2000) and initiatives undertaken within the United States Intermountain region (Kendall 1994). A plan for conserving whitebark pine, while nominally centred around a single species, also ensures the health of a large number of different species and taxonomic groups given its keystone role. Conservation of the keystone species can have ancillary effects to conserve the nature, structure and functioning components of the representative ecosystem (Tomback *et al.* 2001).

Given the potential implications for global climatic change, it is probably best to institute a conservation strategy which provides insurance against the widest possible range of future scenarios (Hanemann 1986). Despite the enormous uncertainty around the nature and scale of the effects of these predicted changes, there are still a few parameters which remain relatively certain: that current climatic zones will shift upward in elevation and northward in latitude. However, the magnitude of changes in hydrological regimes and differential rates of migration,

extinction of different species and ecotypes, and even opportunities for speciation, remain too uncertain to predict.

4.2.2 SHORT-TERM GOALS

One step which has been nearly completed in B.C. is the intensive surveys of whitebark pine populations collecting data on growth and blister rust (Zeglen 1999, 2000). A key goal of these surveys is to identify putatively resistant individuals for future management. In the U.S., a system of blister rust hazard ratings for the range of whitebark pine has also been instituted based on ecological and biogeographical factors (Hoff *et al.* 2001). Extending and adapting this system to B.C. would facilitate blister rust management as well as improving whitebark pine outplanting success for any rehabilitation efforts.

Another facet of active management could include *inter-* and *in situ* measures to complement the *ex situ* program (Brown and Briggs 1991; Ledig 1986; Millar and Westfall 1992). This could include seed collection, germination and outplanting on suitable sites, or sites expected to be suitable within 50-100 years (Ledig and Kitzmiller 1992). This option is extremely labour-intensive and expensive. In years with cone crops, branches with cones in suitable stands must be caged in the early to mid-summer, as soon as access permits, in order to protect seeds from predation. These stands must be revisited in the autumn for cone collections; this step may be more or less complicated depending on whether individual genotypes or mother trees are to be identified or not. The cones must then be transported to a processing facility. Specialized procedures must be followed to extract and identify filled seeds, stratify and germinate them, and then return them to appropriate locations for outplanting. Whitebark pine has been shown to be highly susceptible to a large variety of pathogenic seed and cone storage fungi (Vujanovic *et al.* 2001). While ideally the seedlings would be returned to their original provenances, some seed transfer may be permitted, depending on the nature of the conservation and land use decision, climate change projections, or data from common garden experiments. One trade-off

to take into account in this case is the removal of potential sources of natural regeneration as well as wildlife food; many times the number of seeds must actually be taken compared to the number of seedlings required for the experiments due to the typically very low germination rate.

Virtually nothing is currently known about the extent and nature of adaptive variation in whitebark pine in B.C. Once seed has been procured, common garden experiments should be established to assess important adaptive traits such as budburst date, early growth rates and biomass allocation. Differences among populations and regions could then be used to define appropriate seed and scion transfer guidelines. Seedlings grown for these tests could be utilized for further data collection, including screening for white pine blister rust resistance in highly controlled conditions.

Co-operation among regions where whitebark pine grows would benefit all stakeholders: jurisdictions could share technology and facilities, and develop joint solutions while reducing duplication. Active, ecosystem-based management may also play a role in this conservation strategy (Cole and Landres 1996; Hoff *et al.* 2001). One facet of this currently being explored in the Rocky Mountain National Parks and in the U.S. Intermountain region is the establishment of controlled burns in whitebark pine ecosystems. A century of fire suppression has altered these ecosystems to the degree that their fundamental characteristics have changed dramatically. There is an increased risk of high-severity fires as fuel loads are not periodically burned out by light surface fires; the advanced age of many trees also harbours increased susceptibility to attack by mountain pine beetle (*Dendroctonus pondorosae* Hopkins) and increased occurrence of *Ribes* spp. in the understory is favourable for *Cronartium ribicola*. The lack of surface fires has also allowed for succession and soil development on the talus and thin forest floor, eliminating potential seedbeds for pine and facilitating growth of competing species such as Engelmann spruce (*Picea engelmannii* Parry ex Engelm.), mountain hemlock (*Tsuga mertensiana* (Bong.) Carr.) and subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.). Periodic

moderate to light surface fires also eliminate *Ribes,* the alternate host of the blister rust pathogen, and may moderate local spore densities (Keane *et al.* 1993).

A regime of controlled burns and follow-up monitoring should be instituted in selected areas in B.C. to restore whitebark habitat. Lightning-caused wildfires in remote areas where human habitation and activity would not be at risk should not be suppressed (Wilson and Stuart-Smith 2000). Wildfires of small size that could be controlled at areas that interface with human use or habitation should be allowed to burn, if possible. Nutcrackers have been frequently observed caching pine seeds in recently burned areas, which provide ideal competition-free mineral soil seedbeds for germination (Tomback 1986). Providing the largest possible suitable areas for seedling establishment would help maintain more genotypes as a larger number of cached seeds would germinate (Tomback *et al.* 2001). In cases where a resistant genotype is found, it may be desirable to limit fires to low intensities in those areas, or to suppress local fires until the cones have been collected.

4.2.3 MEDIUM-TERM GOALS

In the case where a unique genotype has been located, such as a rust-resistant tree, it may be desirable to also attempt vegetative propogation or tissue culture, and it is definitely worth the extra effort of intensive surveys and repeated site visits to ensure a supply of seed from these trees (Hoff *et al.* 2001). One possibility is the potential for grafting selected individuals onto western white pine rootstock, also in the taxonomic section Strobi, in order to reduce operational time and increase vigour and growth rates (Arno and Hoff 1989).

Planting seeds or seedlings outside their native provenances may be desirable if short-term extirpation of the species in a region is a more pressing threat than maintaining the indigenous genotypes (Conway 1986). This could also be useful if a consensus has been reached to facilitate species migration through planting northwards or upwards into areas it does not occur at present, as an ameliorating measure against the impacts of climate change (Peters II 1986).

In light of the observed and anticipated rates of climatic change, I believe that planting seedlings in a stepping-stone pattern to the north of their current provenances by up to several hundred kilometres in some cases would be acceptable and that survival would considerably outpace mortality. Long-term reconstructions of historical climate and pollen data show a more contiguous distribution of high-elevation species during warm periods, including whitebark pine (Prentice *et al.* 2000). Nutcrackers also do cache seeds outside of the current species' range, implying that a northward shift facilitated by planting could encourage the birds to expand their range northwards.

Once seed transfer zones have been established, appropriate selections could be made during local mast years, assuming the climate will change at the rates and magnitude outlined by various projections (IPCC 2001a; USEPA 2000; Shafer *et al.* 2001). Trees growing in high-snowpack areas typically survive and regenerate best in clumps, where a microclimate effect causes earlier snowmelt and shallower snowpacks around clumps (Klinka and Chourmouzis 2001). Survival will probably be best if seedlings are planted in clumps adjacent to stumps or coarse woody debris on south-facing, well-drained regosols or calcareous sites where mature potential trees which may be potential competitors are scarce or absent. The possibility that planted seeds may be eaten before they germinate makes the extra effort and expense of planting seedlings worth while.

4.2.4 LONG-TERM GOALS

Once suitable individuals have been located, it may be possible to establish and maintain a few small orchards. It is possible to maintain a representative sample of at least 90% of the most common alleles, i.e., the majority of the genetic potential of a population, with a collection of as few as 10-50 individuals, provided they are collected from a wide range of habitats (Brown and Briggs 1991), especially given the relatively high heterozygosity and fairly low F_{ST} found in this study. One seed orchard could be established per transfer zone in order to keep the

genetic character of each regional population. Priority should of course be given to individuals putatively resistant or tolerant to white pine blister rust. Collecting scion material from these individuals and using controlled pollination would also ensure that the resistance or tolerance characteristics (the most desirable features to conserve) of the parent trees would be expressed.

Establishing a series of range-wide seed orchards for whitebark pine should be a long-term objective due to the enormous expense of collecting and maintaining such an orchard, since it is not a commercially valuable species (Brussard 1990). While only a small number of individuals would be needed to provide genetic resources, the effort required to produce sufficient suitable adults will be very costly and take years. A suitable location for such a collection would also be difficult to find since existing seed orchard complexes in British Columbia are currently located in climates which are likely not suitable for the growth and reproduction of whitebark pine. Seedlings have been successfully grown in nurseries; however, it is not certain that the trees would survive to reproductive age or receive their chilling requirements in any habitat type but the rather extreme ones they now occupy.

While public sentiments in B.C. currently run counter to genetic engineering of forest trees, the potential to mitigate the impact of white pine blister rust and maintain the health of whitebark pine ecosystems may be greatly aided by such technology. They are included in this "long-term goals" section since the technology may take many years of costly and intensive research to be successful and active genetic manipulation of noncommercial forest species is a low priority at this time compared to *in situ* techniques and more conventional strategies. In the event that these techniques do become feasible, some of the applications are listed here. Somatic embryogenesis and tissue culture could rapidly reproduce tolerant or resistant individuals for outplanting in two to three years, instead of waiting dozens of years for seed to grow and reach sexual maturity. In the event that a similar single-gene resistance mechanism is isolated for

whitebark pine to the pathogen as was found for sugar and western white pines, it may be temporarily beneficial until a breeding strategy for producing resistant seedlings is operational. While the comparatively rapid mutation rate of the pathogen may respond to the selection regime imposed by introducing this gene, it could be carefully applied depending on the hazard rating of the outplanting area. In the long term, a multigenic form of either tolerance or resistance would be more beneficial since it would be much more difficult for the pathogen to develop a virulent mutant in such a case (Hoff *et al.* 2001).

4.2.5 REGIONAL CONSERVATION PRIORITIES

When selecting appropriate populations to target for conservation, heterozygosity would be the simplest criterion, aside from blister rust resistance. In terms of heterozygosity, Perkins, D'arcy, Whistler and Lime were particularly high. Paget and Stanley, although they exhibited lower heterozygosity, had slightly higher mean numbers of alleles per locus (there was no real statistical difference for this measure), and more polymorphic loci. Perkins was high in both heterozygosity and allelic richness. For a northwestern seed transfer or conservation quadrant, this population may serve as a genetic reservoir. For the northeast, Stanley may be a suitable population, although the trees in this particular population were on average quite young. Conservation activities should involve both Paget and Stanley, the easiest populations to sample in terms of accessibility, or other populations in this area. In Paget there are many trees of reproductive age but they are suffering heavily from blister rust. This would also tie in with the Rocky Mountain National Parks conservation strategy (Wilson and Stuart-Smith 2000). In the southwest, Whistler and Lime would be suitable candidates with abundant individuals, cone-bearing adults and regeneration, as well as relatively low blister rust impact. In the southeast, I would not suggest a single population should serve as a source population for conservation efforts. Due to the extreme severity of blister rust in this region, selections should be made on an individual-tree basis following comprehensive screening, and resistant genotypes, if

available, could even be imported from the bordering states of Idaho or Montana which share similar climate and topography.

The symbiosis between whitebark pine and Clark's nutcracker is a necessary prerequisite to the shift of the species' range northwards, the bird being the key agent of seed dispersal. While the nutcracker's range can extend beyond that of the tree, it is unknown how dramatically it will shift with a change in climate, and it is also uncertain whether suitable habitat will occur if cache locations shift northward. Assuming that the bird's behaviour will remain consistent when looking for cache locations, optimal germination sites should continue to be a large proportion of bird seed caches. However, that is no guarantee that this will remain the case, or that other species which are components in current whitebark pine ecosystems will accompany this migration (Fisher and Myres 1980; Tomback 2001). Propogule and gamete dispersal by water and wind, as most of the rest of the high-elevation species such as mosses, flowers, shrubs and lichens exhibit, will likely result in a far slower migration rate upwards and northwards. Despite the shorter generation times of these other organisms, it is unlikely that subalpine and timberline communities, as we know them, will remain intact as climate shifts, as historical evidence supports (Davis and Shaw 2001). Introduced and fast-growing, or weedy, species may have an advantage as conditions rapidly change due to short generation times and generally high fecundity (Simberloff 2000). Most species in the genus Ribes, which are the secondary host of the blister rust pathogen, could also easily expand their range or increase in abundance, providing a vector for the disease where it may currently be absent. The fungus itself could also evolve and spread in a similar manner (Ayres and Lombardero 2000).

That said, the most effective insurance policy for high-elevation ecosystems is to set aside a contiguous land base as large as possible, representing as many biogeoclimatic regions as possible, that would support the migration of these species and minimize the effects of fragmentation (Yanchuk 2001; Millar and Westfall 1992; Lester and Yanchuk 1996). In B.C.,

this is still a real possibility, especially at the northern species boundary. In California, Wyoming and Montana, a mosaic of land tenures and uses such as roads, resource extraction, tourist facilities and development have led to a highly fragmented range over much of the mountainous topography. The largely undeveloped, unpopulated and unroaded mountains of B.C. provide the opportunity for this critical first step in conservation.

Currently, the status of whitebark pine conservation in most of B.C. with respect to future climate scenarios is quite good. Despite the spread of blister rust, large tracts of suitable habitat remain unthreatened by human impact. Vast areas of contiguous wilderness are already protected in parks; although some regions do not provide adequate protection yet, they are generally remote, unroaded and inaccessible, providing potential habitat for future range shifts and a rich spectrum of different habitats. Many of these areas are allowed to burn if wildfires occur and provide ideal germination sites for whitebark pine. The species has been designated a high conservation priority, and some resources have been allocated to gather baseline information and develop and implement strategies.

The highest priority should be allocated to identifying rust-resistant individuals. This work has already been initiated. Conducting site hazard assessments for white pine blister rust and collecting seed from as many trees as possible both from these selected individuals as well as targeted populations within regions should be the next most pressing items. Conducting common garden experiments in order to determine adaptive variation and delineate seed transfer zones, followed by outplanting of two- to three-year-old seedlings should be conducted, along with periodic monitoring of both the common garden experiments and the outplanting test sites. All other activities should be assigned lower priorities, and continue if resources allow. Ongoing management including letting wildfires burn in whitebark pine habitat, should be allowed to continue, and to be applied throughout the species' range in B.C., where no human livelihoods are threatened.

Although blister rust is likely to decimate whitebark pine across its northern range in the future, it is anticipated that enough resistant or tolerant genotypes will remain to ensure its survival in the north, although at severely reduced numbers (Hoff *et al.* 2001). Estimates of natural resistance or tolerance rates between one and five percent have been given based on observations of the effects of blister rust on natural populations (Lanner 1996; Hoff *et al.* 2001). The magnitude of this impact will depend largely upon human intervention at the present time; given the incredibly rapid spread of the fungal pathogen, there is no time to lose if our efforts are to make a positive difference (Hoff *et al.* 2001).

4.3 CONCLUSIONS

Due to the effects of fire suppression, introduced fungal disease and mountain pine beetle, whitebark pine ecosystems in B.C. are under threat of drastically reduced population density and even complete extirpation in many southern populations within one generation. It is critical that active range-wide conservation measures be taken as soon as possible. These measures include: (1) screening natural populations for individuals which may be resistant or tolerant to white pine blister rust, (2) collecting seed from accessible targeted populations and supplementing natural regeneration in suitable sites by planting seedlings, except in the southern Rockies where targeting disease-resistant individuals should be paramount, (3) developing appropriate seed transfer zones within B.C. and across jurisdictions to facilitate the transfer of the most suitable materials by implementing common garden experiments to quantify adaptive variation of some important traits, (4) returning wildfires in high-elevation ecosystems to the historical frequencies, and (5) collecting seed and scion materials from putatively resistant individuals and establishing *ex situ* breeding populations in suitable areas. Genetic engineering may have some long-term potential for beneficial effects, but should be considered a lower priority unless no natural resistance to blister rust exists. Sharing research and resources with other jurisdictions included in whitebark pine's range is critical. Extending the

range of whitebark pine to the north in anticipation of accelerated rates of climate change may ensure a seed supply for natural regeneration and adaptation in the next century.

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APPENDIX I - Table of allele frequencies by population

Table A.1.1. Allele frequencies for 17 populations; names and poulation numbers as in Table 3.1.

Pop / Allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
N	19	20	22	27	29	24	19	25	17	30	30	17	20	27	30	29	17
MDH1-1	1.00	1.00	1.00	0.98	1.00	0.95	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	0.93	0.98	1.00
MDH1-2	0.00	0.00	0.00	0.01	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.01	0.06	0.01	0.00
N	20	25	21	32	32	27	19	25	20	30	30	17	24	33	29	30	20
MDH2-1	0.85	0.86	0.88	0.70	0.68	0.74	0.73	0.66	0.75	0.68	0.68	0.58	0.64	0.78	0.75	0.75	0.75
MDH2-2	0.15	0.14	0.11	0.29	0.31	0.25	0.26	0.34	0.25	0.31	0.31	0.41	0.35	0.21	0.24	0.25	0.25
N	20	31	28	32	32	26	19	25	25	28	30	17	24	35	27	30	21
MDH3-1	1.00	0.98	1.00	1.00	0.95	1.00	1.00	0.98	1.00	1.00	0.86	1.00	0.97	0.97	1.00	1.00	1.00
MDH3-2	0.00	0.01	0.00	0.00	0.04	0.00	0.00	0.02	0.00	0.00	0.06	0.00	0.00	0.01	0.00	0.00	0.00
MDH3-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.02	0.01	0.00	0.00	0.00
N	16	13	17	15	21	4	5	25	4	26	30	17	19	20	30	27	8
PGM1-1	0.84	0.80	0.91	0.83	0.54	0.50	0.20	0.54	0.12	0.42	0.75	0.50	0.44	0.57	0.63	0.46	0.50
PGM1-2	0.15	0.19	0.08	0.16	0.45	0.50	0.80	0.46	0.87	0.57	0.25	0.50	0.55	0.42	0.36	0.53	0.50
N	20	24	26	32	28	27	17	25	24	30	30	17	24	34	30	30	18
SKD1-1	0.72	0.87	0.75	0.87	0.83	0.72	0.67	0.66	0.68	0.53	0.68	0.76	0.70	0.60	0.78	0.83	0.88
SKD1-2	0.27	0.12	0.25	0.12	0.16	0.27	0.32	0.34	0.31	0.46	0.31	0.23	0.29	0.39	0.21	0.16	0.11
N	19	21	26	32	30	28	17	25	25	29	30	17	24	34	30	30	18
SKD2-1	0.50	0.61	0.67	0.67	0.48	0.44	0.67	0.62	0.62	0.51	0.58	0.67	0.77	0.63	0.63	0.55	0.72
SKD2-2	0.50	0.38	0.32	0.32	0.51	0.55	0.32	0.38	0.38	0.48	0.41	0.32	0.22	0.36	0.36	0.45	0.27
N	20	2	16	23	16	13	8	25	19	29	30	17	24	35	26	27	21
FDP1-1	0.90	0.50	1.00	1.00	0.93	1.00	1.00	1.00	0.94	1.00	1.00	1.00	1.00	1.00	0.96	0.96	1.00
FDP1-2	0.10	0.50	0.00	0.00	0.06	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.03	0.03	0.00
N	15	17	22	31	22	29	8	25	24	28	30	17	24	35	30	30	19
GDH1-1	0.96	1.00	1.00	1.00	0.95	1.00	1.00	0.84	1.00	0.87	0.83	1.00	0.75	0.95	0.73	0.93	1.00
GDH1-2	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.16	0.00	0.10	0.00	0.00	0.00	0.00	0.03	0.03	0.00
GDH1-3	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.16	0.00	0.25	0.04	0.23	0.03	0.00
N	20	21	18	25	15	29	10	18	26	30	30	17	16	35	30	22	11
LAP1-1	0.90	0.85	0.88	0.60	0.86	0.91	1.00	1.00	0.92	0.83	0.83	0.94	0.93	1.00	1.00	0.93	1.00
LAP1-2	0.10	0.14	0.11	0.40	0.13	0.08	0.00	0.00	0.07	0.16	0.16	0.05	0.06	0.00	0.00	0.06	0.00
N	16	19	10	25	13	28	10	18	21	29	30	17	16	34	30	22	6
LAP2-1	1.00	0.84	0.70	0.50	0.53	0.71	1.00	0.63	0.85	0.62	0.75	0.73	0.81	0.72	0.43	0.79	0.83
LAP2-2	0.00	0.15	0.30	0.50	0.46	0.28	0.00	0.36	0.14	0.37	0.25	0.26	0.18	0.27	0.51	0.20	0.16
LAP2-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00
N	17	16	18	25	32	26	19	25	20	30	30	17	16	25	30	28	21
IDH1-1	0.67	0.65	0.94	0.72	0.82	0.96	0.94	0.54	0.85	0.95	0.95	0.97	0.93	0.92	0.96	0.87	0.95
IDH1-2	0.32	0.34	0.00	0.08	0.17	0.03	0.05	0.46	0.15	0.05	0.05	0.02	0.06	0.08	0.03	0.08	0.02
IDH1-3	0.00	0.00	0.05	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.02
N	15	4	15	11	16	22	8	25	20	30	30	17	12	26	30	30	15
PGI2-1	0.63	0.62	0.63	0.18	0.43	0.63	0.62	0.50	0.72	0.55	0.50	0.47	0.45	0.76	0.55	0.61	0.53
PGI2-2	0.33	0.25	0.20	0.81	0.28	0.22	0.25	0.00	0.20	0.10	0.05	0.08	0.25	0.07	0.16	0.11	0.00
PGI2-3	0.03	0.12	0.16	0.00	0.28	0.13	0.12	0.50	0.07	0.35	0.45	0.44	0.29	0.15	0.28	0.26	0.46

Table A.1.2. Ovule and pollen allele frequencies for Mannning and Baldy; standard deviations in parentheses.

Locus	Allel	Baldy		Manning	
		Pollen	Ovule	Pollen	Ovule
Pgi1	1	.965 (0.014)	.850 (0.037)	.819 (0.052)	.760 (0.053)
	2	.035 (0.014)	.150 (0.037)	.181 (0.052)	.240 (0.053)
Pgi2	1	.852 (0.017)	.833 (0.045)	.917 (0.017)	.900 (0.040)
	2	.148 (0.017)	.167 (0.045)	.083 (0.017)	.100 (0.040)
Idh	1	.916 (0.017)	.820 (0.041)	.953 (0.009)	.941 (0.020)
	2	.002 (0.000)	.016 (0.000)	.002 (0.001)	.020 (0.000)
	3	.082 (0.017)	.164 (0.041)	.045 (0.009)	.039 (0.020)
Pgm	1	.997 (0.000)	.951 (0.020)	.954 (0.024)	.941 (0.029)
	2	.002 (0.000)	.016 (0.000)	.002 (0.000)	.020 (0.000)
	3	.002 (0.000)	.033 (0.020)	.044 (0.024)	.039 (0.029)
6Pg1	1	.279 (0.028)	.164 (0.047)	.232 (0.044)	.216 (0.058)
	2	.002 (0.000)	.016 (0.000)	.002 (0.000)	.020 (0.000)
	3	.720 (0.028)	.820 (0.047)	.766 (0.044)	.765 (0.058)
6Pg2	1	.998 (0.000)	.984 (0.001)	.990 (0.005)	.961 (0.015)
	2	.002 (0.000)	.016 (0.000)	.002 (0.000)	.020 (0.000)
	3	-	-	.008 (0.005)	.020 (0.015)
Mdh1	1	.998 (0.000)	.984 (0.001)	.998 (0.000)	.980 (0.000)
	2	.002 (0.000)	.016 (0.000)	.002 (0.000)	.020 (0.000)
Mdh2	1	.766 (0.043)	.574 (0.063)	.775 (0.031)	.686 (0.075)
	2	.002 (0.000)	.016 (0.000)	.002 (0.000)	.020 (0.000)
	3	.232 (0.043)	.410 (0.063)	.223 (0.031)	.294 (0.075)
Mdh3	1	.998 (0.000)	.983 (0.010)	.998 (0.000)	.980 (0.013)
	2	.002 (0.000)	.017 (0.010)	.002 (0.000)	.020 (0.013)
Mdh4	1	.725 (0.039)	.656 (0.049)	.732 (0.031)	.667 (0.060)
	2	.002 (0.000)	.016 (0.000)	.002 (0.000)	.020 (0.000)
	3	.273 (0.039)	.328 (0.050)	.266 (0.031)	.314 (0.060)

APPENDIX II - Buffer recipes

Table A.2.1. Extraction Buffer – modified from Mitton 1979

Item	Quantity
Polyvinylpyrrolidoninone (PVP-40)	2.00 g
Sucrose	2.00 g
EDTA, Na salt	0.04 g
Dithiothreitol (DTT)	0.03 g
Ascorbic acid, Na salt	0.01 g
Bovine albumin	0.02 g
β-NAD	0.01 g
β-NADP	0.01 g
Pyridoxal-5'-phosphate	0.001 g
β-mercaptoethanol	2 drops

In 20 mL distilled deionized water (ddH_2O), add ingredients sequentially while stirring. Add β-mercaptoethanol in fume hood immediately prior to grinding samples. Keep cold, use immediately. Discard after 24 hours.

Table A.2.2. Morpholine Electrode Buffer

Item	Quantity
Andydrous citric acid	30.74 g
N-3-aminopropyl morpholine	72 mL

In 4 L of ddH_2O, dissolve citric acid while stirring. Add morpholine; adjust pH to 8.0 with morpholine.

Morpholine Gel Buffer

Mix a 1:20 dilution of the electrode buffer.

Table A.2.3. Tris-Citrate Electrode Buffer

Item	Quantity
Tris-HCl	27.00 g
Anhydrous citric acid	16.52 g

in 1 L ddH_2O, dissolve the ingredients while stirring. Adjust pH to 6.3 with 1 M NaOH.

Tris-Citrate Gel Buffer

Item	Quantity
Tris-HCl	1.4521 g
Anhydrous citric acid	0.8646 g

Dissolve ingredients in 100 mL ddH_2O while stirring, then make a 1:15 dilution to make 1.6 L. Adjust pH to 6.7 with 1 M NaOH.

Table A.2.4. Ridgeway Electrode Buffer

Item	Quantity
Boric Acid	11.875 g
Lithium Hydroxide	1.60 g

Dissolve ingredients in 1 L ddH_2O while stirring. Adjust pH to 8.3 with LiOH.

Ridgeway Gel Buffer

Item	Quantity
TRIZMA base	6.20 g
Citric acid monohydrate	1.50 g

Dissolve ingredients in 1 L ddH_2O; then mix a 9:1 dilution of gel:electrode solutions. Adjust pH to 8.3.

Gel fixative

Mix a 1:5:5 solution of glacial acetic acid:methanol:water, soak gel slices for 1-2 hours or until unstained surfaces appear opaque, at least 30 minutes.

APPENDIX III - Locations of all populations sampled

Table A.3.1. List of populations and sampling locations

Pop#	Location	Area	NTS 1:50,000 Mapsheet	Latitude (N) Longitude (W)	Elevation (m)
1	Hudson Bay Mtn	Smithers	Smithers 93L/14	54°56'25" 127°19'15"	1850
2	Higgins Creek	Babine Mtns PP	Driftwood Ck 93L/15	54°54'20" 126°46'55"	1600
3	Sweeney Lake	Houston	Newcombe Lk 93E/14	53°45'25" 127°12'35"	1630
4	Heckman Pass	Tweedsmuir PP	Tusulko R 93C/12	52°32'20" 125°48'40"	1600
5	Perkins Peak	Chilcotin	Tatla Lk 92N/15	51°50'45" 124°59'10"	1700
6	Tchaikazan R	Ts'yl-os PP	Tchaikazan R 92O/4	51°12'00" 123°39'30"	1600
7	Yalakom R	Lillooet	Big Bar 92O/1	51°04'50" 122°27'05"	1900
8	D'arcy	D'arcy	Birkenhead Lk 92J/10	50°31'15" 122°34'35"	1910
9	Van Horlick Ck	Lillooet	Duffy Lk 92J/8	50°16'20" 122°14'45"	2000
10	Whistler Mtn	Whistler	Whistler 92J/2	50°03'45" 122°56'00"	1700
11	Lime Lookout	Clinton	Clinton 92P/4	51°05'25" 121°39'55"	1980
12	Hart's Pass (Washington, U.S.A.)	Okanogan National Forest	USGS 1:24,000 Slate Peak N4837.5 W12037.5/7.5	48°42'30" 120°41'00"	2050
13	Kootenay Pass	Stagleap PP	Salmo 82F/3	49°05'10" 117°02'30"	1940
14	Jumbo Pass	Purcell Mts	Duncan Lk 82K/7	50°20'20" 116°38'00"	2060
15	Stanley Glacier	Kootenay NP	Mt Goodsir 82N/1	51°11'10" 116°04'40"	1850
16	Paget Peak	Yoho NP	Lk Louise 82N/8	51°26'50" 116°21'55"	2240
17	Mt Edith Cavell	Jasper NP	Amethyst Lks 83D/9	52°42'00" 118°03'30"	1750
18	Apex Mtn	Hedley	Penticton 82E/5	49°22'40" 119°55'00"	2170
19	Puddingburn Mtn	Cranbrook	St Mary Lk 82F/9	49°34'00" 116°05'35"	2150
20	Galton Pass	Roosville	Inverted Ridge 82G/2	49°00'45" 114°54'30"	1940
21	Morrissey Ridge	Fernie	Flathead Ridge 82G/7	49°27'00" 114°56'10"	2000
22	Line Ck Mine	Sparwood	Tornado Mtn 82G/15	49°45'50" 114°50'25"	2100
23	Mt Seven	Golden	Golden 82N/7	51°15'50" 116°51'30"	2150
24	Castle Mtn	Banff NP	Castle Mtn 82O/5	51°17'55" 116°56'30"	2200
25	Parker Ridge	Jasper NP	Columbia Icefield 83C/16	52°10'50" 117°04'50"	2200
26	Scout Mtn	Cathedral PP	Ashnola R 92H/1	49°04'40" 120°11'30"	2220
27	Blackwall Peak	Manning PP	Manning Park 92H/2	49°05'35" 120°45'35"	2000
28	Thynne Mtn	Merritt	Tulameen 92H/10	49°42'25" 120°55'50"	1940
29	McBride Mtn	McBride	McBride 93H/8	53°15'00" 120°14'45"	1970

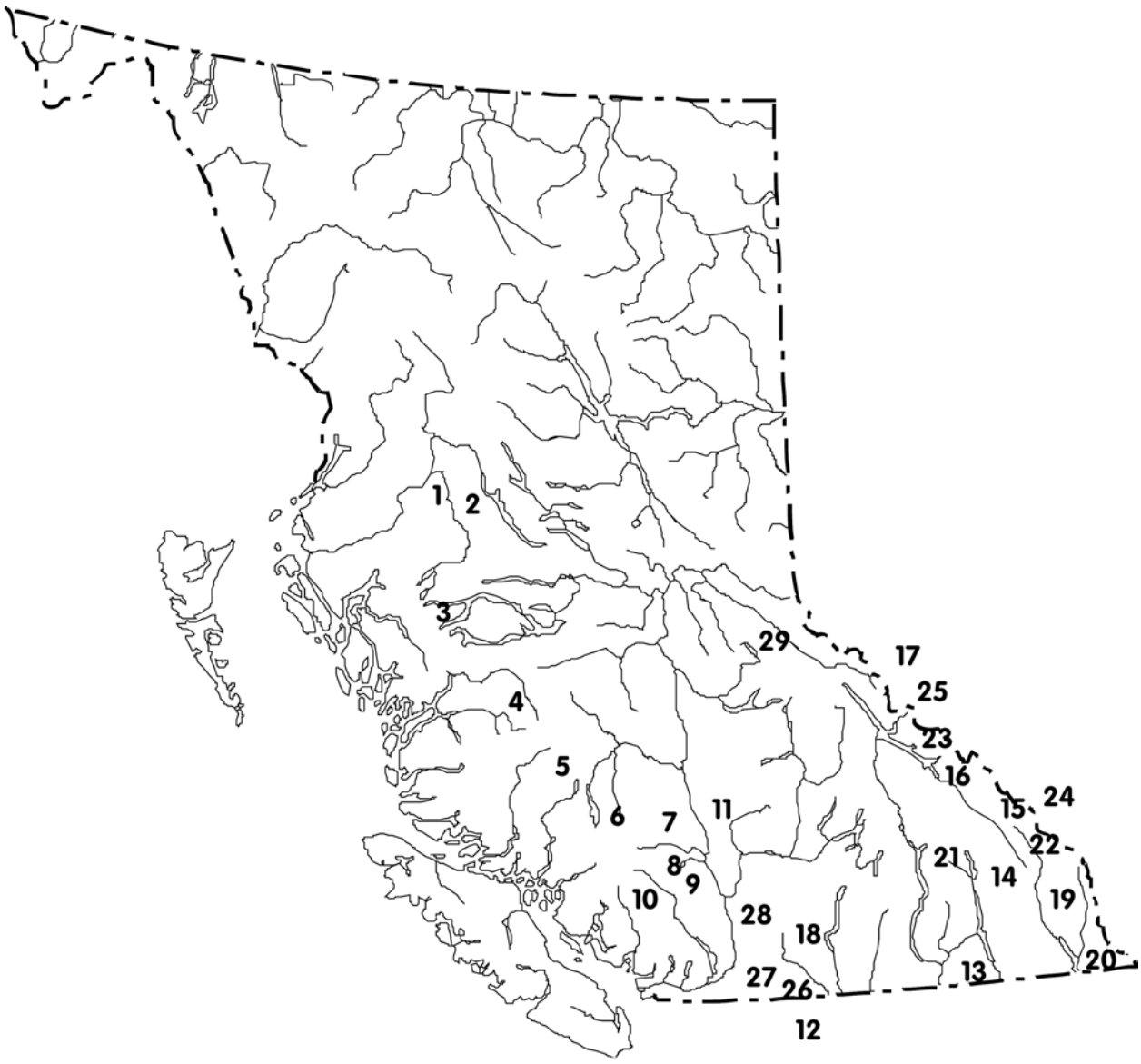

Figure A.3.1. Map of sampling locations. Population numbers as in Table A.3.1.

APPENDIX IV - Zymograms

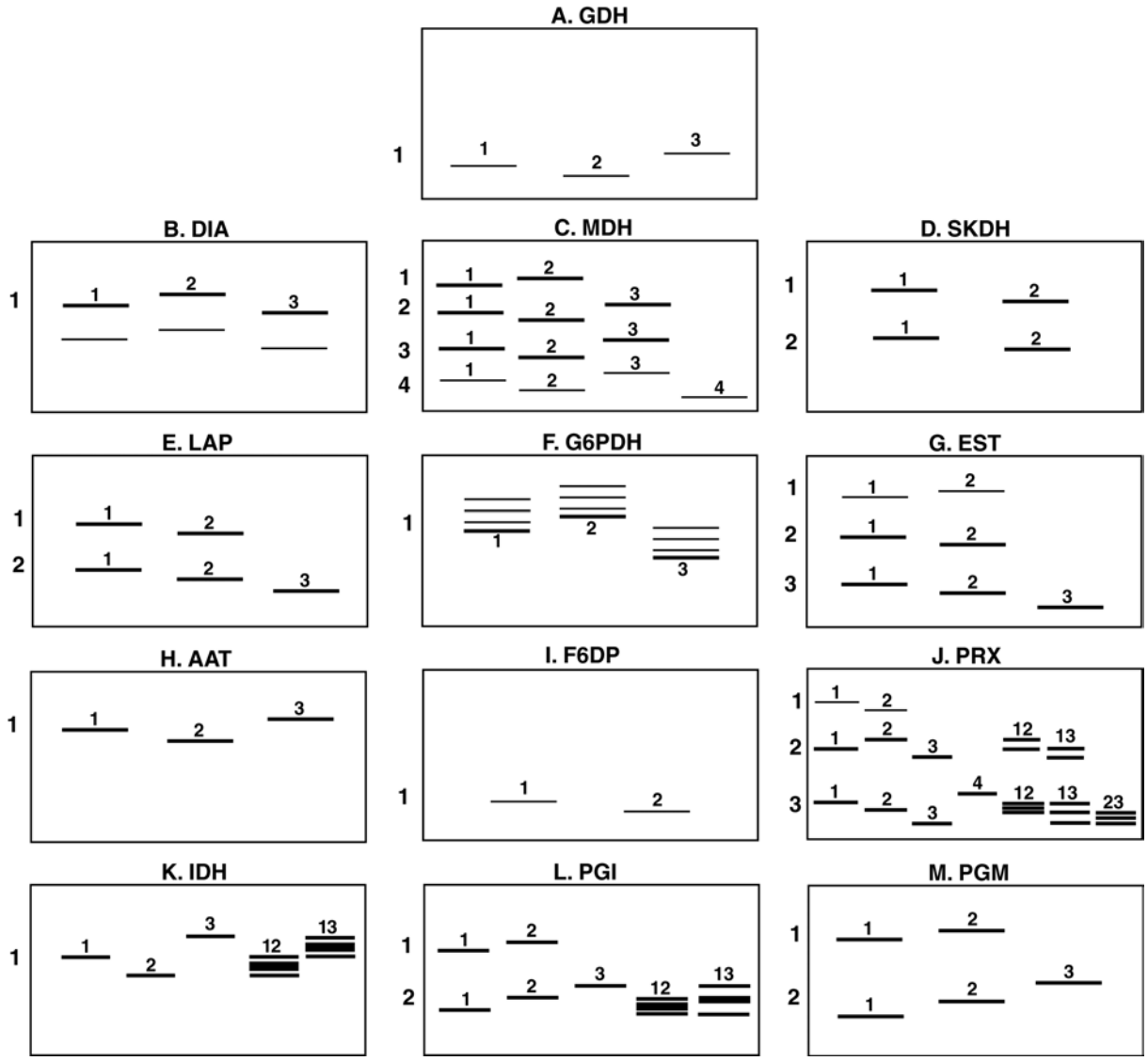

Figure A.4.1. Zymograms.

Zymograms of alleles detected and scored from bud tissue using isozyme analysis. Numbers to the left refer to locus number; numbers atop each banding pattern refer to alleles, or where there are multiple numbers, patterns representing putative combinations of alleles in diploid bud tissue.

Not all enzymes or loci depicted here were used in this study due to inconsistent staining, but are included to aid in interpretation in future studies. All loci which appeared with some consistency are depicted on the zymogram to facilitate interpretation by other researchers; the thickness of the line indicates strength of banding across multiple runs. *Sod*, which had a negative staining pattern, requiring

ultraviolet exposure to score, was not depicted due to the difficulty in achieving consistency in staining and scoring.

APPENDIX V - Tables of genetic diversity and Wright's *F*-statistics supplemental to the text

Statistics for Manning and Baldy are presented separately as they were assayed using seeds, and used different buffers, a slightly different set of loci, had a sample size up to 30 times that of the other populations.

Table A.5.1. Summary of genetic parameters by locus; standard errors of the mean in parentheses. M = Manning, B = Baldy, C = the two populations combined, A = alleles per locus (no criterion), H_e = expected heterozygosity, H_o = observed heterozygosity.

Locus	A			H_e			H_o		
	M	B	C	M	B	C	M	B	C
Pgi1	2	2	2	0.365	0.255	0.312	0.400	0.233	0.345
Pgi2	2	2	2	0.180	0.278	0.238	0.200	0.333	0.273
Idh	3	3	3	0.077	0.255	0.182	0.080	0.233	0.164
Pgm	3	3	3	0.077	0.064	0.071	0.000	0.067	0.036
6Pg1	3	3	3	0.320	0.326	0.326	0.240	0.367	0.309
6Pg2	3	2	3	0.077	0.000	0.036	0.080	0.000	0.036
Mdh1	2	2	2	0.000	0.000	0.000	0.000	0.000	0.000
Mdh2	3	3	3	0.420	0.486	0.467	0.280	0.500	0.400
Mdh3	2	2	2	0.039	0.033	0.036	0.040	0.033	0.036
Mdh4	3	3	3	0.449	0.444	0.451	0.520	0.600	0.564
Mean	2.6 (0.16)	2.5 (0.17)	2.6 (0.16)	0.204 (0.055)	0.218 (0.058)	0.212 (0.055)	0.184 (0.056)	0.243 (0.068)	0.216 (0.060)

Table A.5.2. Summary of Wright's *F*-statistics; standard errors of the mean in parentheses. F = Wright's fixation index, F_{IS} = the reduction in H_e of inbred individuals within subpopulations, F_{IT} = the reduction of H_e of inbred individuals over all populations, F_{ST} = the degree of population subdivision.

Locus	F			F_{IS}	F_{IT}	F_{ST}
	M	B	C	C	C	C
Pgi1	-0.096	-0.176	-0.136	-0.115	0.010	-0.104
Pgi2	-0.111	-0.200	-0.156	-0.151	0.003	-0.147
Idh	-0.042	-0.085	-0.064	0.078	0.046	0.121
Pgm	1.000	-0.034	0.483	0.495	-0.028	0.481
6Pg1	0.250	-0.124	0.063	0.063	-0.019	0.045
6Pg2	-0.042	-	-0.042	-0.023	0.026	0.004
Mdh1	-	-	-	0.000	0.000	0.000
Mdh2	0.333	-0.029	0.152	0.141	0.008	0.148
Mdh3	-0.020	-0.017	-0.019	-0.000	-0.018	-0.019
Mdh4	-0.159	-0.350	-0.255	-0.245	-0.014	-0.263
Mean	0.124 (0.117)	-0.127 (0.040)	0.003 (0.072)	-0.025 (0.025)	0.008 (0.002)	-0.024 (0.027)

Table A.5.3. Genetic diversity statistics for the other 17 populations combined by locus; standard errors of the mean in parentheses. For H_u, corrections for small sample size were included where applicable following Levene (1949) which is equivalent to Nei's (1978) unbiased estimate of H_e.

Locus	Sample size	H_o	H_u	F
Mdh1	804	0.012	0.027	0.541
Mdh2	868	0.355	0.390	0.090
Mdh3	900	0.022	0.035	0.368
Pgm	594	0.599	0.481	-0.246
Skd1	872	0.115	0.387	0.703
Skd2	870	0.481	0.478	-0.005
Fdp	702	0.000	0.039	1.000
Gdh	812	0.106	0.146	0.274
Lap1	746	0.011	0.179	0.940
Lap2	688	0.102	0.416	0.755
Idh	790	0.127	0.237	0.466
Pgi2	652	0.730	0.581	-0.258
Mean	774 (28.4)	0.221 (0.073)	0.283 (0.057)	0.386 (0.124)