CLONALITY, FINE SCALE GENETIC DIVERSITY AND GENETIC STRUCTURE IN NATURAL POPULATIONS OF *Chamaecyparis nootkatensis* REVEALED BY MICROSATELLITE MARKERS

by

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ABSTRACT

Layering, a form of clonal propagation, has been documented to occur in Chamaecyperis nootkatensis (D. Don) Spach (Cupressaceae), but little is known about the frequency and spatial extent of this phenomenon. Clonal reproduction has the potential to greatly affect population genetics parameters such as fine scale genetic variation and genetic structure, and, ultimately, how populations evolve. C. nootkatensis is an economically important coniferous tree species in the forests of the Pacific Northwest. Five microsatellite loci were developed from an enriched genomic library of C. nootkatensis. The developed microsatellite loci showed significant inbreeding in the three natural populations of C. nootkatensis surveyed. A segregation analysis and a test for deviations from Hardy Weinberg equilibrium revealed that one of the five loci was affected by null alleles in high frequency. Interspecific amplifications showed support for a close relationship of C. nootkatensis with members of the genera Cupressus and Juniperus. These microsatellite markers were utilized to infer the frequency and spatial extent of clonally derived individuals in three natural populations of C. nootkatensis and investigate the potential effects of clonal reproduction on fine scale genetic diversity and genetic structure in this species. Clonal extent was found to vary greatly between populations and no clear relationship between clonal extent and specific habitats was detected. Clonal diversity showed that on average ~23% of the trees sampled were clonally derived. Fine scale genetic structure, as detected by genetic spatial autocorrelation, and the coefficient of inbreeding increased with clonal extent. The greatest effect of clonality on fine scale genetic structure was detected between trees separated by approximately five meters or less (the average size of clonal patches). Surprisingly, no relationship was observed between clonal extent and genetic variation. Clonality may play a crucial role by propagating this species, when the effect of purging genetic load in the inbred C. nootkatensis results in extremely low sexual recruitment. Clonality may also be conserving very fit gene combinations from recombination.

Key Words: *Chamaecyparis nootkatensis*, clonality, Cupressaceae, genetic diversity, genetic structure, microsatellite, population genetics, simple sequence repeat (SSR), yellow cedar.

	Page
ABSTRACT	ii
TABLE OF CONTENTS	iii
LIST OF TABLES	v
LIST OF FIGURES	vi
ACKNOWLEDGEMENTS	viii
CHAPTER 1: General Introduction and Literature Review	1
1.1 Plant clonality	1
1.1.1 Introduction	1
1.1.2 Important terms in understanding clonal plants	2
1.1.3 Common patterns of clonal growth in plants	4
1.1.4 Physiological integration and potential evolutionary consequences of clonality.	5
1.1.5 Effective population size in clonal plant populations	7
1.1.6 Genetic diversity, and genetic structure in clonal plants	8
1.1.7 Clonal plants and the accumulation of somatic mutations	11
1.1.8 Clonal assignment	12
1.2 Chamaecyparis nootkatensis	16
1.2.1 Geographic range and ecology of Chamaecyparis nootkatensis	16
1.2.2 Flowering, fruiting, and seed germination in Chamaecyparis nootkatensis	17
1.2.3 Gene flow – pollen and seed dispersal in <i>Chamaecyparis nootkatensis</i>	18
1.2.4 Genetics of Chamaecyparis nootkatensis	19
1.2.5 Human use of Chamaecyparis nootkatensis	20
1.3 Clonality in <i>Chamaecyparis nootkatensis</i> and other tree species	21
1.4 Thesis structure and research hypotheses	23
CHAPTER 2: Isolation, characterization, and cross-species utility of microsatellites in	
Chamaecyparis nootkatensis	25
2.1 Introduction and rationale	25
2.2 Specific objectives	26
2.3 Materials and methods	26
2.3.1 Construction of enriched genomic library	26
2.3.2 Nucleotide sequencing and microsatellite primer design	27
2.3.3 Plant material	27
2.3.4 PCR amplifications	28
2.3.5 Analyses	28
2.4 Results	29
2.4.1 Microsatellite loci recovery and levels of polymorphism	29
2.4.2 Inbreeding	30
2.4.3 Segregation analysis	30
2.4.4 Interspecific amplifications	31
2.5 Discussion	31
2.5.1 Low rates of recovery for microsatellite loci	31
2.5.2 Polymorphism, inbreeding, and detection of clonality	34
2.5.3 Segregation patterns and null alleles	35
2.0.4 interspecine amplifications and taxonomic considerations	00
	30

TABLE OF CONTENTS

,

•

-

CHAPTER 3: Clonal Assignment, Clone Size and Spatial Pattern in <i>Chamaecyparis</i>			
2.1 Introduction and rationala			
3.1 Introduction and rationale			
3.2 Specific objectives			
3.3 Materials and methods			
3.3.1 Collection sites			
3.3.2 Selection and mapping of trees			
3.3.3 DNA isolation and microsatellite genotyping			
3.3.4 Clonal assignment			
3.3.5 Accuracy of microsatellites in clonal assignment	50		
3.4 Results			
3.4.1 Clonal assignment	51		
3.4.2 Accuracy of developed microsatellite markers in clonal assignment	52		
3.4.3 Clonal size and spatial pattern of clonal growth	52		
3.5 Discussion	53		
3.5.1 Clonal assignment and accuracy	53		
3.5.2 Clone size and spatial pattern of clonal growth	56		
3.5.4 Site history and its effect on patterns of clonality	57		
3.6 Conclusion	58		
CHAPTER 4: Clonal Diversity, Fine Scale Genetic Diversity, and Genetic Structure in Clonal Chamaecyparis pootkatensis Populations	67		
4.1 Introduction and rationale	67		
4.2 Specific objectives			
4.3 Materials and methods	68		
4.3.1 Collection mapping genotyping and clonal assignment	68		
4.3.2 Extent of clonality			
4.3.3 Clonal diversity			
4.3.4 Genetic variation	71		
4.3.5 Genetic structure			
4.4 Clopal avtant			
4.4.1 Cional extern			
4.4.4 Genetic structure			
4.5 Discussion			
4.5.3 Clonal extent			
4.5.2 Clonal diversity			
4.5.3 Genetic variation			
4.5.4 Genetic structure			
4.5.4.1 Large-scale genetic structure	83		
4.5.4.2 Genetic spatial autocorrelation excluding clonally derived individuals	83		
4.5.4.3 Clonal reproduction and its effect on fine scale genetic spatial			
autocorrelation	87		
4.6 Conclusion	89		
CHAPTER 5: General Discussion	103		
5.1 Evolutionary perspective on clonality in Chamaecyparis nootkatensis	103		
5.2 Future research needs and conclusion	105		
LITERATURE CITED	108		

LIST OF TABLES

		Page
Table 2.1.	Characteristics of five microsatellite loci in Chamaecyparis nootkatensis	. 39
Table 2.2.	Microsatellite allele number, size range, observed heterozygosity, expected heterozygosity, estimated inbreeding coefficient, and associated estimates of standard error for five loci in 132 individuals in three populations of <i>Chamaecyparis nootkatensis</i> .	. 40
Table 2.3.	Segregation analysis for five microsatellite loci in the 40 offspring (from four families) of <i>Chamaecyparis nootkatensis</i>	. 41
Table 2.4.	Microsatellite allele size range, in bp, and number of alleles (in parentheses) of two individuals in 18 species in the family Cupressaceae sensu lato for five microsatellite markers developed in <i>Chamaecyparis nootkatensis</i>	. 42
Table 3.1.	Description of collection sites including elevation, number of trees sampled per plot, estimated surface area sampled per plot, site description and associated species.	60
Table 3.2.	Number of possible (N_g) and observed multilocus microsatellite genotypes at each plot.	. 61
Table 3.3.	Probabilities of first (p_{gen}) and second (p_{se}) encounters of clonal multilocus microsatellite genotypes for which clonal growth was observed, in all plots	62
Table 4.1.	Clonal extent in <i>Chamaecyparis nootkatensis</i> at three different sites with statistics relating to both ramets and clonal genets	90
Table 4.2.	Clonal diversity in Chamaecyparis nootkatensis at three different sites	. 90
Table 4.2.	Clonal diversity in Chamaecyparis nootkatensis at three different sites	. 91
Table 4.3.	Genetic variation at four microsatellite loci in three populations of <i>Chamaecyparis nootkatensis</i> as defined by the number of alleles per locus, the observed heterozygosity, H_o , the expected heterozygosity, H_e , and the estimated inbreeding coefficient, <i>F</i>	92
Table 4.4.	Genetic structure among sites in <i>Chamaecyparis nootkatensis</i> as estimated by Wright's <i>F</i> statistics	93

LIST OF FIGURES

	. F	' age
Figure 1.1.	Geographic range of Chamaecyparis nootkatensis	24
Figure 2.1.	Allelic variation at five microsatellite loci (Y1E10, Y1F10, Y1G09, Y2C12, and Y2H01) in 24 individuals of <i>Chamaecyparis nootkatensis</i>	43
Figure 3.1.	Locations of collection sites	63
Figure 3.2.	Schematical representations of the spatial locations of <i>Chamaecyparis nootkatensis</i> trees (indicated by dots) within the plots where they were collected.	64
Figure 3.3.	Correlograms at three sites showing kinship coefficients, $\hat{ ho}_{ij}$, inclusive of the	
	genotypes of all ramets sampled and 95% confidence interval around the null hypothesis of no genetic spatial autocorrelation as a function of given distance classes	65
Figure 3.4.	Effect of different numbers and combinations of the microsatellites utilized in this study in detecting clonal structures in <i>Chamaecyparis nootkatensis</i>	66
Figure 4.1.	Clonal extent in <i>Chamaecyparis nootkatensis</i> as measured by A) the number of clonal genets and B) the number of clonal genets per 100 m ² detected in three different sites.	94
Figure 4.2.	Clonal extent in <i>Chamaecyparis nootkatensis</i> as measured by A) the estimated size of clonal genets (m ²), B) the number of ramets per clonal genet, and C) the density of ramets per clonal genets (ramets/m ²).	95
Figure 4.3.	Clonal diversity of <i>Chamaecyparis nootkatensis</i> at three different sites as measured by A) the "proportion distinguishable", <i>G/N</i> , and B) Fager's measure of evenness, <i>E</i>	96
Figure 4.4.	Allele frequencies in four microsatellite loci at the Mount Seymour site	97
Figure 4.5.	Allele frequencies in four microsatellite loci at the Black Tusk site	98
Figure 4.6.	Allele frequencies in four microsatellite loci at the Mount Washington site	99
Figure 4.7.	Correlograms at three sites showing kinship coefficients, $\hat{ ho}_{ij}$, inclusive of	
	only the genotypes of the genets (no genotype replication due to presence of clonal growth), and 95% confidence interval around the null hypothesis of no genetic spatial autocorrelation as a function of given distance classes	100
Figure 4.8.	Correlograms at three sites showing kinship coefficients, $\hat{ ho}_{ij}$, inclusive of the	
	genotypes of all ramets sampled and 95% confidence interval around the null hypothesis of no genetic spatial autocorrelation as a function of given distance classes.	101

Figure 4.9. Correlograms at three sites showing coefficients of kinship, $\hat{ ho}_{ij}$, and their
estimated standard error as a function of given distance classes

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viii

1.1 Plant clonality

1.1.1 Introduction

Clonal growth – the formation of more than one individual of identical genetic composition – is a fundamental ecological adaptation that has far-reaching consequences in population biology, morphology, development, and evolution of such organisms (Jackson et al. 1985; de Kroon and van Groenendael 1997; Fischer and van Kleunen 2002). Since Darwin, development of evolutionary theory has been implicitly constructed from unitary organisms such as fruit flies, birds, and humans. Populations of these species comprise readily distinguishable, sexually derived individuals of determinate adult body size and life span. A rapid survey across a range of taxonomic entities, however, reveals that these traits far from encompass the diversity encountered in life history traits (Jackson et al. 1985). Theorists have largely ignored grasses, vines, sponges, corals, trees, and other clonal organisms, which commonly dominate much of the land and sea and do not display the characteristics described above (Sackville Hamilton et al. 1987; Schmid 1990). This is reflected in the poor understanding of the effect of asexual reproduction on the population dynamics of species which exhibit clonal growth.

Clonality can be described as "growth and propagation by self-replication, either by design or happenstance, such that different portions of genetically unique organisms may function and survive on their own" (Jackson et al. 1985). Clonal organisms are found in a great number of taxa ranging from fungi, animals, to seagrasses and higher plants. A taxonomic bias favoring the plant kingdom is found in the literature and appears to be a direct reflection of the fact that plants have more fully exploited this characteristic mode of reproduction than members of other kingdoms and has therefore been better documented. In fact, Klimeš et al. (1997), in a taxonomical survey of plant form and function, reported that more than 70% of plant species exhibit clonal growth.

Clonality is facultative in most plant species and clonal reproduction interspersed with spurious periods of sexual recruitment is commonly observed. Many plant species use sexual and asexual means of reproduction within the same spatio-temporal framework, leading to an often-complex mosaic of individuals both sexually and asexually derived, and this, within different age classes. A few ecological studies have attempted to understand the environmental and ecological parameters responsible for triggering the switch from a sexual to a clonal mode of reproduction (Eriksson and Fröborg 1996; Peck et al. 1998; Robson et al. 1999; Kettle et al. 2000). Nothing is known about genetic controls behind this choice in reproductive behavior (Eriksson 1993) but evidence of a genetic basis for allocation to both modes of reproduction has been showed (e.g. van Kleunen and Fischer 2001). The evolution of sex has been much debated among evolutionary theoreticians and a great amount of research relating to this topic has been produced (see Michod and Levin 1988; Charlesworth 1989, Redfield 1999). Because clonality has been identified as an ancestral trait (Tiffney and Niklas 1986), the study of clonal reproduction in various taxa with its associated fitness costs and benefits, could clearly offer new perspectives into the evolution of sex.

1.1.2 Important terms in understanding clonal plants

Before trying to understand the population dynamics of clonal organisms and the effect of clonal reproduction on the population genetics of these organisms, one must first delineate clones within a population, hence documenting the spatial architecture (shape) and extent (size and frequency) of clonality. The vast majority of studies to date concerned with clonality have primarily sought to document these characteristics.

First, a few important terms used frequently in clonal plant literature must be defined. Nomenclature found in studies of clonal organisms is often complicated by a lack of consistency between studies as well as the occurrence of a number of terms sharing the same or similar meaning. In an effort to simplify this thesis, I here present and define the terms relating to clonality that will be used throughout the text. My definitions will reflect those commonly used in

most clonal plant studies but may well differ from those used by some authors. They generally follow the definitions given by de Kroon and van Groenendael (1997).

-A *genet* is a single, distinguishable, genetic individual which can be constituted of one or many ramets, stems, or structures.

-A *ramet* is a single physical entity or unit capable of independent growth and reproduction and part of a group of genetically identical entities known collectively as the genet.

-Often, the term *clone* is used and will be used interchangeably with the term genet. An example of this would be a population of sedges where many stems would share the same genotype. These stems would be referred to as multiple ramets of a single genet or clone.

-*Clonality* is defined as the presence of genets containing multiple ramets as a result of asexual reproduction or clonal growth. If all genets within a population were to be present in the form of only one ramet per genet, then no clonality would be observed. -Following this reasoning, a *clonal genet* is defined here as a genet constituted of two or more ramets as opposed to an aclonal genet which would be constituted of a single ramet.

-*Clonal diversity* can be defined as the number of observed genotypes and the fashion in which ramets are distributed within these genotypes. This is, therefore, the result of the joint effect of genetic diversity detected and the amount of clonal growth in a population. If a population demonstrates high clonal diversity, then most ramets have different genotypes and genetic diversity is likely high. Low clonal diversity can be the result of either large clones comprising most of the ramets found in a population or of very low genetic diversity preventing clear clonal assignment.

-The amount of clonal growth or reproduction is termed here *clonal extent*. This is defined here as the number of ramets in clonal genets or more generally as the number of clonal genets detected per unit of surface area. This term is meant as a means to

estimate how important clonal growth is at a given site. Clonal extent can also be examined in the light of space occupied by clonally derived individuals. -Finally, *clonal structure*, or *clonal architecture* describes the spatial arrangement of each ramet within a genet or between genets (e.g. clumped vs. random distribution of ramets) and *clone size* describes the number of ramets within a genet and the approximate surface area occupied by these.

1.1.3 Common patterns of clonal growth in plants

The patterns of growth of modular organisms can be considered as ways in which space is captured. Space corresponds in a broad sense to resources; for example, a space in a tree canopy is a source of light for a photosynthetic organ such as a leaf. The vegetative units of modular organisms represent resource gathering centers; the ways in which they are arranged or packed can determine the efficiency with which resources are garnered by the genet. This is only true, however, of a genet where all constitutive ramets are physically connected and sharing resources. Two characteristic types of clonal growth are often referred to in plants: the phalanx and guerilla types (Lovett Doust 1981). These refer to the spatial arrangement of ramets within a genet. The phalanx growth type can be described as a close cluster of ramets around a mother ramet. Typical asexual modes of reproduction giving rise to this type of structure are layering, short rhizomes (ceaspitose plants), and root sprouting. Such organization derives from the combination of short internodes and frequent branching. Irises (Iris fulva and I. brevicaulis, Burke and Bulger 2000), birches (Betula glandulosa, Hermanutz et al. 1989), alders (Alnus incana, Huenneke 1985), and cacti (Lophocereus schottii, Parker and Hamrick 1992), and many other clonal plant species fall in this category. Many corals, bryozoans, and clonal ascidians also form phalanxes (Coates and Jackson 1985). The contrasting guerilla growth forms are represented by plants like the strawberry (*Fragaria* spp.). creeping buttercup (Ranunculus reptans, Fischer et al. 2000), and sedges (e.g. Carex bigelowii, Jonsson et al. 1996) in which internodes of rhizomes or stolons are long and branching is

infrequent (Harper 1985, Hutchings and Slade 1988). The guerilla growth form produces genets that are spatially spread out and often interdigitating. It must be pointed out that these two types of clonal growth represent extremes of the spectrum of clonal growth patterns and that clonal plants usually fall somewhere between these two extremes.

Apomixis is also a form of asexual reproduction that could be classified as clonal. It is defined as reproduction from gametes, but without fertilization and reportedly occurs especially among lower plants and invertebrate animals. The term parthenogenesis, a common process in apomixis, is usually applied to animals while in plants the terms parthenocarpy and agamospermy are more typical. Apomixis is described as the naturally occurring ability of some plant species to reproduce asexually through seeds. This mode of reproduction is found in many angiosperm families but, interestingly, a case was recently discovered in a gymnosperm species. *Cupressus dupreziana* was demonstrated by Pichot et al. (2001) to have pollen grains which are diploid and develop into an embryo when they land on either the female cones of their own species (uncommon since it is a rare and endangered species) or those of a much more common species of cypress, *C. sempervirens*. In the case of apomixis, mechanisms responsible for seed dispersal will directly affect clonal structure.

1.1.4 Physiological integration and potential evolutionary consequences of clonality

Clonal plants of either phalanx or guerilla type have the ability to spread shoots out, extend and explore the landscape in which they live. To some extent, this behavior can be compared to that of mobility in animals which, if you extend the analogy, provides a mean of foraging (Bell 1984). Obviously, this movement is much slower in plants to our human eyes but when compared in terms of lifespan, clonal plants can move at incredible speed and distance. For instance some clonal poplar species have been documented to have genotypic individuals a few thousand years old that extend for hundreds of kilometers (Kemperman and Barnes 1976). An important difference, however, remains between clonal plants and mobile animals: the physical connection that exists between ramets of the same genet in clonal plants. This

physical connection (roots, stolons, or rhizomes) provides a chance for what has been termed, "physiological integration" (Pitelka and Ashmun 1985). This integration allows individuals to share resources. For example, a ramet located in a shady yet moist microsite can share water with other ramets in dry and sunny locations in exchange for photosynthates. Thus clonal plants may dramatically increase their potential for capturing resources.

Much of the research regarding physiological integration has ignored the evolutionary consequences of this process. Watson (1984) argued that meristem demography would strongly influence the persistence and proliferation of ramets and genets in natural populations. Accordingly, this author urged researchers to "move away from the notion that sexual reproduction is the primary means by which clonal plants achieve fitness" and embrace the view that "ramet persistence and the success of clones at generating new ramets need to be considered as equally important elements in estimations of clonal fitness".

This can be further extended to the idea that the rate of adaptation could potentially be slowed in clonal plants. Adaptation occurs because of selection favoring or "weeding out" phenotypes, and consequently genotypes, owing to their interaction with the environment. By replicating their genotypes and "sending" them in various environments (microhabitats) clonal plants slow down the rate of selection directly affecting genotype frequencies. Genotypes therefore have the potential to persist much longer in clonal plants than in their sexually reproductive counterparts.

This is true also of given gene combinations; clonal reproduction may represent a means for extremely fit gene combinations to persist through time, and escape the shearing effect of recombination. Furthermore, physiological integration adds the extra dimension of resource sharing which can act as a buffer against the effect of local adaptation in clonal plants with large clonal structures. The obvious limitations behind this process are ramet longevity, ramet generation time, and longevity of physical connections between ramets. Finally, this process and its consequences have been suggested to only occur in patchy heterogeneous habitats (Price and Hutchings 1992; Kemball and Marshall 1995).

1.1.5 Effective population size in clonal plant populations

Clonal plants, because of their replication ability may often have fewer genotypes per area than an aclonal counterpart. This can potentially result in a decrease in effective population size (Orive 1993); however, the replication nature of clonal plant, because it increases generation time, may also act as a buffer against negative effects (stochastic and genetic) on the dynamics of populations of small effective size. In addition, repeated consanguineous mating and/or geitonogamy (i.e. crosses between flowers of the same genet) could be favored in clonal plants (reviewed by Charpentier 2002) and if no self-incompatibility mechanisms are involved, this could result in decreased genetic diversity (Loveless and Hamrick 1984) and inbreeding depression (Muirhead and Lande 1997).

Populations of small effective size are under the threat of stochastic environmental and genetic events (Hartl and Clark 1997). Under environmental stress or catastrophic events, these populations have higher probability of becoming extinct because they lack the diversity or genetic potential necessary for adaptation. Furthermore, random genetic drift has been well documented to precipitate populations of small effective size into what has been termed an extinction vortex (Saccheri et al. 1998).

Conversely, in the case of clonal plant species, the replication of genotypes may act in a buffering fashion against stochastic events. Selection, the driving force behind adaptation, acts on phenotypes and not simply on genotypes. These phenotypes are the result of the interaction between genotypes and the environment in which they strive (Falconer and Mackay 1996). Clonal plants have the capacity to produce multiple individuals of the same genotype that will explore and interact with various environments, producing diverse phenotypes, thus material for selection and adaptation to operate. Clonal variation under differing environments is widely known and accepted (Falconer and Mackay 1996). The buffering effect of replicated genotypes may also act against random genetic drift. The actual mechanisms are poorly understood and

the proposed ones are based mostly on genetic surveys of small clonal plant populations (Carter and Robinson 1993; Kettle et al. 2000; Warburton et al. 2000).

Since clonality reduces the number of genotypes in populations then, in the absence of self-incompatibility mechanisms, this may increase the probability of geitonogamy (Handel 1985; Charpentier 2002). This situation could lead to an increase in inbreeding and subsequent loss of genetic diversity (Soane and Watkinson 1979). Traditionally, researchers had believed populations of clonal organisms to be genetically and/or genotypically depauperate (see review by Jackson et al. 1985). The expression of inbreeding depression through the death of many individuals would finally result in the reduction in population size, bringing the population closer to a possible extinction vortex. Clonal plant populations, however, have been documented to have high levels of genetic diversity, a sign that inbreeding is reduced or even prevented (Loveless and Hamrick 1984; Handel 1985; Jackson et al. 1985; Ellstrand and Roose 1987; Eriksson 1993, Eckert 1999). Avoidance of inbreeding through the evolution of selfincompatibility systems seems to have been an important adaptation for the survival of small clonal plant populations. Growth patterns in clonal plants (phalanx vs. guerrilla) also affect mating patterns and thus effective population size. The spatial intermingling of various genotypes in guerrilla-type clonal plants increases the genetic neighborhood and, consequently, the effective population size. Conversely, phalanx-type clonal plants have reduced diversity of genotypes in the neighborhood of each individual (especially when the distance pollen can travel is limited) and may be affected by smaller effective population size.

1.1.6 Genetic diversity, and genetic structure in clonal plants

Clonality creates situations where multiple organisms with identical genotypes strive in close proximity. The laws of probability would predict an increase in chance of mating between ramets of the same genet. This situation would rapidly create inbred populations with depauperate genetic diversity. This theory predicts that all clonally reproducing organisms should have low amounts of genetic diversity when compared to their non-clonal counterparts

(Jackson et al. 1985; Eckert 1999). Surprisingly however, as mentioned above, most studies have shown exactly the opposite trend. Clonal plants (and other clonal organisms) show equal or sometimes higher levels of genetic diversity than sexually reproducing plants (Ellstrand and Roose 1987; Eriksson 1993; Eckert 1999). Caution must be taken in interpreting these trends, however, because these may partly be a consequence of sampling design.

For most plant species, sporadic periods of sexual recruitment have been hypothesized to be sufficient in maintaining, and even increasing, levels of genetic diversity (Soane and Watkinson 1979). Eriksson (1993) developed models exploring the evolution of seed dispersal and clonal and sexual recruitment with the assumption that there is a trade-off between sexual reproduction and clonal growth. The cost for this relationship is often complicated because the expected genetic trade-off between sexual reproduction and growth may be obscured by positive phenotypic correlations where perhaps vigorous individuals do everything well (Roff 1992). Field experiments confirming these findings are few and often confounded by age structure and nebulous identification of sexually derived versus clonally derived individuals (Eriksson and Fröborg 1996). Further explanations for the observed levels of genetic diversity include frequency-dependent selection involving pathogens, somatic mutations (see next section), and gene flow. None of these hypothesized mechanisms, however, have been conclusively proven (Eckert 1999).

Efforts to understand how clonal growth affects genetic diversity have focused largely on average levels of diversity and ignored the repeated observation that clonal species exhibit particularly wide variation in genetic structure among populations (Widén et al. 1994). The ecological and evolutionary causes and consequences of this variation are still unknown (Eckert 1999). In many species, the relative importance of clonal vs. sexual recruitment may be influenced strongly by environmental factors. As a result, populations at the periphery of a species' geographical range may contain relatively little genotypic diversity, thereby limiting the potential for evolutionary expansion of the species' range. Much work remains in order to understand the mechanisms allowing high genetic diversity to persist in clonal plant populations.

Genetic structure can be defined as the spatial distribution of alleles or genotypes. It results from the action of mutation, migration, selection, and genetic drift, which in turn, must function within the historical and biological context of the species (Loveless and Hamrick 1984). A random distribution of genotypes would indicate a lack of genetic structure. Therefore, clonality can clearly become a driving force behind genetic structure in plant populations. The production of multiple individuals of identical genotypes, especially within close proximity of each other, could theoretically increase genetic structure. Furthermore, if these individuals are allowed to mate with each other, a population can quickly become spatially structured (e.g. Berg and Hamrick 1994; Shapcott 1995; Hossaert-McKey et al. 1996; Chung and Epperson 1999). Surprisingly, only a handful of studies (most of them recent) have documented the influence of clonal reproduction on genetic structure in plant populations (e.g. Chung and Epperson 1999; Reusch et al. 1999, 2000).

A relatively new analytical tool capable of detecting fine scale genetic structure known as genetic spatial autocorrelation has fanned this interest (Epperson 1993, 1995). Reusch et al. (1999), in a study of a perennial population of eelgrass, *Zostera marina*, showed a strong influence of the presence of clonally derived individuals on fine scale genetic structure. Upon deletion of these replicated genotypes (due to clonal growth) from the analysis, however, no significant spatial autocorrelation was detected; a surprising finding because gene flow was believed to be extremely limited in this species. The investigation of spatial genetic structure of clonal and sexual reproduction in populations of *Adenophora grandiflora*, a herbaceous perennial, revealed exactly the opposite trends: spatial autocorrelation of genotypes was found in this species, showing significant clustering within distances less than 4 m, but the exclusion of clonally derived individuals showed no significant effect on the results (Chung and Epperson 1999).

Few studies to date have investigated spatial autocorrelation in tree species. Montalvo et al. (1997) showed an increase in genetic spatial autocorrelation due to the presence of clonally derived individuals in their analysis on the oak species, *Quercus chrysolepis*, but the

statistical significance of this increase was not tested. Chung et al. (2000) showed that in the tree species *Rhus javanica*, the clonal growth pattern was clearly of the "guerilla-type" strategy due to the random physical distribution of ramets belonging to the same genets. Because of the long distances between trees of identical genotypes, no significant spatial autocorrelation was attributed to clonal growth in this species. Finally, in the Asian tree named Makino, *Eurya emarginata*, Chung and Epperson (2000) found that clonal reproduction did not contribute substantially to genetic isolation by distance. By contrast, limits to seed and pollen dispersal created considerable levels of genetic structure. The influence of clonal reproduction on the process of isolation by distance may offer new insights into the role of this growth pattern in the evolutionary diversification of some plant groups.

1.1.7 Clonal plants and the accumulation of somatic mutations

Somatic mutations arise in all individuals but are of little significance in organisms where sexual cells are sequestered (e.g. humans). In clonal plants, the situation is quite different because any somatic mutation arising in meristematic tissue has the potential to then be "inherited" in all subsequent ramets of the same genet. Stochastic and spatially explicit models developed by Pineda-Krch and Lehtila (2002) provide strong evidence for the purging of deleterious mutations in stratified shoot apical meristems. The findings of these authors also demonstrate retention of chimeric states for advantageous mutations. Clonal growth may hence influence the long term-evolution of life history by affecting the accumulation theory of clonality". This author argues that, in the absence of sexual recombination, genetic diversity in clonal populations is generated through somatic mutation. As clones age and spread they are likely to accumulate deleterious mutations; a process similar to the mutational meltdown in asexual populations envisioned by Lynch et al. (1993). As mentioned above, evidence for the purging of deleterious mutations exists (Pineda-Krch and Lehtila 2002) but only for stratified shoot apical meristematic organization has been reported in all

angiosperms but only a in a few gymnosperm families (Klekowski 1988). If deleterious mutations are not purged, mutation-selection balance in highly clonal populations is expected to allow the build-up of mutational load that specifically impairs sexual reproduction, because the processes involved in sex contribute little to the fitness of genets. Eckert (1999) suggested that this hypothesis might explain the many observations of infrequent sexual reproduction in highly clonal populations. The somatic mutation theory of clonality has, however, received little empirical investigation (Eckert et al. 1999).

1.1.8 Clonal assignment

In order to study clonality and its potential effects as described above, individuals within populations must be assigned to their respective genets. The classical approach used in investigating clonal architecture and clonal extent in plant species has been to excavate extensive areas to identify connecting structures such as roots, rhizomes or stolons (e.g. Reinartz and Popp 1987; Maddox et al. 1989; Gom and Rood 1999). This procedure allows direct assessment of clonal assignment of ramets to given genets and confirms the physical attachment, and thus potential physiological integration (to be discussed later), of the ramets under observation. Excavation is often time consuming and confusing, due to the profusion of roots of the studied species as well as other species, and can actually lead to an underestimation of clonal extent. Factors such as breakage of underground structures during the unearthing process as well as natural decomposition can also contribute to an underestimation of clone size. In addition, natural grafts of roots from different genotypes may be misleading. In the past twenty years, genetic markers used by population geneticists and molecular ecologists have been applied to resolve clonal identification. The sampling process is often reduced to collecting fresh material from live plant and mapping these using grids or coordinates. After collection, protein or DNA would be extracted and used to fingerprint the individuals. Identical genotypes are then generally assumed to represent ramets of the same

genet or some simple statistical tests can be calculated to estimate the probability of observing a given genotype more than once in a population.

Allozyme electrophoresis has been a popular method for many researchers from the 1970's until present due mostly to the wealth of knowledge on this genetic marker accumulated over the years. Allozyme electrophoresis, which assesses differences in enzyme electrophoretic mobility, has been used to assess clonal identification and diversity in such diverse plant groups as ferns (Parks and Werth 1993; van der Velde et al. 2001), dwarf birches (Hermanutz et al. 1989), columnar cacti (Parker and Hamrick 1992), poplars (Jelinski and Cheliak 1992), grasses (Carter and Robinson 1993), and many more. Allozyme assays are known to be simple, inexpensive, and reliable markers in many population genetics studies. However, the amount or type of tissue required to perform these tests can lead to the destruction of the individuals under observation, a less desirable condition when working on endangered or threatened species. This situation is less likely to occur in plants of significant size where only a few leaves or needles are sufficient. Although relatively inexpensive and simple, isozymes often have low variability, while high levels of variability in molecular markers are desirable when assessing clonal identity.

Restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), as well as amplified fragment length polymorphisms (AFLP) more recently, have all been used in assessing clonality in various plant species (e.g. *Iliamna* spp., Stewart and Porter 1995; *Eucalyptus* spp., Rossetto et al. 1999; *Rhododendron ferrugineum*, Pornon and Escaravage 1999; *Ranunculus reptans*, Fischer et al. 2000; *Asclepias meadii*, Kettle et al. 2000; *Santalum lanceolatum*, Warburton et al. 2000; *Oryza rufipogon*, Xie et al. 2001). These three techniques are DNA-based and rely on cutting with restriction enzymes or amplifying regions of genomic DNA. RFLP markers are co-dominant which provides greater information than their dominant counterparts. This technique, however, requires the use of restriction digests and, consequently, relatively large amounts of high quality DNA. RAPD and AFLP are dominant markers and this dominance results in the loss of information due to the lack of difference

between homozygous dominants and heterozygotes. The high number of loci scored, however, often compensates for the low amount of information provided by each separate locus. RAPD, and more recently, AFLP markers, seem to have been most extensively used in studies of genetic diversity and clonal structure in fungi (e.g. Vasiliauskas et al. 1998).

For the past five to ten years, simple sequence repeats (SSR) markers, or microsatellites, have been the marker of choice for population geneticists as well as conservation biologists interested in genetic diversity, genetic structure and high resolution parentage assignment. These DNA-based markers are highly polymorphic and co-dominant in nature. Because these molecular markers rely on polymerase chain reaction (PCR)-based amplification, minute amounts of DNA are sufficient for genotyping. In addition, microsatellite primers are typically very specific to given species, which greatly reduces the chances of contamination. The amount of information provided by these markers, and consequently the level of resolution in genetic patterns, is often unsurpassed by any other kinds of analyses. The past five years have seen a number of researchers using microsatellites and other PCR-based genetic markers to assess clonality (e.g. Reusch et al. 1999; van der Velde 2001; Ainsworth et al. 2003).

Few studies have compared the usefulness of the different types of markers in correctly assigning clonal identity. Simulations by Harada *et al.* (1997) confirmed what Cheliak and Pitel (1984) had previously suggested: the most desirable characteristic of a molecular marker in assessing clonal identification is its level of variability. Clonal assignment is based on identifying genotypes of all organisms and grouping identical genotypes together into a single genet. Two important factors, however, can cause confusion; 1) genetic relatedness between individuals and 2) the level of commonness of the observed genotypes. Relatedness between nearby individuals can be interpreted incorrectly as clonal growth. Methods using few markers of low variability could erroneously assign full-sibs as ramets belonging to the same genet. In addition, genotypes of high frequencies will have an effect on the level of confidence when assigning clonal identification. Gene frequencies of a population allow the calculation of the

probability of encountering a particular genotype. A common genotype will have a high probability of occurrence in the population while a genotype constituted of less common alleles will have a lower probability of being encountered. This applies directly to the level of confidence when assessing clonal identity. Hence, ramets with an identical genotype that has a high probability of occurrence could be falsely assigned to the same genet. Therefore, a greater number of loci with high variability will strongly reduce the probability of an occurrence of an otherwise common genotype, therefore increasing the confidence level when performing clonal assignment.

The threshold for the probability of genotype occurrence and the establishment of the gene frequency table are two important concerns in analyses dealing with clonal assignment. As mentioned above, the probability of occurrence of a specific genotype, calculated from gene frequency tables, sets a level of confidence in assigning clonal identity. A lack of statistical treatment of this problem, however, has resulted in the absence of a method to establish a threshold at which this probability should indicate high confidence. Most studies appear to set this threshold arbitrarily. Furthermore, very few studies present the probabilities of encounter associated with the genotypes of genets constituted of two or more ramets.

In addition, gene frequency tables are often biased because of the use of *a priori* clonal assignment. For example, if each ramet in a population was genotyped and identical genotypes were all included in the calculations of the gene frequency table this would result in skewed genotype frequencies. Genotypes of clonal individuals would then have high probability of occurrence because of the replicate nature of the clones. To counter this effect, most researchers will simply group identical genotypes into putative genets and include them as a single individual when calculating the gene table frequency. This method clearly involves circular reasoning and has been noted to be a caveat of clonal assignment (Montalvo et al. 1997), however, no alternative method has been proposed to date.

1.2 Chamaecyparis nootkatensis

Chamaecyperis nootkatensis (D. Don) Spach (Cupressaceae), also known as yellow cedar, Alaska cedar, or yellow cypress, is an economically important coniferous tree species in the province of British Columbia. Layering, a form of clonal propagation or growth has been documented to occur in this species (Antos and Zobel 1986; Harris 1990; Hennon et al.1998) but little is known about the frequency, spatial extent, and variation of these parameters across various habitats. This information may be important in understanding population dynamics in *C. nootkatensis* and the effect of clonality on fine scale genetic diversity and genetic structure in this species and other long-lived woody perennial species. Current knowledge of the ecology, genetics and clonal habits of *C. nootkatensis* are reviewed in this section.

1.2.1 Geographic range and ecology of Chamaecyparis nootkatensis

Chamaecyparis nootkatensis is native to western North America where it ranges from Prince William Sound in Alaska in the north to the Siskiyou Mountains in northern California in the south (Fig. 1.1). With the exception of a few isolated stands in the interiors of British Columbia and central Oregon, it is found primarily within 160 km of the Pacific coast. In the states of Washington and Oregon, this species is found in the Cascade Range and the Olympic Mountains. Also, scattered populations are found in the Aldrich Mountains of central Oregon. In British Columbia, and extending to its northern limits, *C. nootkatensis* grows in a narrow strip on the islands and coastal mainland. One exception in British Columbia is the isolated population found near Slocan Lake about 720 km inland. Disjunct populations found in the interior of British Columbia and in Washington and Oregon are interpreted as remnants of preglacial populations (Antos and Zobel 1986; Burns and Honkala; 1990, Alaback 1991).

Chamaecyparis nootkatensis is a resilient tree species which can grow in a variety of habitats. The evolutionary tendency of this species has been persistence through tolerance and longevity. Tolerance is exhibited by the ability of the species to grow in areas of varying levels of shade, frost, and soil moisture, although it favors and is often found thriving in cool and wet

conditions where growing seasons are short. Longevity has been possible through the allocation of carbon to defensive chemicals resulting in wood of very high economic value because of its extractives. Trees more than 1, 000 years-old have been reported to be common (Franklin and Dyrness 1973). Tolerance and longevity, however, come with the cost of slow growth and limited success in sexual reproduction. The reproductive success of *C. nootkatensis* has been limited by extremely poor germination rates and high incidence of cone abortion. These, in turn, confer poor competitive ability to this species which often forces it to inhabit sub-optimal sites where productivity is low.

This late successional species very occasionally grows in pure stands but is usually found singly or in scattered groups mixed with other tree species (Ruth and Harris 1979; Bazzaz et al. 1987; Loehle 1988). In British Columbia, *C. nootkatensis* may be found growing with Pacific silver fir (*Abies amabilis*), western white pine (*Pinus monticola*), or more commonly with western red cedar (*Thuja plicata*), mountain hemlock (*Tsuga mertensiana*), western hemlock (*Tsuga heterophylla*), and shore pine (*Pinus contorta*).

1.2.2 Flowering, fruiting, and seed germination in Chamaecyparis nootkatensis

Chamaecyparis nootkatensis is a monoecious species. Flowering occurs from April in the southern part of the range to June in the north (Owens and Molder 1975; Harris 1990). The male pollen-bearing strobili are inconspicuous and yellow or reddish, while the female cones are green. Both are found at the tips of the branchlets. Pollen becomes mature in the fall, before dormancy. This is an unusual characteristic in conifers and it was suggested to be an adaptation of *C. nootkatensis* to a short growing season because it allows the species to pollinate very quickly after dormancy without having to undergo a period of microsporogenesis and pollen development (Owens et al. 1980). Pollen cone abortion, estimated between 30-60% (Harris 1990), occurs when the mite, *Trisetacus chamaecypari*, feeds on the stalk of the pollen cone, eventually girdling the stalk and killing the cone. Mature ovules exude pollination drops at which point they are receptive for a few days but each seed cone is on average receptive for a

week. Cones generally mature in two years (Fowells 1965), but in the southern part of the range they can mature in one year (Harris 1990; El-Kassaby et al. 1991). Seeds mature and disperse in the third year. Owens et al. (1980) suggested this two-year period of seed-cone maturation following initiation might have arisen as an adaptation to the short growing seasons because of the limited time period to gather resources and allocate them to the seed cones.

Seed germination in C. nootkatensis has historically been the lowest for all British Columbia conifers, averaging 35% in nurseries (Kolotelo 1993). Seed dormancy appears to be responsible for poor seed germination. This problem has received great attention which has resulted in a three-month, combined warm and cold stratification technique to break dormancy. This research has begun to elucidate the biochemical basis for seed dormancy and successful stratification in this species (Tillman-Sutela and Kauppi 1998; Ren and Kermode 1999; Xia and Kermode 1999, 2000; Schmitz et al. 2000). Even with stratification techniques, however, the germination rate of C. nootkatensis seed orchard seeds (8%) has been shown to be much lower than wild seeds (35%) (D. Kolotelo, B.C. Ministry of Forests, Tree Seed Centre, personal communication reported in Anderson et al. (2002)). In a pollination study, Anderson et al. (2002) showed a significant decrease in seeds containing embryos in self-pollinated cones (4%) when compared to open- and cross-pollinated cones (28-33%). Inbreeding has been hypothesized to be an important factor in reduced rates of seed germination in *C. nootkatensis*. Regeneration from seeds in natural populations of this species is generally thought to be uncommon (Antos and Zobel 1984; Harris 1990; Dunsworth 1998; Hennon et al. 1998) and low fertilization success may be the main limitation to sexual recruitment in C. nootkatensis. This hypothesis remains in need of further investigation.

1.2.3 Gene flow – pollen and seed dispersal in *Chamaecyparis nootkatensis*

Little information about distances of seed and pollen dissemination is available for *C*. *nootkatensis*. Both seeds and pollen depend primarily on wind to be dispersed. Unlike some other members of the Cupressaceae, the pollen in this species lacks wings (Owens et al. 1980),

which are assumed to function as flotation mechanisms in the other species. There are of yet no estimates of distance traveled by both pollen grains and seeds. Seeds in this species, however, are heavier than seeds of the closely related Port-Orford-cedar (*C. lawsoniana*), which are reported not to disperse beyond 120m (Harris 1990). Seeds of *C. nootkatensis* therefore have been suggested to travel less (Owens and Molder 1975).

1.2.4 Genetics of Chamaecyparis nootkatensis

Chamaecyparis nootkatensis is generally thought to be a genetically diverse species throughout its geographic range, with high levels of intra-population inbreeding (ca. 30%) when compared to other conifers (Ritland et al. 2001). Genetic diversity in this species has been estimated using various techniques. Common garden studies of morphological and physiological traits have demonstrated that this species has considerable quantitative genetic diversity (Cherry and Lester 1992; Russell 1998). It was suggested that this diversity showed the potential for genetic gains through selective breeding (Russell and Cartwright 1991). Wide ecological amplitude and phenotypic plasticity observed at the population level have suggested that this species had more genetic diversity within populations than among (Russell et al. 1990, Russell 1998). In fact, based on one- to three-year old clonal trials, Russell et al. (1990), showed that only 5-10% of the genetic variability in growth characteristics could be attributable to differences among stands. By contrast, some evidence of among population differences was later demonstrated because of the correlation of some phenotypic traits with latitude and elevation (Russell 1998).

A more recent study of isozyme variation by Ritland et al. (2001) demonstrated the existence of substantial genetic divergence between populations (G_{ST} =0.139) resulting in three distinct phylogeographic groups across the range of the species. The high total gene diversity observed (H_T =0.171), the mean number of alleles per isozyme locus (A=1.68), and the percent polymorphic loci (*PPL*=0.5) confirmed the moderate to high level of genetic diversity. Comparison of the results obtained by Ritland et al. (2001) to previous studies of genetic

diversity and structure in other members of the family Cupressaceae revealed that the strong genetic structure observed in *C. nootkatens* is not uncommon in this group and was suggested to have influenced evolutionary divergence in the Cupressoideae.

1.2.5 Human use of Chamaecyparis nootkatensis

Wood from *C. nootkatensis*, especially in old-growth trees, has been noted for its excellent natural durability as well as milling and manufacturing qualities. This has made *C. nootkatensis*, with its bright yellow and aromatic wood, a commercially very valuable tree (Harris 1990). On a per-unit-volume basis, *C. nootkatensis* has been documented to be the most valuable tree grown in Alaska and British Columbia and commands the highest price (Louisier 1991). This wood is used for boat building and other marine purposes, interior molding, cabinets, furniture, fence posts, bleacher seats, saunas, musical instrument, and carving (Hennon et al. 1998). Most of the harvested wood is exported to countries along the Pacific Rim, especially Japan, where it is used in home construction and is prized for making ceremonial boxes and restoring temples and shrines (Frear 1982).

Many of the characteristics that have led *C. nootkatensis* to be evolutionarily successful have presented both benefits and problems in human use of this species. Provenance trials and operational plantations have been discouraged by the slow growth rate and poor reproductive success of this species (Barker 1991). Forest managers have voiced concern over *C. nootkatensis*' unusual lack of natural regeneration from seeds after harvesting (Hennon et al. 1998). This is even true of sites where *C. nootkatensis* was an important component of the previous old-growth stand. Perhaps, slow growth and poor competitiveness in this species are overlooked when natural regeneration is evaluated. Recent efforts of reforestation using this species have focused on the ability of *C. nootkatensis* to reproduce asexually through layering by using plant material originating from rooted cuttings. These vegetative propagules, known as "stecklings", are obtained from young and vigorous vegetative material taken from hedged orchards with a five- to ten-year cycle to maximize production (Russell and Grossnickle 1989;

Russell et al. 1990). In coastal British Columbia, stecklings have become the primary mean of artificial regeneration of *C. nootkatensis* (Karlsson and Russell 1990).

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1.3 Clonality in *Chamaecyparis nootkatensis* and other tree species

Vegetative reproduction by layering in *C. nootkatensis* has been qualitatively documented in a study of populations in southern Washington, Oregon, and California (Antos and Zobel 1986) which suggested that layering (as estimated by the presence of prostrate growth form observed in the field) occurred mostly in dry open stands and that vegetative reproduction increased with altitude. These authors also suggested that snow cover might influence clonality in *C. nootkatensis* by forcing lower branches to the ground, therefore favoring the formation of adventitious roots. A more recent study conducted on stands of *C. nootkatensis* found in southeast Alaska (Hennon et al. 1998) described layering as a process mostly occurring in wet boggy sites and absent from well-drained sites. It was proposed by Dunsworth (1998) that vegetative reproduction in this species may have compensated for poor sexual reproduction and allowed more successful adaptation to moist sites, avalanche disturbance, and volcanic tephra. Hypotheses relating to the amount or degree of clonality in *C. nootkatensis* in relation to 1) altitude, 2) moisture, and 3) snow cover remain untested. The answers to which types of habitats favor clonality in *C. nootkatensis* may be employed for natural reforestation purposes and better management of this species.

The spatial extent of clonally derived structures or clonal genets in this species has received minor attention and is often anecdotal; however, a handful of studies have partially addressed this aspect. Antos and Zobel (1986), used foliage characteristics (juvenile characters are reportedly only observed in young stems derived from a seed), and documented that in 10 stands, for a total of 3137 trees surveyed, an average of 65% of these trees had originated through layering. Further dissection of their results showed that only 9% of the trees under 0.3 m in height and very few trees taller than 2 m were asexually derived from layering. The number of clonally derived individuals reported in the second group is obviously

underestimated because of the loss of juvenile foliage characteristics in older and taller trees. These authors conclude that in the Cascades range, *C. nootkatensis* seedlings are few and most stems come from layering, especially on open sites. Harris (1990), in a description of the rooting habit of the species, reported that root systems have been observed to extend to 100 feet (~30 m). Hennon et al. (1998) reported that stems, often prostrate in form, within single patches, are typically connected to the same root system, suggesting that clonal extent in *C. nootkatensis* is likely to be distributed in close aggregates or clumps.

The studies presented above provide anecdotal and conflicting views relating to the types of habitat correlated with clonal growth in *C. nootkatensis* and the extent of clonal reproduction in this species. Furthermore, the role and effect of clonal reproduction and growth on the genetic diversity and genetic structure of this species remain unknown.

Tree species, in comparison to herbaceous plants, have been poorly studied with regards to their ability to clonally reproduce (reviewed by Peterson and Jones 1997). To date, clonal trees species receiving the most attention have been aspens and cottonwood (*Populus* spp., e.g. Kemperman and Barnes1976; Jelinski and Cheliak 1992; Gom and Rood 1999), Oaks (*Quercus* spp., e.g. Berg and Hamrick 1994; Montalvo et al. 1997; Ainsworth et al. 2003), birches (*Betula* spp. e.g. Hermanutz et al. 1989) and a few tropical trees (e.g. Hamrick et al. 1993; Chung et al. 2000; Chung and Epperson 2000). Furthermore, even fewer studies of clonality in conifers have been published. Examples of these include *Cryptomeria japonica* (Moriguchi et al. 2001) and *Pinus pumila* (Tani et al. 1998). Characteristics distinguishing woody plants from herbaceous plants such as size, longevity, and presence of wood may modify several patterns observed in clonal plants. The greater size of woody plants may predispose them to self-thinning within ramets of the same or different genets (e.g. de Kroon and Kalliola 1995). Also, the longevity and woody composition of below ground structures among ramets belonging to the same genet may allow the persistence of physiological integration (resource sharing among interconnected ramets) through long periods of time.

Clonality in C. nootkatensis is poorly described and understood, and the effect of

clonality on fine scale genetic diversity and genetic structure in this species is unknown. In addition, as presented above, information about clonality in tree species (especially conifers) is scarce and largely limited to a few species. Greater knowledge about clonal reproduction and its effect on population dynamics and population genetics of clonal plant species is needed from a range of taxa to better understand the role of clonality in the evolution of clonal organisms.

1.4 Thesis structure and research hypotheses

In this thesis, I will undertake a population genetic study of C. nootkatensis in the light of its ability to clonally reproduce. In the second chapter of this thesis, I will describe the isolation and characterization of microsatellite markers from a genomic library of C. nootkatensis and discuss trends in microsatellite recovery rates in conifers. In chapter three, I will use the developed microsatellite markers and measure their performance in performing clonal assignment. The following hypothesis will be tested: a small number of microsatellite loci can accurately assess clonal identity in C. nootkatensis. This chapter will also investigate size and spatial patterns of clonal growth. This will be performed to test 1) the frequency and spatial scale of clonal reproduction in C. nootkatensis, and 2) whether this species can be classified as a phalanx-type clonal plant which exhibits clumped distribution of ramets, and 3) whether C. nootkatensis shows high levels of variation in clonal extent. The fourth chapter will examine variation in clonal extent, clonal diversity, genetic diversity and genetic structure in this species and investigate their potential relationship with the patterns of clonal growth reported in the previous chapter. Two hypotheses will be tested in this chapter; 1) fine scale genetic variation is inversely related to clonal extent in C. nootkatensis, and 2) fine scale genetic structure as estimated by genetic spatial autocorrelation is positively related to clonal extent in C. nootkatensis. Finally, the fifth chapter will present a general discussion of the results obtained in this thesis with an emphasis on the evolutionary consequences of clonality in C. nootkatensis and describe future research needs with regards to C. nootkatensis and clonal plants in general.



Figure 1.1. Geographic range of Chamaecyparis nootkatensis.

CHAPTER 2: Isolation, characterization, and cross-species utility of microsatellites in Chamaecyparis nootkatensis

2.1 Introduction and rationale

Microsatellites, or simple sequence repeats (SSRs), are tandemly repeated DNA motifs of 1–6 nucleotides found in all prokaryotic and eukaryotic genomes analyzed to date. They have been isolated from both coding and non-coding regions and are typically characterized by a high degree of length polymorphism. The origin(s) of such levels of polymorphisms is (are) still debated although slippage events during DNA replication (Schlötterer and Tautz 1992) appear to be the most accepted explanation to date (Zane et al. 2002). In addition to being highly variable, microsatellites have been noted for their co-dominant inheritance, short lengths, and ease and reliability of scoring. The explosion of interest in microsatellite markers has even resulted in the creation, in 2002, of a sister journal to *Molecular Ecology* named *Molecular Ecology Notes* which has been mostly concerned with publishing microsatellite primer notes.

Previous genetic studies in *C. nootkatensis*, as noted in Chapter 1, have been limited to quantitative traits in common garden experiments (Cherry and Lester 1992, Russell 1998) and isozyme markers in natural populations occurring across the range (Ritland et al. 2001). Isozyme markers have been successful in range-wide assessment of genetic diversity and genetic structure in many organisms, but do not typically demonstrate the level of polymorphism required in studies of fine scale genetic diversity, genetic structure, and clonal assignment.

Plant clonal reproduction, by multiplying given genotypes in a population, has the potential to affect population mating patterns and genetic parameters such as genetic diversity and genetic structure. This may, ultimately, affect the evolutionary trajectory of clonal plant species. To investigate plant clonality, plants within populations under study must first be assigned to their respective genet. Resolution of clonal associations and investigation of fine scale genetic diversity and genetic structure requires the use of highly variable molecular markers such as microsatellites. To this end, robust and highly polymorphic microsatellite loci

were isolated and characterized for *C. nootkatensis*. Loci considered robust amplify reliably, are easily scored, and are not affected by null alleles. Because microsatellite marker recovery is typically low in conifers (e.g. Smith and Devey 1994; Kostia et al. 1995; Pfeiffer et al. 1997), the developed markers were tested for transferability across several species within the family Cupressaceae.

2.2 Specific objectives

The specific objectives of the work described in this chapter were to:

i) Isolate microsatellite loci from an enriched genomic library in C. nootkatensis.

ii) Develop robust polymorphic microsatellite markers.

iii) Characterize the level of polymorphism and segregation patterns of the developed microsatellite markers in natural populations of *C. nootkatensis*.

iv) Test the amplification potential of the developed microsatellite primers across a number of species within the family Cupressaceae *sensu lato*.

2.3 Materials and methods

2.3.1 Construction of enriched genomic library

The construction of the genomic library was performed by C. Newton of the British Columbia Research Institute (Vancouver, BC). The modified biotin-enrichment strategies of Kijas et al. (1994) were used to isolate microsatellite markers from *C. nootkatensis* genomic DNA. Total genomic DNA (0.5 to 1.0 μ g) was digested with *Hae*III and *Psb*AI; individual fragments were ligated to double stranded oligonucleotide adapters (M28 5'

CTCTTGCTTGAATTCGGACTA and M29 5'TAGTCCGAATTCAAGCAAGAGCACA) on their 5' and 3' ends, respectively. Adapter ligated fragments were then denatured, hybridized with 5' biotin labeled (AC) ₂₄ or (TC) ₂₄ and enriched by selection with magnetic streptavidin affinity support (Dynal M280 beads Promega, Madison, Wis.). Biotin-selected genomic fragments were amplified using primer M30 (5'CTCTTGCTTGAATTCGGACTACC) and the resulting mixture

was digested with *Eco*RI and ligated into a standard cloning vector (pGEM3Z+, Promega) for propagation in *E. coli* (strain SURE TM Stratagene, La Jolla, Calif.). Individual microsatellite-containing clones were detected by colony hybridization with ³²P-labelled (AC) ₁₂ or (TC) ₁₂ and positive clones were transferred into 15% glycerol yeast trypton stock for long-term storage and further characterization.

2.3.2 Nucleotide sequencing and microsatellite primer design

192 clones were sequenced, for both DNA strands, directly from glycerol stock using SequiTherm EXCEL[™] II Long-Read DNA Sequencing Kits-LC (Epicentre Technologies) on a LiCor 4200 (LiCor Inc. Lincoln, NB). Seventy-five clones were chosen on the basis of having ten or more repeats and flanking regions of 15 bp or more to design microsatellite primer sets. These were designed using the Oligo 6.3 program (Molecular Biology Insight Inc., Cascade, CO). For each primer pair, one of the primers (chosen randomly) was tailed with an M-13 sequence primer (Oetting et al. 1995), which aids with the labeling of the microsatellite amplicon for subsequent scoring.

2.3.3 Plant material

To assess levels of polymorphism, three natural populations of *C. nootkatensis* were sampled from different locations in British Columbia. Fresh foliage was collected from 50 trees of a population near the summit of Mount Seymour, 49 trees of a population found on a broad ridge extending southward from the Black Tusk, in Garibaldi Provincial Park, and 52 trees emerging from a bog found at the foothills of Mount Washington, on Vancouver Island. Characteristics of these sites are further detailed in the following chapter. To assess Mendelian segregation, four full-sib families, each with 10 offspring, were obtained from the breeding and testing program of the B.C. Ministry of Forests (courtesy of John Russell, British Columbia Ministry of Forests). Finally, to assess cross-species amplification, individuals from 18 species of the family Cupressaceae were collected: fresh needles were sampled from *C. thyoides*
(Lebanon State Forest, Ocean County, New Jersey) and from *C. lawsoniana* (Institute of Forest Genetics, Placerville, California), seeds were obtained from *C. obtusa* (Tree Seed Laboratory, Taiwan Forestry Research Institute) and grown to the seedling stage, and foliage from 14 other species (see Table 2.4) were obtained from the collection of John Russell at the Cowichan Lake Research Station, Vancouver Island, BC. Care was taken to ensure that two individuals from genetically distinct clones were collected for each species.

2.3.4 PCR amplifications

Total genomic DNA from *C. nootkatensis* and the other species was isolated following the CTAB method described by Doyle and Doyle (1990). Polymerase chain reactions (PCR) amplifications were then performed using 10 μ l total reaction volumes. Reactions consisted of 1X *Taq* buffer (10mM Tris, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3; Roche, Mannheim, Germany), 1.0 pmol dNTP, 0.5 pmol each of forward and reverse primers, 0.5 pmol M13 infrared labeled primer, 1.0 Unit Taq DNA polymerase (Roche), and 10 to 20 ng of genomic DNA template. Samples were amplified using a PTC-100 thermal cycler (MJ research Inc. Waltman, MA). The PCR profile involved an initial 5 min denaturation at 95°C followed by 34 cycles consisting of a 45 s denaturing step at 95°C, a 45 s annealing step at various temperatures (see Table 2.1), and a 45 s extension step at 72°C. These cycles were followed by a final 5 min extension step at 72°C. Following amplification, 3 μ l of loading/stop dye (LiCor Inc. Lincoln, NB) was added to each reaction. Microsatellite loci were finally analyzed on a LI-COR 4200 sequencer (LiCor Inc. Lincoln, NB) using 7% polyacrylamide gels (Long RangerTM, BioWhittaker Molecular Applications, Rockland, ME).

2.3.5 Analyses

Identical genotypes were of clonal nature with high probability, and such clonal replicates were excluded from the polymorphism analysis. Estimates of observed heterozygosity, expected heterozygosity, and the inbreeding coefficient were obtained following

Hedrick (2000). The associated standard errors were obtained by performing 100 bootstrap estimates, using a FORTRAN 95 program written by K. Ritland. In the full-sib families, chi-square tests for deviations from expected segregation ratios were performed, and the results at each locus were summed over the four families.

2.4 Results

2.4.1 Microsatellite loci recovery and levels of polymorphism

Of the 75 microsatellite primer pairs designed, 41 produced PCR products but only 21 of these exhibited patterns suggesting polymorphic microsatellite loci. Of these 21, eight primer pairs produced amplifications with multiple stutter bands, four amplified erratically (showing a high number of alleles and very few heterozygotes), suggesting the presence of null alleles at high frequency, three showed multiple bands or dark monomorphic bands that overlapped with alleles to be scored, and one was affected by a locus duplication where alleles from both loci overlapped. Of the initial 75 microsatellite primer pairs designed, five amplified reliably and showed interpretable polymorphism (Table 2.1).

Once all trees from the three populations were genotyped, 16 groups of individuals were identified as clonally derived on the basis of having identical microsatellite fingerprints. Removing identical genotypes resulted in the reduction of individuals included in the polymorphism analysis from 50 to 45 in the Mount Seymour population, from 49 to 37 in the Black Tusk population, and from 52 to 45 in the Mount Washington population. The number of alleles observed ranged from 3 to 20 with a mean of 13.67 (Table 2.2). Allele size ranges corresponded to that predicted by the original sequence from which primers were designed (Table 2.1 and Fig. 2.1). Observed heterozygosities were generally lower than expected heterozygosities except in the case of two different populations in each of two loci (Y1F10 and Y2C12) (Table 2.2). Deviation from Hardy Weinberg equilibrium was detected in loci Y1E10, Y1F10, and Y1G09, three of the five developed microsatellite loci. Deviations in these three loci were the result of heterozygote deficiency. Locus Y1G09 was affected by the largest deviations

from Hardy Weinberg equilibrium in all three populations surveyed, a possible symptom of the presence of a null allele.

2.4.2 Inbreeding

Estimates of the inbreeding coefficient were usually positive (Table 2.2). Inbreeding coefficient averaged over all loci was 0.202 (SE=0.048) in the Mount Seymour population, 0.158 (SE=0.043) in the Black Tusk population, and 0.125 (SE=0.043) in the Mount Washington population. Y1G09 was the only locus with a cumulative inbreeding coefficient over all populations (*F*=0.393; SE=0.064) significantly different from zero (a=0.05). The overall inbreeding coefficient (*F*=0.161; SE=0.025) was also found to be significantly different from zero (a=0.05). This significance was due in part to the high estimate of inbreeding at locus Y1G09. When this locus was excluded from the analysis, however, the overall inbreeding coefficient (*F*= 0.074; SE=0.028) was still significantly different from zero (a=0.05).

2.4.3 Segregation analysis

The segregation analysis revealed that all loci surveyed behaved as typical co-dominant markers and segregated in a Mendelian fashion. Null alleles were observed directly in three of the five loci (Y1F10, Y1G09 and Y2C12) (Table 2.3). Null alleles in one of the two parents in families 11 and 26 (locus Y1F10) explain the observed segregation patterns in these families. Only family 11 in locus Y2C12 was found to be significantly different from Mendelian expectations via the chi-square test. When chi-square values were summed over all families within a locus, however, observed segregation ratios did not deviate significantly from expected Mendelian segregation ratios at any locus (Table 2.3). At locus Y2H01, families 4, 11, and 26 showed no segregation as the parent trees had identical homozygous genotypes. This locus, however, displayed single-locus segregation in family 34 as well as when analyzed for polymorphism.

2.4.4 Interspecific amplifications

Cross-species amplifications using microsatellite primers developed for *C. nootkatensis* were most successful in *Cupressus* and *Juniperus* (Table 2.4). The microsatellite primers did not readily amplify other members of *Chamaecyparis* except in the case of locus Y1E10. Both locus Y1F10 and Y2H01 are specific to *C. nootkatensis*; they did not amplify most (Y2H01) or all (Y1F10) other species. On the other hand, Y1E10 amplified in most species. No products, however, were obtained in *C. obtusa, Fokienia hodginsii, Cupressus arizonica* and *C. bakerii.* The last two species were the only members of *Cupressus* tested in this study (out of eight) that did not readily amplify with any of the developed primers. Most species in which polymorphic amplified bands were detected showed the typical stutter band pattern usually present in dinucleotide repeat microsatellite loci.

2.5 Discussion

2.5.1 Low rates of recovery for microsatellite loci

The well-documented attributes of codominance, reproducibility and high resolution have been important in the popularity of microsatellites as genetic markers in molecular ecological studies (Powell et al. 1996; Zane et al. 2002; Squirrell et al. 2003). At each step in the development of working microsatellite primer pairs, however, there is a potential to "lose" loci, a process termed "attrition rate" (Squirrell et al. 2003). Attrition rates in 71 studies in which microsatellites were isolated from plant species, have been reviewed and classified in causal groups by Squirrell et al. (2003). The percentage of primers that revealed polymorphic loci in this study (~28%) is low compared to the average found in the review by Squirrell et al. (2003) (49%). The percentage of primers that produced no, or poor, amplification (49%) and multiple or uninterpretable bands (39%) in this study, are high compared to the average found from the comparison of 25 plant studies (Squirrell et al. 2003) (19.5% and 24.1%, respectively). This high attrition rate may be explained by the large genome size of conifers and their highly repetitive nature.

Highly repetitive DNA, typically associated with large genome size such as those encountered in conifers, has been reported to be the major cause of low recovery of microsatellites from genomic DNA (Fischer and Bachmann 1998). This has been well documented in *Pinus* (Smith and Devey 1994; Kostia et al. 1995; Pfeiffer et al. 1997; Fisher et al. 1998). In my study, I found this problem extends to the Cupressaceae; of the 192 *C. nootkatensis* insert-containing clones sequenced, only 75 produced a sequence from which primers could be designed and of those, only 5 primer pairs were reliable in amplifying interpretable microsatellite loci. Similarly low microsatellite loci recovery was found in *C. obtusa* (Nakao et al. 2001), where 500 clones were screened for the presence of microsatellites, using the (CT)₂₀ probe, revealing 155 positive clones. Of these, 135 were sequenced, and of 44 designed primer pairs, nine proved to amplify interpretable polymorphic loci.

Large genome sizes can be due to polyploidy such as in the ferns, but chromosome number is relatively small and constant both in the Pinaceae (12 pairs) and in the Cupressaceae (11 pairs) (Murray and Leitch 2001). "C-values" represent the amount of DNA in picograms (pg) in the non-replicated haploid or gametic nucleus of an organism, irrespective of the ploidy level of the taxon (Swift 1950). C-values for the genus *Chamaecyparis* have a mean of 13.27 pg while in the genus *Pinus* they average 23.64 pg (Murray and Leitch 2001). In comparison, the angiosperm model organism *Arabidopsis thaliana* has a C-value of 0.18 pg while angiosperm trees such as *Acer carpinifolium* and *Fraxinus excelsior* have C-values of 0.38 pg and 0.98 pg, respectively (Bennett and Leitch 2001). These are comparable to the C-values for the model tree genus *Populus* which can be estimated at ~0.50 pg (450-550 Mbp) (Taylor 2002). Thus, while possessing a smaller genome than pines, *Chamaecyparis* still has a relatively large genome, which poses problems for molecular genetics research, such as microsatellite locus isolation and identification.

Because genome size should be positively correlated with the number of microsatellite regions, it is assumed that the size of an organism's genome should not affect the likelihood of finding suitable regions to design microsatellite primers. As the examples above demonstrate,

however, this assumption is often found not to be true. Garner (2002) proposed two mechanisms to explain this phenomenon. Firstly, template concentration is typically considered in terms of total nuclear DNA, but if genome sizes are large, the relative proportion of target to non-target DNA is reduced. Secondly, as genome size increases, the number of DNA regions complementary to any 20-bp region (the average length of microsatellite primers) also increases. This, in turn, increases the amount of nonspecific binding of primers, therefore decreasing the available pool of primers available for PCR. Both of these proposed mechanisms have dilution effects on the rate of recovery of microsatellite primers.

Recently, methods have been developed successfully that increase recovery of microsatellite markers from organisms with large genomes. Scott et al. (1999) increased efficiency of enrichment by 100% in Pinus elliottii using the DIG detection system (Roche, Basel, Switzerland). Their method uses numerous probes labeled at their 3' end with Digoxigenin-11-ddUTP to detect clones containing microsatellite inserts (van Miltenburg et al. 1995). Their strategy, however, resulted in a high level of redundancy in the detected microsatellites of Araucaria cunninghamii (only 16 unique sequences in 49). Elsik and Williams (2001) have developed a method using low-copy enrichment and filter-hybridization of tri- and tetra-nucleotide repeat motifs. Although their method revealed lower enrichment and higher redundancy in the low-copy libraries than in the total-genome library in the first stages, the final results showed greater number of polymorphic markers retrieved from the low-copy library. Finally, Zhou et al. (2002) used undermethylated DNA fragments to construct a microsatelliteenriched copy library. This last method resulted in higher recovery of microsatellite markers but lower diversity than with regular genomic DNA libraries. The above-mentioned methods offer higher number of retrieved microsatellite loci at the expense of lower diversity and greater redundancy. The higher number of microsatellite loci obtained with these methods could be of interest in increasing the power of analyses in studies concerned with large-scale genetic diversity and genetic structure, but the reduced variability of such markers decreases the ability

33

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to resolve fine scale patterns of genetic structure, genetic diversity, parentage assessment, and the identification of clonal groups.

2.5.2 Polymorphism, inbreeding, and detection of clonality

Ritland et al. (2001) studied allozyme variation at 10 loci in 17 natural populations of *C. nootkatensis* across its range. Their study revealed one to three alleles per locus with a mean of 1.68. Expected heterozygosity in each population ranged from 0.062 to 0.198 and averaged 0.147. In comparison, this study of microsatellite marker diversity showed, albeit with just three populations, a mean of 13.67 alleles per locus and an expected heterozygosity of 0.592 averaged over five loci. The diversity shown by the five microsatellite markers is thus much greater than that shown by ten allozyme loci. Such high levels of variation will be helpful in studies of genetic diversity and genetic structure at fine spatial scales.

Highly polymorphic markers are necessary for clonal assignment to confidently identify clonal structures. Genetic relatedness between individuals and the level of commonness of the observed genotypes are the most important factors that can induce error. These can be countered with the efficient use of even just a few highly variable molecular markers. In this study, the great degree of polymorphism of the developed microsatellite markers allowed the successful detection of putative clones in the population surveyed, and their elimination from statistics of genetic diversity.

The mean inbreeding coefficient over all loci and populations found in this study (F=0.161; SE= 0.025) is similar to that found by Ritland et al. (2001) in their isozyme study of *C. nootkatensis* (F=0.184; SE=0.022). These values would be caused by a self-fertilization rate of 30-35% (assuming inbreeding equilibrium). Highly localized genetic substructure may also increase homozygosity. This seems unlikely, however, because conifers generally show low levels of local genetic structure due to long distance pollen flow by wind. The relatively high inbreeding coefficient is somewhat surprising for a conifer, but in a review of genetic studies of species related to *C. nootkatensis*, Ritland et al. (2001) documented that it is not uncommon,

34

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and even characteristic, of many Cupressoid species. High inbreeding coefficients and deviations from Hardy Weinberg equilibrium due to heterozygote deficiency can also be the consequence of the presence of null alleles (Chakraborty et al. 1992; Pemberton et al. 1995). Although deviations from Hardy Weinberg were significant across three of the five loci (Y1E10, Y1F10, and Y1G09), only one locus (Y1G09) showed an *F* that was significantly different from zero. It is unlikely that null alleles are present at all of these loci. Subtraction of the locus with the highest F (Y1G09) from the analyses still provided estimates of the overall inbreeding coefficient that were significantly different from zero, showing that this is not due to the effect of a single locus and that these populations as a whole are affected by some level of inbreeding. For these reasons, it is unlikely that loci other than Y1G09 are affected by the presence of null alleles.

2.5.3 Segregation patterns and null alleles

Null alleles are the result of a lack of amplification of a product or allele during PCR, often due to non-conserved priming sites (Garner 2002). If not identified, they can lead to the false assignment of heterozygotes as homozygotes. This can result in the overestimation of inbreeding coefficients and genetic structure for the loci where null alleles are present.

In the segregation analysis, direct observation of null alleles was made in loci Y1F10, Y1G09, and Y2 C12 but at very low frequency (Table 2.3). Within-locus cumulative chi-square values also showed no significant deviations from expected Mendelian segregation ratios. Observed levels of heterozygosities in the natural population were high for both Y1F10 and Y2C12 loci in the polymorphism analysis (Table 2.2) and the estimated inbreeding coefficients were found not to be significantly different from zero. These observations suggest that heterozygote deficiencies in loci Y1F10 and Y2C12 are the consequence of inbreeding and genetic structure and not the presence of null alleles. Therefore, the direct observation of null alleles in the segregation analysis was likely the product of poor quality DNA samples resulting

in imperfect amplifications. For these reasons, these loci are considered to be microsatellite markers that can be used confidently in a study of genetic diversity and genetic structure.

Locus Y1G09 consistently showed deviations from Hardy Weinberg equilibrium as well as inbreeding coefficients that were significantly different from zero across all three populations. Furthermore, null alleles were directly observed in two of the four families tested in the segregation analysis with a mean frequency of 0.09. Assuming null homozygotes are ignored or not observed in the sample, which applies here, null allele frequency can be estimated as $(H_e - H_o)/(1 + H_e)$, where H_e and H_o denote the expected and observed heterozygosity, respectively (Brookfield 1996). The mean frequency of the null allele in locus Y1G09 across the three populations was estimated at 0.16, a high frequency for the microsatellite allele of a locus with an average number of alleles of 5.33 (Table 2.2). For these reasons, locus Y1G09 should be used cautiously and with the knowledge of the existence of a null allele. Studies concerned with genetic diversity should correct estimates of heterozygosity accordingly while mating system studies should avoid the use of this microsatellite marker.

2.5.4 Interspecific amplifications and taxonomic considerations

Evolutionary conservation of regions that flank microsatellite loci allows microsatellite primers developed for one species to be used for related species. This approach has been used successfully in animals (Moore et al. 1991; Schlötterer et al. 1991; Roy et al. 1994; Blanquer-Maumont and Crouau-Roy 1995; Pépin et al. 1995; Kayser et al. 1996) and plants (Kijas et al. 1995; Brown et al. 1996; Peakall et al. 1998; Westman and Kresovich 1998) and has increased the cost-effectiveness of these genetic markers. Studies investigating interspecific amplification potential of microsatellite markers in conifers have mostly been limited to *Pinus* (e.g. Powell et al. 1995; Cato and Richardson 1996; Fisher et al. 1998; Echt et al. 1999; Kutil and Williams 2001) and *Picea* (e.g., van de Ven and McNicol 1996; Hodgetts et al. 2001; Rajora et al. 2001). An exception to that is the study of Nakao et al. (2001) on *C. obtusa*.

36

For these reasons, other members of the family Cupressaceae were tested with the microsatellite primers developed for *C. nootkatensis*.

Recent molecular phylogenies of conifers show a close relationship of *Chamaecyparis* to *Juniperus* and *Cupressus* (Tsumura et al. 1995; Kusumi et al. 2000; Gadek et al. 2000). Furthermore, Gadek et al. (2000) showed considerable support for the paraphyly of *Chamaecyparis*, placing *C. nootkatensis* within *Cupressus* and *Juniperus* while *C. lawsoniana* and *C. obtusa* were placed outside of this group with *Fokienia sp.* Results of my cross-species amplifications are in concordance with Gadek et al.'s (2000); primers developed for *C. nootkatensis* were most successful at amplifying members of *Cupressus* and *Juniperus*, and other members of the genus *Chamaecyparis* gave largely unsuccessful amplification. Also, microsatellite primers developed for *C. obtusa* (Nakao et al. 2001) yielded negative results when tested on *C. nootkatensis* for interspecific amplification.

Previous studies in pines have shown very low levels of transferability of microsatellite loci between hard and soft pines, which are different subgenera within *Pinus* (Fisher et al. 1998; Echt et al. 1999). Greater transferability has been observed in triplet repeats (especially perfect ones) (Kutil and Williams 2001) and microsatellites isolated from the chloroplast genome (Powell et al. 1995; Cato and Richardson 1996), but at the expense of lower variability. Microsatellite loci in *Picea* have been documented to be successfully transferable within the genus (van de Ven and McNicol 1996; Hodgetts et al. 2001; Rajora et al. 2001) but have performed poorly when tested on species of *Pinus* (van de Ven and McNicol 1996). The results obtained here demonstrate similar trends in that successful transferability of the microsatellite loci isolated in *C. nootkatensis* to other species within the family Cupressaceae was limited to very closely related species.

The microsatellite primers developed here for *C. nootkatensis* demonstrate high potential for use in genetic diversity studies in members of *Cupressus* and *Juniperus*. These must be used with caution, however, because microsatellite primers used interspecifically can show greater numbers of null alleles, due to mutation of the primer binding sites. Also, as

demonstrated by Westman and Kresovich (1998) some PCR products obtained from interspecific microsatellite primer pairs may not actually contain simple sequence repeats even if they are of the expected size range. Additional tests such as hybridization, DNA sequencing, or single-primer assays should be performed to determine whether the products contain microsatellites.

2.6 Conclusion

In this chapter, I presented the development of primer pairs to amplify five highly variable microsatellite loci from an enriched *C. nootkatensis* genomic library. Although microsatellite recovery rate was low, it was not atypical for organisms with large genomes. The developed microsatellite loci showed significant inbreeding in the three populations of *C. nootkatensis* surveyed. A segregation analysis and a test for deviations from Hardy Weinberg equilibrium revealed that one of the five loci was affected by high frequency null alleles. Finally, interspecific amplifications show support for a close relationship of *C. nootkatensis* with members of the genera *Cupressus* and *Juniperus*.

Locus	Primer sequence (5' to 3')	Motif	Ta	Expected size (bp)	Genbank accession no.
Y1E10	F ^a :GGCTAGAGGCACACCTAACTA	(GA) ₂₅	58	209	AY141043
	R [₺] :ATGTTGATAATTGGGTGAATC				
Y1F10	F ^a : GTGCATATGCCCTACTACTT	(CT) ₂₇	62	168	AY141044
	R [♭] : GCAAATAACGACTTACAGGCT				
Y1G09	F ^a : ATGTCACCCCTTCTCTCT	(CT) ₁₁	50	133	AY141045
	R [♭] : GGGTTGATTGGCTACTTATCC				
Y2C12	F ^a : GCATAGATTCCAAATGTGTGT	(GT) ₂₀ (GA) ₁₂	58	185	AY141046
	R⁵: GTAATGCCCAAACAAGACCTC				
Y2H01	F ^a : CCTTCGTCACCCTCTGTGTGT	(GT) ₁₈	64	116	AY141047
	R [♭] : TAATCATAGAACTGGCCTAAA				

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Table 2.1. Characteristics of five microsatellite loci in Chamaecyparis nootkatensis.

Note: T_a, annealing temperature. ^a Forward primer ^b Reverse primer

Table 2.2. Microsatellite allele number, size range, observed heterozygosity, expected
heterozygosity, estimated inbreeding coefficient, and associated estimates of
standard error for five loci in 132 individuals in three populations of *Chamaecyparis*
nootkatensis.

Locus	Population	No. of alleles	Size range (bp)	N	H _o	H _e	(SE) ^a	F	(SE) ^b
Y1E10	Seymour	20	182-232	41	0.829*	0.898	(0.018)	0.076	(0.059)
	Black Tusk	13	184-230	25	0.640*	0.894	(0.018)	0.284†	(0.093)
	Mt Washington	17	176-236	33	0.818	0.838	(0.033)	0.024	(0.070)
	_	16.67	182-236	33.0	0.762*	0.877	(0.013)	0.050	(0.043)
V1E10	•		404 400	45	0 750+	0.004	(0.040)	0.404	(0.070)
11110	Seymour	14	161-193	45	0.756*	0.904	(0.013)	0.164	(0.072)
	Black Tusk	12	161-191	37	0.865	0.832	(0.027)	-0.040	(0.068)
	Mt Washington	18	151-205	45	0.756*	0.862	(0.019)	0.123	(0.064)
		14.67	151-205	42.3	0.792	0.866	(0.011)	0.042	(0.039)
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Y1G09	Seymour	5	127-137	44	0.432*	0.746	(0.025)	0.421 [†]	(0.101)
	Black Tusk	6	127-139	37	0.324*	0.631	(0.054)	0.486 [†]	(0.119)
	Mt Washington	5	127-137	44	0.523*	0.717	(0.019)	0.271 [†]	(0.111)
	-	5.33	127-139	41.7	0.426*	0.698	(0.019)	0.393†	(0.064)
V2C12	Sourcour	11	101 007	45	0.644	0.616	(0.055)	0.047	(0.062)
12012	Block Tuck	17	191-237	4J 27	0.044	0.010	(0.000)	-0.047	(0.002)
	DIACK TUSK	17	197-241	57	0.005	0.000	(0.020)	0.024	(0.040)
	wit wasnington	10	191-239	44	0.773"	0.851	(0.021)	0.092	(0.079)
		14.07	191-241	42.0	0.701	0.704	(0.018)	0.023	(0.036)
V2H01	Sourcour	3	122 136	13	0 116	0 102	(0.064)	0 205	(0.241)
121101		5	122-100	40 25	0.110	0.192	(0.004)	0.030	(0.241)
		Д	120-124	30	0.200	0.290	(0.002)	0.034	(0.109)
	wit wasnington	4	120-120	43	0.250	0.289		0.113	(0.130)
		3.33	120-130	40.3	0.219	0.259	(0.037)	0.101	(0.102)
Arithme	tic mean	13.67		40.0	0.592	0.697	(0.001)	0.161 [†]	(0.025)

Note: N, sample size; H_0 , observed heterozygosity; H_e , expected heterozygosity; F, estimated inbreeding coefficient.

^aEstimated standard error of expected heterozygosity.

^b Estimated standard error of inbreeding coefficient.

* Significant deviation from Hardy Weinberg equilibrium (a=0.05).

[†] Significant deviation of inbreeding coefficient from zero (a=0.05).

Locus	Family	Expected segregation ratio	Observed segregation ratio	Chi-square values	Probability	Null Alleles (direct observation)
Y1E10	4	1:1:1:1	0:5:3:2	5.20	0.16	No
	11	1:1:1:1	0:5:3:2	5.20	0.16	No
	26	1:2:1	2:5:3	0.20	0.90	No
	34	1:1	7:3	1.60	0.21	No
				12.20	0.43	
Y1F10	4	1:1:1:1	2:3:2:3	0.40	0.94	No
	11	1:1:1:1	0:5:1:4	6.80	0.08	Yes*
	26	1:1	3:7	1.60	0.21	Yes*
	34	1:1	5:4	0.20	0.65	Yes
_				9.00	0.62	
Y1G09	4	1:1:1:1	3:4:0:3	3.60	0.31	No
	11	1:1	7:3	0.20	0.21	No
	26	1:1:1:1	1:1:3:4	2.80	0.42	Yes
	34	1:1	5:2	1.8	0.18	Yes
				6.60	0.31	
Y2C12	4	1:1:1:1	4:4:1:1	3.60	0.31	No
	11	1:1:1:1	5:0:0:5	10.00	0.02 [†]	No
	26	1:1:1:1	4:1:4:0	5.20	0.16	Yes
	34	1:1:1:1	2:3:2:3	0.40	0.94	No
				19.20	0.20	
Y2H01	4	-	-	-	-	No
	11	-	-	-	-	No
	26	-	-	-	-	No
	34	1:1	6:4	0.40	0.53	No
				0.40	0.53	

 Table 2.3. Segregation analysis for five microsatellite loci in the 40 offspring (from four families)
 of Chamaecyparis nootkatensis.

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* Null allele found in one of the two parents.
 * Significant deviation from expected Mendelian segregation ratio

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	Locus				
Species	Y1E10	Y1F10	Y1G09	Y2C12	Y2H01
Chamaecyparis lawsoniana	202(1)	ı	ı	ı	ı
Chamaecyparis obtusa	•	ı	,	ı	ı
Chamaecyparis thyoides	202(1)	ı	ı	235(1)	ı
Chamaecyparis formosensis	166-174 (2)	ı	ı	ı	ı
Chamaecyparis pisifera	206(1)	ı	ı	ı	ı
Fokienia hodginsii	·	ı	ı	ı	ı
Cupressus arizonica	•	·	ı	·	·
Cupressus bakerii	ı	ı	·	ı	ı
Cupressus goveniana	180-222 (3)		121(1)	211-255(4)	ı
Cupressus macrocarpa	176-224 (3)	ı	121(1)	161-173(2)	140(1)
Cupressus nevadensis	212-244 (4)	·	121(1)	161-197 (4)	ı
Cupressus montanensis	194-230 (2)	ı	121(1)	233-255(2)	ı
Cupressus sargentii	178-220 (4)	ı	117-121 (2)	175-211 (2)	ı
Cupressus sempervirens	222-236 (2)	·	121-131 (2)	•	ı
Juniperus communis	182-232 (2)	ı	121-127 (2)	197-235(3)	146(1)
Juniperus occidentalis	198-210 (2)	ı	121-127 (2)	205-223 (3)	ı
Juniperus scopulorum	190-238 (4)	I	121-129(2)	235(1)	146(1)
Juniperus virginiana	206-224 (4)		121-131 (2)	203-235(3)	

 Table 2.4. Microsatellite allele size range, in bp, and number of alleles (in parentheses) of two individuals in 18 species in the family

 Cupressaceae sensu lato for five microsatellite markers developed in Chamaecyparis nootkatensis.

Note: - = failed amplification.



Figure 2.1. Allelic variation at five microsatellite loci (Y1E10, Y1F10, Y1G09, Y2C12, and Y2H01) in 24 individuals of *Chamaecyparis nootkatensis*. Lane 1 and 14 (indicated by arrows) are molecular ladders (Licor Inc., Lincoln, NE).

CHAPTER 3: Clonal Assignment, Clone Size and Spatial Pattern in Chamaecyparis nootkatensis

3.1 Introduction and rationale

Clonal growth or reproduction, by multiplying given genotypes, directly affects allele frequencies in a population and, depending on clonal growth patterns (phalanx vs. guerilla) may spatially clump identical genotypes. Because of close proximity, genetically identical individuals may have increased potential of mating together, resulting in effective geitonogamy and increasing the potential for inbreeding. Also, clonal plants, through underground physical connections, may be capable of sharing resources (physiological integration). This could potentially influence selection in these populations and favor clonally derived individuals because of their ability to capture greater arrays of resources. The potential consequences of plant clonality presented above may greatly affect population genetic parameters such as fine scale genetic diversity and genetic structure, and, ultimately, the evolution of clonal plant species. In order to investigate the potential effect of clonal reproduction on these parameters, identification or delineation of clonal associations must first be assessed accurately.

Clonal reproduction has been reported to occur in *C. nootkatensis* through layering, where low lying branches come in contact with the soil and form adventitious roots. This form of asexual reproduction is highly suggestive of a phalanx type of growth. Information regarding the spatial extent and importance of this mode of reproduction in *C. nootkatensis*, however, has been anecdotal, and variation in the amount of clonal reproduction in different habitats has been mostly reported in qualitative terms.

In this chapter, the microsatellites developed and presented in Chapter 2 of this thesis were utilized to perform clonal assignment in three *C. nootkatensis* stands, and the accuracy of this assignment were evaluated. Based on this clonal assignment, spatial patterns of clonal growth in *C. nootkatensis* sites were documented in each site.

3.2 Specific objectives

The specific objectives of the work reported in this chapter were to:

i) Perform clonal assignment in three different sites of *C. nootkatensis* using the microsatellite markers developed and documented in the previous chapter.ii) Assess the efficiency of microsatellite markers in performing clonal assignment.

iii) Document the size and spatial pattern of the detected clones in three different sites.

To understand the role or influence of clonality in the population dynamics and evolution of plant species, clonal assignment must first be performed. Clonal assignment is a critical initial step in documenting the frequency and spatial scale of clonal reproduction in different populations or species. Inaccurate or biased clonal assignment may distort or even prevent the detection of trends, patterns, or relationships between clonality and various ecological and genetic parameters. Assignment based on unearthing of physical connections may be biased due to the decomposition of such structures and potential root grafting. Therefore, clonal assignment inferred from microsatellite loci genotypes was performed. Inferences from molecular markers, however, possess their own biases and consequently also need to be assessed for accuracy.

3.3 Materials and methods

3.3.1 Collection sites

Chamaecyparis nootkatensis trees were sampled from three sites in southwestern British Columbia (Fig. 3.1). These sites are the same as used in Chapter 2. The sites were chosen to vary in altitude and moisture regime (Table 3.1). The Mount Seymour site is found approximately 200 m below the summit of the mountain in Vancouver's north shore mountains and most *C. nootkatensis* individuals found at this site were ~10 to 25 m tall mature trees. Almost no prostrate form of this tree was found and the understory was generally open. The Black Tusk site is found south of Whistler, on a broad southwest-oriented shoulder flowing from

the geologic landmark named Black Tusk. Most *C. nootkatensis* individuals found at this site were of moderate height (characteristic of this elevation) for the species (~10-15 m) except when found in wet depressions where they were short and prostrate. The Mount Washington site lies at the base of the mountain on the east coast of Vancouver Island, south and west of the town of Courtenay, BC. Trees found at this site were generally stunted (~1-3 m in height) except for a few individuals (~10-15 m) found in the middle of drier hummocks. The prostrate growth form prevailed and consequently, tree (stem) density was far greater than at the two previously described sites.

At each site, three plots along an approximately linear transect were selected 80-100 m apart. Transects and plots were selected on the basis of apparent similarity in vegetative association, density, and the prevalent growth form of *C. nootkatensis* within sites.

3.3.2 Selection and mapping of trees

At each plot, a tree was marked as the center. All trees taller than 1 m were tagged following concentric circles of increasing size until approximately 50 trees were included. The last circle was then completed to include all trees in it. For each tree tagged, fresh young foliage was sampled using pruning shears in small trees and a slingshot and rocks on taller trees. The foliage was kept cool until DNA isolation. The spatial location of each tree was recorded using compass and measuring tape. Polar coordinates were plotted using Sigmaplot for Windows Version 5.00 (1999, SPSS Inc.) to produce maps of the spatial distribution of all sampled trees within the plots (Figure 3.2). Cartesian coordinates were calculated from polar coordinates for use in the spatial autocorrelation analyses.

3.3.3 DNA isolation and microsatellite genotyping

Total genomic DNA from the fresh foliage of *C. nootkatensis* was isolated and genotyped following the methods described in Chapter 2. The five microsatellite markers described in the previous chapter were used to genotype all sampled individuals.

3.3.4 Clonal assignment

Clonal assignment can be defined as the description of the genetic identity of the sampled trees. The presence of identical microsatellite genotypes in different trees can be the result of either 1) closely related siblings sharing the same alleles for the given loci, 2) a genotype being composed of alleles commonly found in the population surveyed, or 3) clonal reproduction of the individuals in question. For these reasons, clonal assignment was performed first with a statistical test, and then confirmed with an analysis of genetic spatial autocorrelation.

The aim of clonal assignment is to establish, with a certain degree of confidence, whether trees were sexually or asexually derived, and in the latter case, group together members of the same "clonally derived progeny". Clonal reproduction in *C. nootkatensis* has been documented to occur through layering. Because a great proportion of individuals of this species are very long-lived, the low lying branches, responsible for the formation of adventitious roots, often die through a process of self-thinning and the underground structures are often found to decompose. This die-off process results in the disappearance of a physical link between the "parental plant" and its asexually produced "clonal progeny". Therefore, an inferential method involving the use of genetic markers was preferred in assessing clonal assignment.

The initial statistical test is based on calculations relating to probabilities of genotype occurrence. The preliminary allele frequency tables were calculated after removing all replicated microsatellite genotypes based on the assumption that these were clonally derived. To carry out clonal assignment, genotype frequencies and their probability of occurrence must be calculated based on observed allele frequencies. These are typically estimated from a dataset where all replicated genotypes are removed on the basis of being clonally derived. This circular reasoning has been described as a caveat of studies using clonal assignment (Montalvo

et al. 1997) but remains commonly in use. The total number of potential unique genotypes in each site (Cheliak and Pitel 1984) is

$$N_{g} = \prod_{i=1}^{L} \frac{(a_{i}(a_{i}+1))}{2}$$

where *a_i* is the number of alleles at locus *i*, and *L* is the number of loci analyzed for a particular site. Multilocus genotypes constituted of low frequency alleles have low probability of occurrence in randomly mating populations. Conversely, multilocus genotypes that have common alleles at each locus have a much higher probability of occurring more than once.

The following equations were used to determine the probability of a given multilocus genotype being drawn at random from a given population and its probability of being drawn a second time. Assuming linkage equilibrium, the probability of a multilocus genotype (Parks and Werth 1993) is

$$p_{gen} = \prod_{i=1}^{L} g_i$$

where *g_i* is the Hardy Weinberg expected frequency of the genotype at locus *i* and *L* is the number of loci. Linkage disequilibrium between all pairs of loci was tested using the program Arlequin version 2.000 (Schneider et al. 2000). Since the data consisted of genotypes and no information about the gametic phase was available, a likelihood ratio test was performed (Slatkin and Excoffier 1996, Excoffier and Slatkin 1998) to estimate gamete frequencies. In this test, the likelihood of the data *not* assuming linkage equilibrium is obtained by applying an expectation-maximization (EM) algorithm to estimate haplotype frequencies. In order to better approximate the underlying distribution of the likelihood-ratio statistic under the null hypothesis of linkage equilibrium, the program uses a permutation approach where alleles between individuals are permuted within each locus.

For any given single multilocus microsatellite genotype (with probability of occurrence p_{gen}), the probability of a second encounter of the same genotype among *G* genotypes (individuals of differing genotype) (Parks and Werth 1993) is

$$p_{se} = 1 - (1 - p_{gen})^G$$

Separate individuals of identical genotypes were treated as a single clone if the probability of second encounter of the same genotype (p_{se}) was found to be less than 5% and if these individuals were from the same plot.

A second method based on spatial autocorrelation of multilocus genotypes was used to confirm clonal assignment. All genotypes, including all ramets of putative genets, were included in the analysis. Estimates of multilocus coefficient of coancestry (kinship) were calculated as described in Loiselle et al. (1995) using the program SPA*GeDi* 1.0 (Hardy and Vekemans 2003). These were calculated as a function of the physical distance between pairs of individuals within given distance intervals, relative to the allele frequencies of the sample population. At each locus, the frequency of a particular allele in individual *i* (*pi*) can be 0 (not present), 0.5 (present in heterozygous state), or 1 (present in homozygous state). The correlation of this frequency with the frequency of the same allele in individual *j* (*pj*) is estimated as (Loiselle et al. 1995)

$$\hat{\rho}_{ij} = \frac{\sum_{ij} (p_i - \overline{p})(p_j - \overline{p})}{k\overline{p}(1 - \overline{p})} + \frac{1}{2(N-1)} \qquad (i < j)$$

where \overline{p} is the site mean allele frequency, *k* is the total number of possible pairwise connections between individuals located a particular distance apart, and *N* is the total number of individuals in the sample population. Results from each locus were weighted by their polymorphic index, Σp_i (1- p_i), and combined to provide a multilocus estimate of spatial genetic structure. Under the assumption that individuals are not inbred (*F* = 0), Wright's coefficient of relatedness is ~2 $\hat{\rho}_{ij}$. The estimated coefficient of kinship, $\hat{\rho}_{ij}$, ranges from 0 to 1 with a value of 0 for unrelated pairs, 0.25 for pairs of full-siblings, 0.5 for comparisons among clonal ramets, and 1 for populations subdivisions at fixation. An estimated value of kinship coefficient of 0.5 is only expected to be obtained for clonal ramets in outbreeding plant populations.

To test whether spatial genetic structure was significant, 1000 permutations of intact individual genotypes for all spatial position recorded were performed in the program SPA*GeDi* 1.0. In doing so, the spatial character of the observed genetic structure is destroyed and 95% confidence intervals around the null hypothesis of no genetic structure are obtained.

3.3.5 Accuracy of microsatellites in clonal assignment

Duplicate samples originating from the same tree were collected and genotyped from ten trees at the Mount Seymour site to initially assess reproducibility of results and to test the robustness of microsatellite markers at genotyping.

To investigate the effect of the number of loci being used and to verify the accuracy of the markers used, clonal assignment was performed using all possible combinations of the five microsatellite markers. Assignment was performed using one to five markers at all nine plots and clonal genotypes were recognized only on the basis of sharing identical genotypes. The total number of distinct genotypes identified using a given number and combination of loci was finally divided by the number of distinct genotypes identified when using all loci to provide an estimate of efficiency. Standard errors of the sample were calculated within each class of number of loci. A saturation-like curve would indicate the number of loci needed to get an estimate similar to that of a higher number of loci. It would therefore constitute evidence for an accurate assessment of clonal assignment. Alternatively, a curve where saturation would not be reached would demonstrate that the number of loci used, or their level of polymorphism, were insufficient at providing accurate clonal assignment.

3.4 Results

3.4.1 Clonal assignment

Because the initial statistical test assumes linkage equilibrium, all pairs of loci were tested for significant disequilibrium. Two loci were found to be significantly linked in the Mount Washington population. Because the above test assumes Hardy Weinberg proportions of genotypes, and because the sample size was greatly reduced in this population due to a high proportion of clonally derived individuals, these departures from linkage equilibrium are assumed here to be of little significance. Therefore, the five loci were included in the analyses concerned with clonal assignment.

The number of observed distinct genotypes at all sites ranged from 55 to 24 with a mean of 41.7 (Table 3.2). In comparison, the calculated number of possible genotypes, N_g , based on the number of alleles observed in the given populations ranged from 87,664,500 to 3,457,792,800 with an arithmetic mean of 1,486,296,487 (Table 3.2).

The probability of a given multilocus genotype being drawn at random from a given population was calculated for each multilocus genotype that was found more than once in a plot $(p_{gen} \text{ in Table 3.3})$. This probability in all plots varied from 6.04E-11 to 1.79E-4 with a mean of 9.00E-6. The probability of a second encounter of a given genotype with a previously estimated probability of occurrence among a given number of genotypes was also calculated $(p_{se} \text{ in Table 3.3})$. This probability ranged from 2.72E-9 to 9.77E-3 with a mean of 4.30E-4. Because no replicated multilocus genotypes had a probability of second occurrence higher than 0.05, all were assigned to putative clones that shared the same genotype. No genotypes were found to be identical between plots or between sites.

The spatial genetic autocorrelation analysis presented a similar spatial pattern of clonality in *C. nootkatensis* (Fig. 3.3). Kinship coefficients within the first three meters varied from ~0.2 to 0.4 and decreased drastically by five meters, where they hovered around the upper limit of the 95% confidence interval around the hypothesis of no genetic structure. The estimated coefficient of kinship intercepted the 95% confidence interval around the null

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hypothesis of no genetic structure at ~10 m in the Mount Washington site, at ~20 m in the Black Tusk site, and did not intercept this confidence interval within 20 m in the case of the Mount Seymour site. Assuming that most of the genetic autocorrelation observed at the lower distance classes is due to clonality (more details will follow), most of the clonality occurs within 3 m (±0.5 m), after which point values of estimated coefficients of kinship suggest the structure observed is due to the presence of half-sibs or a possible combination of clonal offspring and unrelated individuals.

3.4.2 Accuracy of developed microsatellite markers in clonal assignment

Duplicates of material from the ten trees sampled produced identical banding patterns within samples originating from the same tree indicating both robustness and reproducibility of the microsatellite markers.

The proportion of clonally derived structures detected increased with an increase in number of loci used for clonal assignment (Fig. 3.4). The standard errors associated with the proportion of detected clonality decreased as the number of loci increased. The shape of the curve obtained is similar in nature to a saturation curve where at a certain threshold the number of loci will not significantly improve the accuracy of clonal assignment. Because the curve flattens considerably when four loci are used, this suggests that four to five microsatellite loci was probably an optimal number of markers in detecting clonality in *C. nootkatensis* (Fig. 3.4). It should also be noted that clonal assignment using three or four of the *most* variable loci provided results of clonal assignment very close to that obtained with five loci.

3.4.3 Clonal size and spatial pattern of clonal growth

Clonal size, as described by the distance between the two most distant ramets of a genet averaged $3.45 \text{ m} (\pm 2.75)$. This size can be translated into a surface area estimated by a circle with a diameter equal to the two most distant ramets within a genet, which averaged 9.35

 m^{2} (±5.93). Interdigitating clones and large clones spanning up to ~15 m (e.g. Mount Seymour, plot 3) (Fig. 3.2) were observed at all three sites.

Genets consisted on average of 1.12 (SE=0.02) ramets or trees at the Mount Seymour site, 1.27 (SE=0.09) at the Black Tusk site, and 1.74 (SE=0.42) at the Mount Washington site, thus the majority of genets were represented in these population by only one tree. The percentage of clones that were constituted of more than one ramet was 7.9% (SE=0.7) at Mount Seymour, 14.9% (SE=4.1) at Black Tusk, and 20.9% (SE=3.8) at Mount Washington. These numbers suggest a high degree of variation in clonal extent among the three sites studied.

The spatial patterns of clonal growth observed at the three sites are shown in Figure 3.2. Patterns shown in this figure should be interpreted cautiously because the scale varies between plots. Clonal genets showed clumping of their constitutive ramets. A majority of the clonal genets detected here were linearly arranged as opposed to a circular pattern. Finally, most plots showed clones interdigitating since clonal genets were often found interspersed with other aclonal genets.

3.5 Discussion

3.5.1 Clonal assignment and accuracy

To perform clonal assignment, genotype frequencies and their probability of occurrence must be calculated based on observed allele frequencies. These, in turn, are estimated from a dataset where all replicated genotypes are excluded on the basis of being clonal in nature. This circular reasoning has been used in other studies of clonal organisms (e.g. Parks and Werth 1993; Montalvo et al. 1997; Ivey and Richards 2001) and represents a flaw in studies using clonal assignment. The highly variable nature of the microsatellite markers used in this study, however, provided large numbers of potential genotypes at each plot in comparison with the number of observed genotypes. Hence, the assumption of unique fingerprint per genet is reasonable and alleviates some of the bias inherent in the process used for clonal assignment. An approach using maximum likelihood estimates may be better suited to assess clonal

identification, but none are available at present and the development of such an analytical tool is beyond the scope of this thesis.

The presence of two identical genotypes in a plot can be the consequence of asexual reproduction or simply the sharing of a common genotype between two closely related individuals. The probability of encountering two individuals with varying levels of relatedness who share identical genotypes can be calculated using methods related to assessing identity by descent. The probability of observing two genetically identical individuals at five loci, when on average 32% of the loci are homozygous in an array of full-sibs, is 0.024. In the case of an array of half-sibs, this probability becomes 0.0012, and the same probability between parent and offspring is 0.0079. Because inbreeding was found to be high in the populations sampled, it should be considered in the calculation of these probabilities. The probability of encountering two genetically identical individuals in an array of self-sibs is 0.067, which is much higher than the probabilities above. This indicates that in highly inbred populations, clonal assignment can suffer from an upward bias. Anderson et al. (2002), however, showed that selfing in *C. nootkatensis* produced very low number of full seeds when compared to wind pollinated controls, thus markedly reducing the potential to observe a high number of self-sibs compared to other types of siblings.

The number of loci and the number of alleles detected at each locus are important in performing clonal assignment. This situation is similar to that of parentage studies, in that highly variable markers are a key component to reaching high levels of confidence when assigning parentage or clonal association. The effect of the number and combination of microsatellite loci in detecting clonality in *C. nootkatensis* (Fig. 3.4) showed that with the average number of alleles observed here (13.2 ± 1.4) , only four to five of the microsatellite loci were sufficient for performing an accurate clonal assignment in the sampled populations of *C. nootkatensis*. A decrease in number of alleles may be easily alleviated by an increase in number of loci. This appears to be especially true in the lower range of number of loci. A comparison between accuracy of clonal assignment (as presented here), using various molecular markers but within

the context of the effort needed to develop these markers, could be very helpful in choosing the most efficient method to perform clonal assignment.

Comparing efficiency or accuracy in clonal assignment between different molecular markers is difficult because most studies of clonal organisms show varying levels and forms of clonality as well as different levels and distributions of genetic diversity. This study found much higher allelic diversity in C. nootkatensis than the study of Ritland et al. (2002) where allozymes were used. This suggests that microsatellite markers may be more efficient than allozymes at detecting clonal structures. This hypothesis is supported by other studies where various molecular markers were used on the same samples. In a study of the genetic structure of a Pinus pumila population using allozymes, randomly amplified polymorphic DNA (RAPD), and microsatellite markers, Tani et al. (1998) demonstrated that DNA markers were approximately 3.5 times more effective than allozymes for identifying clones. Sydes and Peakall (1998) reported congruence between allozymes and randomly amplified polymorphic DNA (RAPD) in the clonal and endangered shrub Haloragodendron lucasii. Work on the rare grass Calamagrostis porteri ssp. insperata by Esselman et al. (1999) showed, however, that microsatellite markers detected far more genotypes in clonal populations than allozymes did and even more than RAPDs in one population. Using RAPDs and amplified fragment length polymorphisms (AFLPs), Moriguchi et al. (2001) investigated the clonal structure of the Japanese conifer Cryptomeria japonica and suggested that because AFLPs can detect several hundred markers simultaneously, they may be more efficient than microsatellite markers for clonal assignment. This hypothesis, however, remains untested, and the authors failed to mention the loss of information when using a dominant marker (AFLP) compared to a codominant marker, especially in subsequent analysis of genetic diversity and genetic structure.

The popularity of microsatellites has been mostly due to their high level of variability, a consequence of mutation rates in the order of 10^{-5} to 10^{-2} , that is, two to three orders of magnitude higher than values known for allozymes. This high mutation rate, however, comes at the cost of back mutations, resulting in homoplasy: alleles become identical in state (IIS) as

opposed to identical by descent (IBD). Size homoplasy in microsatellite markers has been known to introduce bias in studies of genetic diversity, genetic differentiation (Hedrick 1999; Balloux et al. 2000), genetic relatedness, and assignment methods (Cornuet et al. 1999; Estoup et al. 2002) thus, some caution should be taken when interpreting the results of this clonal assignment analysis. Estoup et al. (2002) reported that assignment scores are greatly influenced by marker variability, the best scores being obtained with the most variable markers. Marker variability was found to be very high in this study. In addition, size homoplasy would most likely occur between alleles of very similar sizes and at very few loci at the same time. Close inspection of the genotypes between genets shows clear differences in distances between allele sizes and in the number of loci showing dissimilarity. These observations provide strong evidence against erroneous clonal assignment due to homoplasy.

3.5.2 Clone size and spatial pattern of clonal growth

Clonal genets with more than one ramet averaged ~4 m in size (distance between the two farthest ramets within a genet). This distance was further confirmed in the genetic spatial autocorrelation (Fig. 3.3). This short distance, the physical clumping of the ramets of single genets, and the fact that intermingling of clones was not so diffuse that their boundaries could not be defined, confirms that layering is the most likely mode of asexual reproduction in *C. nootkatensis*. Alternative modes of asexual reproduction such as basal sprouting and suckering could also explain this pattern but have never been documented in *C. nootkatensis* and were not observed in the field during this study.

Clonal size in *C. nootkatensis* documented here differs from the information available in the literature. Harris (1990) states that layering in this species has been observed to extend, at high elevation sites, upwards of 100 feet (~30 m) (after Arno 1966). Clonal genets detected in this study were on average much smaller and even the largest structures were just half the size proposed by Harris (1990). This discrepancy may be a result of differences in the type of sampling area and/or sampling strategy. This is likely because in most plots, sampling did not

extend beyond 30 m. The evaluation of reasons explaining this discrepancy, however, is limited by the lack of details from previous studies. Because the sample size used in this study is small in terms of its geographical extent, it is highly plausible that larger clones exist in different parts of this species' range.

3.5.4 Site history and its effect on patterns of clonality

Because ramets were often discontinuously distributed within clones or genets, a possible hypothesis would involve the history of clonal reproduction by layering and subsequent thinning resulting in the death of many clonal ramets within a genet. Size and pattern of clonal growth in *C. nootkatensis* as reported in this study represent only a "snapshot" or static picture of a dynamic system where events of sexual and asexual reproduction, intra- and interspecific competition, selection, disease, and stochastic events are at play in a complex spatio-temporal matrix. These events and forces shaping the populations vary themselves through time and seral stages of the ecosystem. The only hard limits existing in this system are life-history traits of *C. nootkatensis* for which little is known and the approximate timeframe, because of the last glaciation 10,000 ybp, in which the patterns we are observing today had time to evolve.

With the knowledge of approximate time between layering events (an asexual generation time) and approximate distance on average between a "mother" tree and its asexually derived progeny, *C. nootkatensis* clones could be aged. This was first done in the alpine plant *Carex curvula*, which revealed a clone of approximately 2000 years of age (Steinger et al. 1996). A similar approach was used in the woody species *Rhododendron ferrugineum* where the age of clones was estimated using their spatial extent and the annual growth rate of shoots (Pornon and Escaravage 1999). Most likely, the estimated age of a *C. nootkatensis* clone would be underestimated due to the death of the primary ramet since its original establishment. Clonal age could allow reconstructing the chronology of genet establishment at each site. Drawbacks of this method to estimate clone age would be that 1) the smallest clone may not necessarily be the youngest clone but rather a relict of bigger and therefore older clone

and 2) competition between clones (de Kroon et al. 1992) could have limited the growth of the involved clones, resulting in misleading estimates of age. In addition, aging *C. nootkatensis* trees is difficult because of "basal ring width asymmetries" (Brubaker 1982), reports of false and missing rings from this and other Cupressaceae species (Dobrý and Kincl 1990; Laroque and Smith 1999), annual rings of very small density difference (Jozsa 1992; Laroque and Smith 1999) and old trees reported to have more than 1824 annual rings (Pojar and MacKinnon 1994).

Size of clonal structures varied extensively but, generally, the larger clones were present in the plots where the largest trees were found (see Mount Seymour, Table 3.4). This pattern is likely the result of a longer history of C. nootkatensis in such plots, giving increased possibility of clonal spread. This is an important consideration when investigating the influence of clonal reproduction on the mating system in C. nootkatensis. Because this species is wind pollinated, the physical location of clonal ramets will partially dictate the potential for selfing. In a large C. nootkatensis clone constituted of small or short ramets, the probability of selfing is increased because pollen flow is limited and the nearest neighbors are genetically identical. To decrease geitonogamy, a tree species such as C. nootkatensis would benefit from limiting the spatial extent of clonal spread and maximizing the ratio of tree height to clonal size. A threshold for this ratio, however, is impossible to estimate here because of the lack of information on pollen dispersal in C. nootkatensis. Similarly, seed dispersal will interact with the clonal architecture in defining the mating system. Limited seed dispersal in conjunction with extensive clonal spread will increase the probability of consanguineous mating when the offspring arising from the seed becomes sexually mature and mates with a clonal ramet of its mother, resulting, effectively, in a back cross. Again, more information about seed dispersal in C. nootkatensis is required prior to a conclusion.

3.6 Conclusion

The five highly variable microsatellite loci developed and used in this thesis accurately performed clonal assignment in three populations of *C. nootkatensis*. Clonally derived trees

sharing identical genotypes were found to grow in close proximity, often in clumped and linear arrangements . Their spatial arrangement (especially in relation to sexually derived *C. nootkatensis* individuals) may not significantly contribute to increasing inbreeding through effective geitonogamy. Among-site differences in spatial size and number of ramets of clonal genets were not observed, suggesting these parameters are not strongly affected by environmental factors. This observation, however, may be strongly influenced by stand history and age structure. Interpretation of the results presented in this chapter, in the light of information on gene flow, genetic variation, and genetic structure, will likely provide a clearer perspective of the role of clonality in the population ecology, genetics and evolution of *C. nootkatensis*.

Table 3.1. Description of collection sites including elevation, number of trees sampled per plot, estimated surface area sampled per plot, site description and associated species.

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Site	Elevation (m)	N number of trees sampled per plot	Estimated surface area sampled (m ²) per plot	Site description	Associated species
Mount Seymour	980	50, 50, 57	2034, 5655, 2274	-Well drained -Mild to steep slopes	Thuja plicata Tsuga heterophylla
				-South aspect	Vaccinium sp.
				-Well drained mixed with wet depressions	Thuja plicata
Black Tusk	1480	49, 60, 51	558, 628, 827	-Mild slope	Vaccinium sp.
				-Northwest aspect	Alnus crispa
				-Wet to boggy	Carex sp.
Mount Washington	520	52, 56, 61	223, 146, 177	-Level	Lysichiton americanum
				-Most trees found on hummocks	Menziesia ferruginea

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Site - Plot	Ng	Observed genotypes
Mt Seymour - 1	130 977 000	45
Mt Seymour - 2	3 457 792 800	46
Mt Seymour - 3	3 200 319 360	49
Black Tusk - 1	131 085 864	35
Black Tusk - 2	3 228 583 050	55
Black Tusk - 3	2 321 610 984	39
Mt Washington - 1	533 725 200	45
Mt Washington - 2	284 909 625	37
Mt Washington - 3	87 664 500	24
Arithmetic mean	1 486 296 487	41.7

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Table 3.2. Number of possible (N_g) and observed multilocus microsatellite genotypes at each plot.

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Site - Plot	clone #	p _{gen}	pse	Site - Plot	clone #	p _{gen}	pse
Mt Seymour - 1	1992	1.83E-08	8.22E-07	Black Tusk - 3	2179	4.84E-07	1.89E-05
-	1973	2.01E-08	9.05E-07	(cont'd)	2221	3.97E-07	1.55E-05
	1986	1.86E-08	8.35E-07		2188	5.20E-09	2.03E-07
	1999	7.43E-09	3.34E-07		2174	1.12E-07	4.37E-06
Mt Seymour - 2	2023	1.97E-09	9.07E-08	Mt Washington - 1	1409	4.76E-09	2.14E-07
	2047	3.89E-06	1.79E-04		1405	4.10E-09	1.84E-07
	2015	6.26E-10	2.88E-08		1461	5.12E-09	2.30E-07
	2103	4.17E-09	2.05E-07		2247	6.04E-11	2.72E-09
Mt Seymour - 3	2092	1.07E-09	5.25E-08		2225	1.01E-08	4.54E-07
	2109	6.98E-09	3.42E-07		1484	3.94E-06	1.77E-04
	2071	3.84E-06	1.88E-04	Mt Washington - 2	2285	1.10E-08	4.07E-07
Black Tusk - 1	1938	3.54E-05	1.24E-03		2274	3.87E-08	1.43E-06
	1944	1.10E-04	3.84E-03		2281	1.66E-07	6.16E-06
	1929	3.03E-08	1.06E-06		2254	3.94E-08	1.46E-06
	1927	1.89E-09	6.62E-08		2293	2.43E-08	8.99E-07
	1903	5.14E-06	1.80E-04		2249	3.37E-07	1.25E-05
	1951	9.61E-10	3.36E-08		2271	1.67E-06	6.19E-05
	1911	4.87E-08	1.71E-06		2294	3.01E-08	1.11E-06
	1918	6.52E-10	2.28E-08		2256	3.12E-09	1.15E-07
Black Tusk - 2	2149	1.79E-04	9.77E-03	Mt Washington - 3	2339	1.18E-06	2.84E-05
	2131	1.80E-07	9.90E-06		2353	2.28E-08	5.47E-07
	2167	1.04E-04	5.71E-03		2329	6.84E-10	1.64E-08
	2121	1.91E-07	1.05E-05		2343	8.82E-09	2.12E-07
<u></u>	2163	2.68E-08	1.47E-06		2333	2.95E-08	7.08E-07
Black Tusk - 3	2217	1.10E-07	4.27E-06		2308	5.73E-09	1.37E-07
				Arithmetic mean		1.77E-05	8.46E-04

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Table 3.3. Probabilities of first (p_{gen}) and second (p_{se}) encounters of clonal multilocus microsatellite genotypes for which clonal growth was observed, in all plots.

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Figure 3.1. Locations of collection sites. A star indicates the three sites where Chamaecyparis nootkatensis trees were sampled.


Figure 3.2. Schematical representations of the spatial locations of *Chamaecyparis nootkatensis* trees (indicated by dots) within the plots where they were collected. Polygons drawn to illustrate clonal assignment. Note that scale varies among plots.



Figure 3.3. Correlograms at three sites showing kinship coefficients, $\hat{\rho}_{ij}$, inclusive of the genotypes of all ramets sampled and 95% confidence interval around the null hypothesis of no genetic spatial autocorrelation as a function of given distance classes.



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Figure 3.4. Effect of different numbers and combinations of the microsatellites utilized in this study in detecting clonal structures in *Chamaecyparis nootkatensis*. The maximum number of loci used in clonal assignment in this thesis was five, for which, the proportion of clonal structures detected is one or 100 percent.

CHAPTER 4: Clonal Diversity, Fine Scale Genetic Diversity, and Genetic Structure in Clonal Chamaecyparis nootkatensis Populations

4.1 Introduction and rationale

Asexual reproduction through clonal growth is a common feature of many plant taxa. In fact, because of the late specialization of the plant cell, its totipotency, and its modular construction where each module contains somatic and meristematic tissues, all plants are potentially clonal (van Groenendael et al. 1996). Clonal reproduction, or clonality, increases the number of identical genotypes in plant populations and, therefore, potentially decreases the effective population size after intra-specific (or inter-clonal) competition (Orive 1993). Furthermore, clonality, especially in plant species that exhibit phalanx growth (where ramets of a given genet are found in clumps), increases the number of near neighbors sharing an identical genotype. This, in turn, can potentially increase the probability of selfing (e.g. Barrett and Harder 1996) unless processes are in place that would create a barrier to self-pollination or increase long-distance pollen movement.

If processes promoting self-incompatibility do not exist in clonal plant species, inbreeding, through selfing and consanguineous mating, could potentially lead to a further decrease in genetic diversity. Species where clonality is a dominant mode of reproduction have been historically hypothesized to possess depauperate levels of genetic diversity (reviewed by Jackson et al. 1985). Recent reviews agree, however, that on average, clonal plants do not seem to be less genetically variable than non-clonal plants (Ellstrand and Roose 1987; Widén et al. 1994), but relatively few studies have investigated intra-specific variation in reproductive mode (i.e., clonal vs. sexual) and the effect of clonal growth on genetic structure (e.g. Montalvo et al. 1997; Chung and Epperson 1999; Reusch et al. 1999; Chung and Epperson 2000). Moreover, such studies are rare in tree species, especially conifers. Clonal reproduction in phalanx species associated with limited gene flow, two life-history characteristics found in the

coniferous tree *Chamaecyparis nootkatensis*, has the potential to drastically increase levels of genetic structure.

Ritland et al. (2001) demonstrated high levels of inbreeding in *C. nootkatensis* as well as a high degree of large-scale genetic structure between populations, reflected by the strong genetic differentiation found between the major phylogeographic groups described. Limited gene flow and a high tolerance to selfing, in association with clonal reproduction and phalanx growth, may be important factors responsible for the patterns reported by Ritland et al. (2001).

In this chapter, clonal extent, clonal diversity, genetic diversity, and genetic structure at a fine scale were investigated within populations in three stands of *C. nootkatensis*. The effect of varying levels of clonal reproduction on genetic diversity and genetic structure were also explored.

4.2 Specific objectives

The specific objectives of the work reported in this chapter were to:

i) Measure and compare clonal extent between sites.

- ii) Measure and compare clonal diversity between sites.
- iii) Estimate fine scale genetic diversity and genetic structure within and between sites.

iv) Investigate the relationship between the extent of clonal reproduction in C.

nootkatensis and levels of genetic diversity and genetic structure.

4.3 Materials and methods

4.3.1 Collection, mapping, genotyping, and clonal assignment

Details of these methods are described in Chapter 3. *C. nootkatensis* trees were sampled from three sites in southwestern British Columbia: Mount Seymour, on Vancouver's north shore mountains, Black Tusk, found south of Whistler, and Mount Washington, on the east coast of Vancouver Island (see Chapter 3, Fig 3.1 and Table 3.1). At each site, three plots along a linear transect were selected 80-100 m apart. At each plot, a tree was marked as the center and all trees taller than 1 m were sampled and mapped following concentric circles of increasing size until approximately 50 trees were included.

Total genomic DNA from the fresh foliage of *C. nootkatensis* was isolated and genotyped using five microsatellite markers (see Chapter 2).

Clonal assignment was first performed using an initial statistical test based on calculations relating to probabilities of genotype occurrence and then confirmed using a method based on spatial autocorrelation of multilocus genotypes.

4.3.2 Extent of clonality

To provide an estimate of the spatial density of clonal genets within the plots, clonal genet number (the number of genets which exhibited clonal growth) was standardized by the estimated surface area sampled. The size of the clonal genets was estimated by the surface area of a circle with a diameter equal to the distance between the two farthest ramets found within each genet. The number of ramets per clonal genet was averaged in each plot and was then divided by the size of the clonal genet to estimate the ramet density per clonal structure.

Differences in number of clonal genets detected and number of clonal genets per 100 m² at each site were tested using an analysis of variance (PROC ANOVA) in SAS 8.02 (SAS Institute Inc.). Estimated size of clonal genets, number of ramets per clonal genets, and density of ramets within clonal ramets were tested for differences between the three sites using a general linear model analysis of variance (PROC GLM) in SAS because of the unbalanced nature of the data.

4.3.3 Clonal diversity

Clonal diversity has been assessed in plant populations using methods and indexes developed by ecologists to measure species diversity and evenness (Parker 1979; Ellstrand and Roose 1987; Jelinski and Cheliak 1992; Parker and Hamrick 1992; Eckert and Barrett 1993;

Montalvo et al. 1997). Simpson's diversity index modified for finite sample size (Pielou 1969) can be used to measure the probability that two trees selected at random in a population of N trees will have different multilocus genotypes (Ellstrand and Roose 1987). This index yields a measure of multilocus genotypic diversity and is calculated as

$$d = 1 - \sum \left\{ \frac{[n_i(n_i - 1)]}{[N(N - 1)]} \right\}$$

where n_i = number of individual trees of genotype *i*. A high value of *d* is interpreted as high genotypic diversity of clones or low number of clonal structures sharing the same genotype. Following Ellstrand and Roose (1987), a standardized measure of equitability of which stems are distributed among genotypes is calculated using Fager's (1972) measure of evenness,

$$E = \frac{d}{d_{\max}}$$

where,

$$d_{\max} = \frac{\left[(k-1)N \right]}{\left[k(N-1) \right]}$$

and k = number of clones. Simpson's index is bounded by 0 and d_{max} . Evenness, ranging from 0 to 1, provides a mean to standardize Simpson's index for different values of d_{max} and allows inter-population comparisons (Montalvo et al. 1997).

The number of distinct genotypes (*G*) relative to the number of stems sampled (*N*) is another frequently used measure of clonal diversity and was calculated here. This statistic (*G/N*) was termed "proportion distinguishable" by Ellstrand and Roose (1987). In the case where all stems sampled have unique genotypes, G/N = 1 and there is maximum diversity. In other words, all genets are constituted of a single ramet, and no clonally derived individuals are found.

Values of evenness (*E*) and proportion distinguishable (*G/N*), once calculated for each plot, were tested for statistical differences between the three sites using analysis of variance (PROC ANOVA) in SAS 8.02. The unit of observation was evenness (*E*) and proportion distinguishable (*G/N*) at each plot within sites and the means were compared among sites.

In addition to ecological measures of clonal diversity, different measures of genetic diversity and genetic structure at various spatial scales were estimated to provide a clearer picture of the population genetics of *C. nootkatensis*.

4.3.4 Genetic variation

In the analysis of genetic variation, each identified clone was treated as a single sample. Locus Y1G09 was omitted from this analysis as well as subsequent analyses because it was found to have a null allele in high frequency (see Chapter 2 of this thesis).

The following four measures of genetic variation were calculated for the populations of trees at each site and each locus: (1) the average number of alleles per locus was calculated, estimates of (2) observed heterozygosity, (3) expected heterozygosity, and (4) the inbreeding coefficient were obtained following Hedrick (2000). Their associated standard errors were estimated by performing 100 bootstrap estimates, using a FORTRAN program written by K. Ritland. Allele frequencies in each site were plotted for all loci at the three sites. In addition, Hardy Weinberg equilibrium was tested using the algorithm developed by Guo and Thompson (1992) for exact test of Hardy Weinberg proportions in the program Genepop (Raymond and Rousset 1995). Estimates of inbreeding coefficient were tested for deviation from zero (F = 0) using two-tailed t-tests (Sokal and Rohlf 1995 p.169).

Clonal ramets were excluded from the analysis because they would artificially increase the frequency of certain genotypes and alleles, therefore biasing allele frequency-dependent estimates of genetic variation such as observed and expected heterozygosity and inbreeding coefficient.

4.3.5 Genetic structure

To evaluate genetic structure among sites or populations, Wright's (1951) *F*-statistic was calculated using the program SPA*GeDi* 1.0 (Hardy and Vekemans 2002). In this program, the estimation procedure is based on a nested ANOVA following Weir and Cockerham (1984). These statistics were computed among sites for an estimate of genetic structure within the *C*. *nootkatensis* populations sampled. Estimates of standard error for the intra-class correlation coefficients of allelic states for genes within individuals relative to all populations (*F*_{*it*}), genes within individuals relative to a population (*F*_{*is*}), and genes within populations relative to all populations relative to all population (*F*_{*st*}) were obtained from a jackknife routine performed on the four microsatellite loci in SPA*GeDi* 1.0.

In addition, an analysis of spatial autocorrelation of multilocus genotypes, as described in section 3.3.4, was performed to describe fine scale genetic structure within the three sites. This technique has been described as a powerful tool in detecting the nature and scale of genetic differentiation across a range of different spatial scales (e.g. Sokal and Oden 1978; Cliff and Ord 1981). Autocorrelation techniques differ from statistics based on allele frequencies such as F_{st} because they make fewer assumptions regarding underlying population genetic model (Heywood, 1991). Also, no information is lost by pooling individuals into arbitrary sampling areas for subsequent comparison of gene frequencies among subpopulations. In autocorrelation analyses, each individual's spatial location, relative to the others, can be utilized (Heywood 1991, Epperson 1993). Consequently, these techniques can possess superior power over methods based on nested samples of gene frequencies for detecting spatial patterns (Epperson and Li 1996; Epperson and Tian-Quan 1997). Furthermore, few studies concerned with the effect of clonal reproduction on fine scale genetic structure in plant species have used spatial autocorrelation techniques (Montalvo et al. 1997; Chung and Epperson 1999; Reusch et al. 1999; Chung et al. 2000; Chung and Epperson 2000) and can therefore be directly compared with this study.

All clonally derived individuals were excluded from the analysis and the center of mass of each genet was used for spatial location. Again, spatial genetic structure was tested for significance by performing 1000 permutations of intact individual genotypes for all spatial position recorded in the program SPA*GeDi* 1.0. Ninety-five percent confidence intervals around the null hypothesis of no genetic structure were estimated and plotted alongside the estimated coefficients of kinship at given distance classes.

To investigate the influence of clonal or asexual reproduction on genetic structure in *C. nootkatensis*, estimates of kinship coefficients obtained above were compared to those obtained from the dataset inclusive of all individuals. Standard errors associated with estimates of kinship coefficient from both datasets were calculated from the jackknife procedure used in SPA*GeDi* 1.0 following Sokal and Rohlf (1995, p.821).

4.4 Results

4.4.1 Clonal extent

Clonal extent was measured by 1) the absolute number of clonal genets detected, 2) the number of clonal genets detected per 100 m², 3) the estimated size of clonal genets, 4) the number of ramets per clonal genet, and 5) the spatial density of ramets in genets. These values for each plot within sites are reported in Table 4.1.

The mean number of clonal genets detected per plot was 5.6 (SE=0.8) and was not significantly different between sites (F=4.16, p=0.074, df=2) (Fig. 4.1A). After standardizing for surface area sampled by calculating the number of clonal genets detected per 100 m², however, the difference between sites was found to be highly significant (F=11.70, p=0.009, df=2) (Fig. 4.1B). The average values for this variable were 0.14 (SE=0.04) clonal genets/100 m² at Mount Seymour, 0.94 (\pm 0.25) at Black Tusk, and 4.02 (SE=1.01) at Mount Washington.

Differences between sites were not significant for the estimated size of clonal genets (F=2.24, p=0.117, df=2) (Fig. 4.2A), the number of ramets per clonal genets (F=1.38, p=0.261,

df=2) (Fig. 4.2B), or the spatial density of ramets in clonal genets (F=0.92, p=0.404, df=2) (Fig. 4.2C).

4.4.2 Clonal diversity

The number of trees or ramets (*N*) per plot averaged 54.0 (SE=2.8) while the number of clones or genets (*G*) averaged 41.7 (SE=4.5), showing that on average ~23% of trees sampled were clonally derived (Table 4.2). The proportion distinguishable averaged 0.777 (SE=0.072), Simpson's diversity index averaged 0.974 (SE=0.012), while the measure of evenness averaged 0.980 (SE=0.012). The high evenness shows that, combined with the fact that the occurrence of clonality is low to moderate, the number of different genotypes in all clonal genets is very high, resulting in a very minimal "loss" of genetic diversity due to clonal reproduction. The proportion distinguishable, "*G/N*", was not significantly different among sites (F=2.17, p=0.195, df=2) (Fig. 4.3A) and the same was true of Fager's measure of evenness "*E*" (F=1.21, p=0.362, df=2) (Fig. 4.3B). Plot 2 of the Black Tusk site was found to have higher clonal diversity than the other two plots found within this site (Table 4.2).

4.4.3 Genetic variation

The number of alleles per locus averaged 14.4 (\pm 1.7), while observed and expected heterozygosities averaged 0.66 (SE=0.04) and 0.78 (SE=0.02), respectively, over all populations and loci (Table 4.3). Significant deviations from Hardy Weinberg proportions were consistently found across populations in loci Y1F10 and Y1E10. Across loci, all three populations were found to deviate from H-W proportions with high significance. Estimated coefficients of inbreeding deviated significantly from zero consistently across sites in loci Y2C12 and Y1E10. The Mount Seymour site had the highest inbreeding coefficient when averaged over loci (0.26; SE=0.06), while the Black Tusk site had an intermediate value (0.18; SE=0.04), and the Mount Washington site had the lowest estimated coefficient of inbreeding (0.06; SE=0.04).

Loci Y1F10 and Y2C12 exhibited allele frequencies suggestive of a bimodal distribution (Figs. 4.4-4.6). Distributions of allele frequencies at locus Y1E10 and Y2H01 more closely resembled a normal distribution. All loci except Y2H01 were affected by a very high number of alleles present at very low frequencies. A large number of these alleles were rare alleles (i.e., frequency < 0.05).

4.4.4 Genetic structure

 F_{st} was found to be low but significantly different from zero (0.023±0.008, Table 4.4). Average F_{it} and F_{is} values, after the jackknife routine, were high (0.168±0.029 and 0.148±0.030, respectively) and suggest that high levels of inbreeding within sites can explain most of the genetic structure observed.

Fine scale genetic structure was spatially explored using genetic spatial autocorrelation analyses. In the first step, all clonally derived individuals were taken out of the analysis. Most genetic correlation that could be attributed to the presence of half-sibs (where $\hat{\rho}_{ij}$ =0.25) disappears at the Mount Seymour site in distance classes higher than two to three meters (Fig. 4.7). This structure was not observed in the other two sites since the highest coefficient of kinship was 0.07 at Black Tusk and 0.14 at Mount Washington.

Significant spatial genetic structure, as measured by the lack of interception of the estimated coefficient of kinship with the 95% confidence interval (which corresponds to the null hypothesis of no genetic structure) varied between sites. The disappearance of significant genetic structure occured in the Mount Washington site at ~10 m and at ~13 m at the Black Tusk site (Fig. 4.7). It did not appear within the 20 m specified in the analysis at the Mount Seymour site. These observations suggest that genetic structure spans long distances at Mount Seymour but is more spatially restricted at Black Tusk and Mount Washington, disappearing within a short distance of ~10 to 15 m.

To investigate the effect of clonality on spatial genetic structure, a genetic spatial autocorrelation analysis inclusive of all ramets was performed. Kinship coefficients within the

first three meters varied from ~0.2 to 0.4 and greatly decreased by five meters, where they hovered around the upper limit of the 95% confidence interval (Fig. 4.8). The estimated coefficient of kinship intercepted the 95% confidence interval around the null hypothesis of no genetic structure at ~10 m in the Mount Washington site, at ~20 m in the Black Tusk site, and did not intercept this confidence interval within the 20 m specified in the analysis in the case of the Mount Seymour site. A subsequent analysis revealed that the interception of the confidence interval occurred at ~35 m (results not shown).

In addition, to directly compare the data set inclusive of all genotypes and the data set excluding all replicated genotypes, these were plotted together with their respective standard errors for their estimated coefficient of kinship (Fig. 4.9). In all three sites, the inclusion of all clonally derived individuals resulted in more genetic structure, especially between the distance classes of one and five meters. Results from Mount Seymour showed that clonality had little effect on the spatial genetic structure except at the distance class of three meters. At the Black Tusk site, the effect of clonality on structure became non-significant at approximately five meters while in the Mount Washington site, this occurred at around seven meters. The effect of clonality was most notable at the Mount Washington site, with a shift in kinship coefficient of \sim 2.3 in the smallest distance classes while this shift is smaller at \sim 1.0 at the Black Tusk site and not significant at the Mount Seymour site.

4.5 Discussion

4.5.3 Clonal extent

Differences in clonal extent between sites were only significant when measured as the number of clonal genets per 100m² (Fig. 3.5B). This measure showed that clonal extent was greatest at Mount Washington and least at Mount Seymour site. The total number of clonal genets detected (another measure of clonal extent) showed a similar trend but the differences were not statistically significant (Fig. 3.5A). Although no data for soil moisture were collected, site description, vegetative associations, and differences in clonal extent found in this study

suggest that clonal reproduction in *C. nootkatensis* may be more important in moist habitats and decreases with an increase in drainage. This is in agreement with the findings of Hennon et al. (1998) in stands of *C. nootkatensis* in southeast Alaska where the authors estimated layering based on the observation of prostrate form of the tree species. Hennon et al. (1998) stated that layering was not so common in more productive, healthy forests. Observations from this thesis of multiple large clones composed of more than a single ramet of very large size in the mature and productive site of Mount Seymour suggests otherwise. These findings, however, must be interpreted cautiously because patterns of clonality at the three different sites could be biased due to the small sample size and because the growth and dispersal patterns at early colonization and aging of the habitat need to be considered.

4.5.2 Clonal diversity

Clonal diversity, as described by the genotypic diversity of all ramets, was calculated here using Simpson's diversity index "*d*", Fager's measure of evenness "*E*", and the proportion distinguishable "*G/N*". The latter measure of clonal diversity was more variable, most likely an artifact of its sensitivity to the number ramets and genets sampled. Evenness was on average very high (0.980), showing that Simpson's index of diversity usually approached d_{max} . Measures of *d* and *E* were high because of the highly variable nature of the microsatellite markers used in this study. The lowest values of clonal diversity were found in plot one of the Black Tusk site and plot three of the Mount Washington site. Incidentally, the largest clones comprised of the greatest number of ramets were also found in these plots (see Chapter 3, Figure 3.2 and this chapter, Table 4.1).

Studies concerned with clonal reproduction in tree species are rare as evidenced by the presence of only one tree species (*Populus tremuloides*) out of a total of 27 plant species in the review by Ellstrand and Roose (1987). A more recent review (Widén et al. 1994) showed a slight increase in the number of woody species investigated but conifer representation is still very low. Hence, some comparisons are possible but must be made with caution. Clonal

diversity was high in *C. nootkatensis* relative to most of the species reviewed by Ellstrand and Roose (1987) where the mean values of *d* and *E* were 0.62 and 0.68, respectively. The proportion distinguishable was higher in this study than that revealed by RAPD and AFLP in the closely related Japanese conifer *Cryptomeria japonica* (*G*/N=0.103) (Moriguchi et al. 2001), which also clonally reproduces by layering. The clonal diversity found in *C. nootkatensis* was slightly lower than that found in trees such as *Quercus chrysolepis* (*G*/N=0.97) (Montalvo et al. 1997) and *Populus tremuloides* (*G*/N=0.92) both using allozyme loci (Jelinski and Cheliak 1992) and is closer to that of *Quercus geminata* (*G*/N=0.62) using microsatellite loci (Ainsworth et al. 2003). All of these tree species are known to use root suckering as their primary means of clonal reproduction. Finally, clonal diversity found in this study was much higher than that typically found in most woody shrubs such as *Betula glandulosa* (*G*/N=0.13), as defined by multilocus allozyme loci and RAPD (Sydes and Peakall 1998), and *Rhododendron ferrugineum* (*G*/N=0.08), using AFLP (Pornon and Escaravage 1999).

It should be noted that comparison of clonal diversity between studies is difficult. Sampling methods, number of samples, type of marker used, and mode of clonal or asexual reproduction may greatly influence the end results, thus the comparisons made above should be interpreted cautiously and understood to be only *estimates* of clonal diversity. A number of studies have illustrated this point with the comparison of clonal diversity obtained from different molecular markers for the same samples (e.g. Sydes and Peakall 1998; Tani et al. 1998; Moriguchi et al. 2001).

Clonal diversity was highly variable between plots as evidenced by the range observed (Table 4.1). Due to this great variation and the small sample size (N=3), no difference in clonal diversity was detected between the three sites. The lack of statistical difference in clonal diversity between sites may be the result of ecological heterogeneity within sites, resulting in increased statistical variance. This factor is evidenced by the low clonal diversity detected in plots one and three of the Black Tusk site where wet depressions and prostrate form of *C*.

nootkatensis, suggestive of clonal reproduction, were found. In future studies, greater care should be taken when making site selection to avoid such heterogeneity. An approach using floristic survey and subsequent principal component analysis (PCA), to test discrimination between sites and ecological similarity between plots, could have alleviated this problem. Similarly, increasing the number of plots at each site could help to reduce the variance. The power $(1-\beta)$ of the analysis of variance for the proportion distinguishable and Fager's measure of evenness was 0.26 and 0.17, respectively (Sokal and Rohlf 1995, p. 264). The small sample size resulted in very low power; therefore, the lack of significant statistical difference should be viewed less as a lack of true difference and rather a lack of discrimination power. It is also possible, however, that trends in clonal diversity operate at a scale different than the one for which it was tested in this study. With the estimates of variance and given a certain statistical power $(1-\beta)$ and error rate (a), a sample size required to obtain significance can be calculated. For the proportion distinguishable "G/N", if we assume that the largest differences between means is unchanged and for α =0.05, the sample size should be equal to 9 for a power of 0.83 and equal to 25 for a power of 0.99. For Fager's measure of evenness "E", the sample size should be 14 for a power of 0.82 and 40 for a power of 0.99. These are very high sample sizes, especially when the amount of work necessary to generate this data is taken into account. These observations suggest that significant differences in clonal diversity in *C. nootkatensis*, given the current methods available, may be difficult to obtain and will require more extensive sampling.

4.5.3 Genetic variation

The four microsatellite loci used in this study to assess genetic variation were polymorphic at all sites and plots within sites. No genotypes of genets were found to be duplicated. The mean number of alleles per locus (12.8, SE=1.2) was high compared to that found in isozyme loci (Ritland et al. 2001) and comparable to that found in nine microsatellites in *C. obtusa* (10.3, SE=1.7) (Nakao et al. 2001). Genetic variation as estimated by heterozygosity

expected under Hardy Weinberg equilibrium (H_e) averaged 0.78 (SE=0.02). This value was much higher than that obtained by Ritland et al. (2001) (H_e =0.147, SE=0.003), a likely consequence of the much greater number of alleles found at the microsatellite loci. Nakao et al. (2001) obtained a very similar value from nine microsatellite loci in one population of 18 individuals (H_e =0.77, SE=0.05). Ritland et al. (2001), in their study, compared estimated values of genetic diversity from allozyme data between *C. nootkatensis* and seven other closely related species. In their comparison, *C. obtusa* was found to be more genetically diverse (H_e =0.202) (Uchida et al. 1997) than *C. nootkatensis*. The close similarity in genetic variation between this study and the study of Nakao et al. (2001) is likely due to the limited number of individuals sampled by Nakao et al. (2001). Sampling of multiple populations of *C. obtusa* would most likely result in a greater detected genetic variation, which, in turn, would render the result of the comparison between microsatellite data more similar to that found in isozyme data.

Genetic variation, as estimated by 1) the mean number of alleles per locus and 2) the average observed and expected heterozygosities, did not differ significantly between collection sites (Table 4.3). Classical descriptions of genetic patterns in clonal plants predicted that in populations or species where clonality occurs, genetic and/or genotypic diversity would be lowered or even reach depauperate levels. Most recent studies comparing levels of genetic variation between species and intraspecific populations varying in clonal extent, however, have demonstrated comparable-to-high levels of genetic variation (reviewed in Ellstrand and Roose 1987; Widén et al. 1994). This study, showing varying levels of clonal extent, yet no significant difference in genetic variation between sites, is in agreement with the most recent findings. Clonal growth, or asexual reproduction, has been demonstrated, through simulations, to erode genetic diversity, but these simulations have also shown that even periodic episodes of sexual recruitment can maintain genetic diversity in clonal organisms (Watkinson and Powell 1993). In addition, clonal growth can potentially also conserve genetic diversity by increasing the life span of genotypes with rare alleles.

The overall inbreeding coefficient reported in this chapter (F=0.17, SE=0.05) (Table 4.3) was high but very similar to that found by Ritland et al. (2001) (F=0.184, SE=0.022) and to that reported in Chapter 2 of this thesis (F=0.161, SE=0.025) (Table 2.2). This result is surprising for a wind pollinated tree and even more so for a conifer. If selfing alone was responsible for the inbreeding coefficient and if F was constant among generations, it would require (after Ritland et al. 2001) a selfing rate of 2F/(1+F)=0.29 to explain this inbreeding coefficient value in C. *nootkatensis*. Mortality caused by inbreeding depression before the census would raise the inferred rate even higher. Such high levels of inbreeding appears to be a common feature of many species in the family Cupressaceae. In fact, species such as C. *thyoides* (F=0.200) (Kuser et al. 1997) and *Cupressus forbesii* (F=0.285) (Truesdale and McClenaghan 1998) have higher levels of inbreeding than *Chamaecyparis nootkatensis*.

Interestingly, and contrary to expectations, the trend in estimated inbreeding coefficient averaged over loci in the three sites (Table 4.3) appears to be inversely associated with the observed clonal extent (Table 4.1); sites with high inferred levels of clonal reproduction such as Mount Washington have low estimated inbreeding coefficients. Because the sample size was so small (N=3), caution must be taken when interpreting this trend. High levels of clonal reproduction, by multiplying identical genotypes, should theoretically increase the probability of selfing. This is especially true in species, such as *C. nootkatensis*, that exhibit phalanx clonal growth and have limited pollen dispersal, because genetically identical individuals would exist in close proximity and share self-pollen. This could be due to differential selection regimes at each site as well as, or in association with, different age structures of the stand. In addition, the amount of clonal reproduction may have been historical periods of high inbreeding may have purged deleterious alleles or genes tightly associated with genes involved in sexual reproduction and, therefore, favored in genes or gene combinations associated with genes promoting clonal reproduction. Simultaneous selection for heterozygosity may have led to

populations in which highly clonal individuals may be now responsible for conserving highly heterozygous genotypes and perhaps gene combinations responsible for high levels of fitness. These possible explanations point towards further investigations in *C. nootkatensis* and other clonal plants which may shed light on the evolutionary role, costs, and benefits of clonal reproduction.

4.5.4 Genetic structure

Plant genetic structure results from the combined action of mutation, migration, selection and drift, which operate within the confines of the species' historical and biological context. As illustrated by Loveless and Hamrick (1984), ecological factors affecting reproduction and dispersal are likely to be especially important in determining genetic structure. The apparent high level of inbreeding in *C. nootkatensis* should increase genetic correlations between uniting gametes, reduce recombination, and maintain gametic phase disequilibrium. This should, in turn, result in increased divergence among populations due to drift and a reduction in gene flow. Within populations, heterozygosity and within-family genotypic diversity should consequently be reduced, and between-family genetic variation should be increased. This situation would lead to high population subdivision (Loveless and Hamrick 1984).

At a fine spatial scale, clonality in *C. nootkatensis* may become an important factor affecting genetic structure. Facultative clonal reproduction may slow the decay of genetic variance (Levin and Kerster 1971), producing aggregated genotype distributions, and altering genotypes' fecundity schedules (Loveless and Hamrick 1984). By increasing longevity in some genotypes, clonality can increase generation time. This, in turn, may result in the increase of the effective population size due to the increase in time of overlapping generations, and the subsequent inhibition of population subdivision. In self-compatible plants, however, spatial clumping of identical genotypes may give rise to a reduction of the effective population size and, therefore, promote local subdivision. Finally, clonal reproduction also prevents recombination, thus allowing a perpetuation of gametic phase disequilibrium, an increase in population

divergence, and a slowing of the population's approach to equilibrium. Because *C. nootkatensis* appears to tolerate a high amount of inbreeding, reproduces clonally, and is hypothesized to have limited gene flow, genetic structure should be high at all scales observed.

4.5.4.1 Large-scale genetic structure

Genetic structure among sites, as described by Wright's (1951) F_{st} , was found to be lower (F_{st} =0.023, SE=0.008) than that found by Ritland et al. (2001) (G_{st} =0.139). This is probably the result of sampling only three populations, all of which are found in only one of the three different phylogeographic clades described by Ritland et al. (2001). Although F_{st} was small, it was significantly different from zero, showing evidence of genetic divergence among populations operating at this scale, for this species. This finding is not surprising given the putatively short dispersal range of both seed and pollen and the disjunctive nature of *C*. *nootkatensis* tree distribution across the landscape.

Values of F_{it} and F_{is} over loci (Table 3.7) averaged 0.168 (SE=0.029) and 0.148 (SE=0.030), respectively. These high values, which are directly related to the estimated inbreeding coefficient, are indicative of the important role played by inbreeding in defining genetic structure in *C. nootkatensis*. High inbreeding drastically increases genetic differentiation among populations. As reviewed by Ritland et al. (2001), high levels of inbreeding appear to be present in many taxa within the Cupressaceae; this characteristic may have played an important role in the evolution and diversification of members of this family. Inbreeding may decrease allelic variance within populations and, in combination with random genetic drift, increase genetic differentiation among population.

4.5.4.2 Genetic spatial autocorrelation excluding clonally derived individuals

A spatial autocorrelation approach was used to investigate genetic structure at a fine scale. Clonal ramets were removed from the analysis to investigate fine scale genetic structure

in *C. nootkatensis* without the influence of clonal structures. In doing so, only the effect of the present clonal ramets is excluded; the effect over time of clonal reproduction on mating between close relatives and like genotypes and their effect on the spatial distribution of genes cannot be excluded, but only observed.

In their study of clonal growth and limited gene flow in eelgrass, *Zostera marina*, Reusch et al. (1999) used the nearest neighbor (between a ramet of a clonal genet and a genetically different neighboring ramet) when calculating kinship in a spatial autocorrelation analyses. These authors suggest that this method is more representative of the potential gene flow than using the center of mass of a clonal genet (e.g. Montalvo et al. 1997 and this thesis). In the case of *C. nootkatensis*, however, genet sizes (area occupied by a genet) were smaller or similar to the height of most trees. Because this species is wind-pollinated, using the nearest neighbor as opposed to using the center of mass would not significantly affect the results.

The highest estimate of both kinship and inbreeding were found at the Mount Seymour site. Mount Seymour had the largest and tallest trees in this study. Because taller trees can potentially spread their seeds and pollen further than a shorter counterpart, this high degree of genetic correlation between trees at this site is surprising. The scale and tree density found at this site may however, explain this finding.

The trees were larger at the Mount Seymour site than at the other two sites, but they were further apart as evidenced by the high surface area needed to sample ~50-60 trees (see Chapter 3, Fig. 3.2). This lowered density of trees may significantly decrease the proportion of outcrossing events in this population and thus favor selfing. Also, the size and spatial distribution of the trees and the associated vegetation found at this site was suggestive of a late successional forest ecosystem. The high kinship value estimated at this site could be the result of a long history of selection favoring a few highly related individuals. At Mount Seymour, a longer history or presence of *C. nootkatensis* versus the other two sites may have also allowed more time at this site since colonization by this species and, subsequently, greater potential for successful mating events between closely related individuals. The observation of greater

genetic structure at the Mount Seymour site, in itself, provides evidence for old age because theoretical predictions show that old populations should have more spatial genetic structure than recently established populations (Epperson 1990, 1992). Alternatively, the population found at Mount Seymour may have experienced an early bottleneck, due to its high elevation and consequent harsher climate, which could have left only a few highly related individuals standing. Similarly, a founder effect during the initial colonization in this species following the last glaciation, 10, 000 ybp, could potentially explain this high kinship value.

Genetic structure, as measured by the coefficient of kinship, disappears in the Mount Washington and Black tusk sites at approximately 10-15 m. This observation suggests limited amount of gene flow within these two populations. Alternatively, and because no seedlings were observed at the sampling sites, this pattern could be the consequence of strong local adaptation at a small spatial scale. This explanation is, however, rather unlikely because of the scale and heterogeneity of the landscape in most forest ecosystems which would simply not favor such a narrow clumping of similar genotypes. Conversely, at Mount Seymour, positive and significant pairwise relatedness appeared to extend beyond the 20 m limit. A further analysis of this site showed this genotypic structuring to become non-significant at ~35 m. Given the scale of tree density and size of clonal genets, these differences are substantial. Tree height is an important factor in determining the distance potentially traveled by pollen and seeds because these are wind-disseminated in *C. nootkatensis*. The findings relating to genetic structure presented here attest to this trend because of the association between average tree height at each site and the boundary at which genetic correlation between individuals disappears.

The shape of the curves in the correlograms at Mount Seymour and Mount Washington (Fig. 4.7) show a clustered distribution of closely related genotypes at short distance intervals. This pattern of clustered and highly positive correlations suggests a case of isolation-by-distance in these populations (Sokal and Wartenberg 1983). In contrast, the clinal pattern of the curve in the correlogram for the Black Tusk site is more suggestive of either directional

migration or selection (Sokal et al. 1997). This observation could potentially be further investigated using correlograms that include the directionality of the autocorrelation.

Assuming random mating, the coancestry estimator is a measure of the inbreeding coefficient between related individuals with an expected value of 0.5 between clonal ramets, 0.25 for full-sibs, and 0.125 for half-sibs. Coefficients of kinship observed within the first ~1-3 m in the Black Tusk and Mount Washington sites (~0.07-0.14, Fig. 4.7) are much less than that expected for full-sibs, suggesting that although some proportion of seed dispersal may be localized, there is substantial mixing of seed shadows. In this regard, the coefficients of kinship observed at small distances at the Mount Seymour site suggest the presence of half-sibs within close proximity of each other. This is surprising for a site where the tallest trees were observed. It is likely, however, that a putatively longer period since colonization, and thus, of selection at this site, may have favored the aggregation of highly related individuals.

Hamrick et al. (1993) documented measures of genetic similarity at a spatial scale in two wind dispersed tree species, *Alseis blackiana* and *Platypodium elegans* from the tropics. Positive significant genetic structure was revealed in *Alseis* and *Platypodium* juveniles out to distances of 30 m and 100 m, respectively. By contrast, this structure was not found among adults, presumably due to mortality during recruitment. These observations demonstrate that gene flow is very limited in *C. nootkatensis* when compared to other wind pollinated tree species. Interestingly, differences in genetic structure between juveniles and mature trees appear to be contradictory to the patterns observed in *C. nootkatensis*. Admittedly, tree size is often not an adequate surrogate for age, but stand characteristics in the *C. nootkatensis* sites are suggestive of an older stand in the Mount Seymour site and a younger stand at the Mount Washington site. For both sites, significant genetic correlation among individuals was observed and, in fact, Mount Seymour exhibits a more extensive genetic neighborhood. It would be of great interest to age all trees sampled and compare genetic structure between age classes, but aging *C. nootkatensis* trees is difficult (see Chapter 3).

4.5.4.3 Clonal reproduction and its effect on fine scale genetic spatial autocorrelation

The presence of multiple individuals of identical genotypes within close proximity should increase the mean kinship coefficient over small distance intervals. In addition, time, lack of self-incompatibility, and short dispersal distances, should result in an increase in spatial scale and amplitude of genetic autocorrelations.

At the three sites, at short distance intervals, mean kinship was greater when all ramets (including clonally derived one) were included, and this, at all three sites. This agrees with expectations and is congruent with results obtained in the oak, Quercus chrysolepis (Montalvo et al. 1997), the herbaceous perennial, Adenophora grandiflora (Chung and Epperson 1999), the eelgrass, Zostera marina (Reusch et al. 1999), and the Asian tropical tree, Rhus javanica (Chung et al. 2000). The study of Eurya emarginata (Chung and Epperson 2000), a dioecious, insect-pollinated tree which combines sexual reproduction and clonal spread, is the only study I know of that showed no significant effect of clonal reproduction on the fine scale genetic structure in the populations studied. The difference in mean kinship coefficient between datasets was noted to be greater at the Black Tusk and Mount Washington sites. The relatively smaller difference between correlograms at the Mount Seymour site was an artifact of a smaller number of observations in small distance classes as evidenced by the high standard errors reported. In addition, the patterns of clonal growth observed at Mount Seymour (see Chapter 3, Fig. 3.2) suggest greater self-thinning of clonal structures or clonally derived ramets. This would negatively affect the mean kinship coefficients observed in small distance classes by decreasing the number of identical genotypes. The Mount Washington site exhibited the greatest difference in mean kinship coefficients between the dataset including all genotypes and the dataset excluding all replicated genotypes. This site was also found to exhibit the greatest amount of detectable clonal reproduction as evidenced by the number of clonal structures per unit surface area (Table 4.1, Fig. 4.1B). In fact, the difference between correlograms within sites appears to

be associated with clonal extent. This observation suggests that clonal reproduction may have an important effect on fine scale genetic structure.

Differences between correlograms obtained from the two datasets appear to become non-significant after a short distance (~5-10 m). This confirms that clonal size in *C. nootkatensis* is small, a common trend in most woody clonal plants asexually reproducing by layering which are typically classified as phalanx type clones (e.g. *Xanthoxylum americanum*, Reinarzt and Popp 1987; *Gleditsia tricanthos*, Schnabel and Hamrick 1990; *Quercus laevis* and *Q. margaretta*, Berg and Hamrick 1994; *Q. havardii*, Mayes et al. 1998). Also, this suggests that there is a general absence of very large clonal patches in this species.

Trends observed at all three sites may offer an insight into the role, over generations, of clonal reproduction in modifying fine scale genetic structure. Aggregation of identical genotypes due to clonal growth should theoretically increase genetic autocorrelation, as illustrated above. The proximity of the genetically identical individuals will most likely cause an increase in the incidence of consanguineous mating, further increasing genetic autocorrelation. Over generations, this process, similar in fashion to a feedback loop, will repeatedly accelerate the development of genetic isolation by distance. The data presented here, fit this theory. The putatively older site (Mount Seymour) exhibits less clonal extent (Table 4.1, Fig. 4.1B) but isolation by distance, as measured by correlograms, is more pronounced as well as more spatially extensive than in the other two sampled sites (Figure 4.7). More information regarding age of trees as well as site history or the three sites sampled may provide further evidence and support for this hypothesis.

The observations reported above demonstrate that sampling must be performed with care in clonal plants. By sampling all individuals at given sites, in this study, clonal individuals were revealed. The inclusion of all individuals in the spatial autocorrelation analysis demonstrated an effect of clonal individuals on fine scale genetic structure. Therefore, a sampling scheme, which does not acknowledge the presence and spatial distribution of clonal genotypes, may result in biased estimates of genetic variation and genetic structure. Although

this situation appears obvious to circumvent in phalanx clonal plants, it may be more troublesome in plants exhibiting guerilla-type growth where identical genotypes may be scattered throughout a population.

4.6 Conclusion

Clonal extent differed significantly between sites but significant differences in clonal diversity and genetic variation between sites were not detected. The low number of ramets per clonal genet and the overall high genetic variation detected by the microsatellite loci are likely explanations for these findings. Inbreeding coefficients were high for a conifer but not uncharacteristic of other members of the family Cupressaceae. Genetic structure detected among sites was low compared to previous studies but still significantly different from zero, showing that even within a restricted part of the species' range, geographical genetic structuring does exist. Finally, clonality in *C. nootkatensis*, as in a few other plant species, was shown to significantly increase fine scale genetic structure at short distance ranges. Based on these observation, it was hypothesized that, over time, clonality in *C. nootkatensis* would be an important factor in increasing mean pairwise genetic autocorrelation, even between physically distant individuals. This, in combination with high inbreeding in this species, may contribute locally to within stand increased fine scale genetic structure, and more globally to among population differentiation.

Table 4.1. Clonal extent in Chamaecyparis nootkatensis at three different sites with statistics relating to both ramets and clonal genets.

		Stat	tistics relating t	o genets	Statistics rela	ating to ramets
Collection site	Plot	No. clonal genets detected	No. clonal genets detected per 100m ²	Mean estimated size of clonal genets (m ²) (SE)	Mean number of ramets/genet (SE)	Mean spatial density of ramets in genets (ramets/m ²) (SE)
	-	4	0.20	5.08 (3.66)	2.25 (0.25)	1.78 (0.79)
Mount Seymour	2	с	0.05	14.69 (10.83)	2.33 (0.33)	0.40 (0.18)
	S	4	0.18	31.32 (24.28)	2.55(1.00)	0.41 (0.25)
	t	8	1.43	8.37 (3.33)	2.75 (0.62)	2.74 (1.90)
Black Tusk	2	5	0.79	1.66 (0.81)	2.00 (0.00)	7.69 (5.29)
	3	5	0.60	3.83 (1.64)	3.40 (1.17)	1.34 (0.29)
	٢	9	2.73	2.07 (1.07)	2.17 (0.17)	6.41 (4.41)
Mount Washingtor	2	6	6.00	3.29 (0.95)	3.11 (0.70)	3.01 (1.64)
	ġ	6	3.33	13.80 (6.76)	7.17 (2.50)	2.48(1.64)

Site	Plot	Trees or ramets (N)	Clones or genets (G)	G/N	σ	d _{max}	Е
	-	50	45	0.900	0.995	0.998	0.997
Mount Seymour	2	50	46	0.920	0.996	0.998	0.998
	3	57	49	0.860	0.989	0.997	0.992
Arithmetic mean (SE)		52.3 (2.3)	46.7 (1.2)	0.893 (0.018)	0.993 (0.002)	0.998 (0.000)	0.996 (0.002)
	ł	49	35	0.714	0.974	0.992	0.983
Black Tusk	2	60	55	0.917	0.997	0.998	0.999
	3	51	39	0.765	0.973	0.994	0.979
Arithmetic mean (SE)		53.3 (3.4)	43.0 (6.1)	0.799 (0.061)	0.982 (0.008)	0.995 (0.002)	0.987 (0.006)
	٢	52	45	0.865	0.994	0.997	0.997
Mount Washington	2	56	37	0.661	0.969	0.991	0.979
	3	61	24	0.393	0.877	0.974	0.900
Arithmetic mean (SE)		56.3 (2.6)	35.3 (6.1)	0.640 (0.137)	0.947 (0.036)	0.987 (0.007)	0.958 (0.030)

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Table 4.2. Clonal diversity in Chamaecyparis nootkatensis at three different sites.

Note: *G/N*= proportion distinguishable; *d*=Simpson's diversity index, *d_{max}*=Simpson's diversity index, maximum possible value; *E*=Fager's measure of evenness.

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Collection site	Locus	No. alleles per locus (SE)	H _o (SE)	H_e (SE)	F (SE)
	Y1E10	23.0(2.5)	0.75 (0.05)	0.94 (0.01)	0.20 (0.05)***
	Y1F10	17.0(2.1)	0.70 (0.05)	0.90 (0.01)	0.23 (0.05)***
	Y2C12	16.3 (3.5)	0.75 (0.05)	0.85 (0.02)	0.11 (0.05)*
	Y2H01	5.7 (1.5)	0.23 (0.04)	0.46 (0.05)	0.49 (0.10)***
Mean within site		15.5(2.0)	0.61 (0.05)	0.79 (0.02)	0.26 (0.06)***
	Y1E10	21.0(4.9)	0.81 (0.04)	0.94 (0.00)	0.14 (0.04)***
Alach Tuick	Y1F10	16.3 (2.6)	0.74 (0.04)	0.92 (0.01)	0.19(0.04)***
	Y2C12	17.3(0.3)	0.77 (0.04)	0.88 (0.01)	0.12 (0.04)**
	Y2H01	4.3 (0.9)	0.34 (0.05)	0.48 (0.04)	0.28 (0.08)
Mean within site		14.8(1.8)	0.67 (0.04)	0.80 (0.02)	0.18(0.04)***
	Y1E10	20.3(1.7)	0.80 (0.04)	0.94 (0.01)	0.15(0.04)***
Mt W/achinaton	Y1F10	15.0(1.5)	0.84 (0.03)	0.83 (0.02)	-0.01 (0.04)
	Y2C12	12.0(2.0)	0.88 (0.03)	0.88 (0.01)	0.01 (0.03)
	Y2H01	4.0 (0.6)	0.30 (0.04)	0.33 (0.04)	0.07 (0.08)
Mean within site		12.8(1.2)	0.70 (0.03)	0.74 (0.02)	0.06 (0.04)
Arithmetic mean amo	ng sites	14.4 (1.7)	0.66 (0.04)	0.78 (0.02)	0.17 (0.05)***

Note: *a*=0.05; *0.05≥p >0.01; *0.01≥p >0.001; ***p <0.001

Table 4.4. Genetic structure among sites in *Chamaecyparis nootkatensis* as estimated by
Wright's *F* statistics. Estimates of F_{it} , F_{is} , and F_{st} for 4 microsatellite loci calculated
according to the methods of Weir and Cockerham (1984).

Locus	F _{it}	F _{is}	F _{st}
Y1E10	0.175	0.163	0.014
Y1F10	0.173	0.136	0.043
Y2C12	0.102	0.085	0.018
Y2H01	0.298	0.292	0.008
All loci	0.171	0.151	0.023
Jackknifed estimators (over loci)		
Arithmetic mean (SE)	0.168 (0.029)	0.148 (0.030)	0.023 (0.008)

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Figure 4.1. Clonal extent in *Chamaecyparis nootkatensis* as measured by A) the number (±1 SE) of clonal genets and B) the number (±1 SE) of clonal genets per 100 m² detected in three different sites.

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Figure 4.2. Clonal extent in *Chamaecyparis nootkatensis* as measured by A) the estimated size (±1 SE) of clonal genets (m²), B) the number (±1 SE) of ramets per clonal genet, and C) the density (±1 SE) of ramets per clonal genets (ramets/m²). Note; MS=Mount Seymour, BT=Black Tusk, MW=Mount Washington. Numbers used next to these abbreviations stand for plot number.



Figure 4.3. Clonal diversity of *Chamaecyparis nootkatensis* at three different sites as measured by A) the "proportion distinguishable", *G/N*, (±1 SE) and B) Fager's measure of evenness, *E* (±1 SE).



Figure 4.4. Allele frequencies in four microsatellite loci at the Mount Seymour site.



Figure 4.5. Allele frequencies in four microsatellite loci at the Black Tusk site.



Figure 4.6. Allele frequencies in four microsatellite loci at the Mount Washington site.


Figure 4.7. Correlograms at three sites showing kinship coefficients, $\hat{\rho}_{ij}$, inclusive of **only the genotypes of the genets** (no genotype replication due to presence of clonal growth), and 95% confidence interval around the null hypothesis of no genetic spatial autocorrelation as a function of given distance classes.



Figure 4.8. Correlograms at three sites showing kinship coefficients, $\hat{\rho}_{ij}$, **inclusive of the genotypes of all ramets** sampled and 95% confidence interval around the null hypothesis of no genetic spatial autocorrelation as a function of given distance classes.



Figure 4.9. Correlograms at three sites showing coefficients of kinship, $\hat{\rho}_{ij}$, and their estimated standard error as a function of given distance classes. Results from the dataset inclusive of the genotypes of all ramets and the dataset excluding clonally derived genotypes are shown to illustrate the effect of multi-clonal genotypes on fine scale genetic structure.

CHAPTER 5: General Discussion

5.1 Evolutionary perspective on clonality in Chamaecyparis nootkatensis

Because clonal spread results in the proliferation of identical ramets, it directly affects the distribution and frequency of both genotypes and phenotypes within and among populations, thus, ultimately, the fashion in which populations and species evolve. Clonal reproduction was shown in this thesis to be an important mode of reproduction in *Chamaecyparis nootkatensis*. With its suspected limited gene flow, phalanx clonal growth, and apparent lack of self-incompatibility mechanisms, this species was expected to show high levels of inbreeding. Inbreeding in this species was, in fact, high for a conifer, but *C. nootkatensis*, as well as other members of the Cupressaceae appear to tolerate such high levels. The reasons behind this tolerance to high inbreeding remain unclear. Also, low genetic variation would be expected in clonally reproducing species. This was, however, not the case in *C. nootkatensis* as well as in many other of the clonal plants presented in the fourth chapter's discussion. What could explain the apparent contradictory trends that are high inbreeding, moderate to high clonality and high genetic variation in *C. nootkatensis*?

A well-known consequence of inbreeding is the accumulation of mutation-caused deleterious alleles in homozygous state, a condition termed "genetic load" (Charlesworth et al. 1990). When a certain number of deleterious alleles (often found in a recessive state) occur together in a zygote, death ensues. The proportion of the population that suffers genetic death is called the "load" (Falconer and Mackay 1996). Death of these individuals and, therefore, loss of these alleles is referred to as "purging" the genetic load. Obviously, a pre-requisite for purging to occur is sexual reproduction and expression in the homozygous state if they are recessive. Extremely low seed viability and sexual recruitment in *C. nootkatensis* constitute strong evidence for a high genetic load. Clonal reproduction may offer an escape from purging this load. Asexual reproduction such as clonality offers reproductive assurance and the species or population can escape from a severe decline in number due to load when successful sexual

reproduction is impeded. These mutations, however, could also negatively affect clonal reproduction. I hypothesize that in species that are highly sensitive to genetic load and are facultative clonal reproducers, clonal reproduction could be a life history trait ultimately favored by selection to avoid severe population bottlenecks. These bottlenecks may lead to decreased genetic variation that, in turn, could even push species to extinction. This hypothesis may explain the high frequency and strong persistence of clonality in many plant species and is in need of further research.

Also, in addition to being an instrument in avoiding genetic purging, clonal reproduction may also be a means preserving extremely fit gene combinations, which would otherwise be broken down by recombination during meiosis (Sisyphean fitness). Perhaps, in cases where fitness may be related to heterozygosity (see review in Savolainen and Hedrick 1995), this preservation of fit genotypes may also explain high levels of genetic variation in clonal species. There may be "optimal" levels of clonality for given life history characteristics that would limit the effects of purging, but without limiting evolutionary potential. Evidence for selection for high heterozygosity and greater clone size has been shown in the clonal canyon live oak, *Quercus chrysolepis* (Montalvo et al. 1997). These hypotheses, relating to the preservation of fit gene combinations and high genetic variation, may prove hard to investigate in a species such as *C. nootkatensis* where sexual reproduction rarely results in successful offspring due to poor seed germination. They could, however, prove important to our understanding of the role of clonal reproduction, as a life strategy, in the evolution and perpetuation of species. Perhaps, certain clonal plant species may be better suited than *C. nootkatensis* in becoming "model clonal plants".

The ideas explored above may offer insight into the evolutionary forces driving the switch from sexual to asexual modes of reproduction. The paleobotanical account of the evolution of plant clonality by Tiffney and Niklas (1986) suggests that clonality is the ancestral growth form. These authors also demonstrated, through the investigation of fossil records, an evolutionary trend towards a decrease in the prevalence of clonal growth. This appears to be

driven by the appearance of more derived non-clonal species and by the extinction of highly clonal plant species. Do these findings imply that clonality is an evolutionary dead-end? Clonality might be declining in representation across plant families, but because it offers reproductive assurance, it is a life-history strategy which allows more flexibility to plant species, especially when it is facultative. Also, because identical genotypes can give rise to various phenotypes under varying environmental conditions, even strict clonal reproduction still offers material for selection to operate upon.

5.2 Future research needs and conclusion

This thesis is exploratory in nature and a product of such work is often the realization that much more research needs to be performed in order to better understand the putative processes under observation. The research topics which should receive the most attention are: 1) developing a greater knowledge of seed and pollen dispersal in *C. nootkatensis*, 2) using a sound experimental design to attempt to ascribe clonal extent in this species to specific habitats or biotic/abiotic factors, 3) aging trees and clones to further understand the dynamic of clonality through time, and finally, 4) performing controlled crosses and survival tests on the offspring to measure fitness and test the hypothesis of clonality as a mean to avoid purging.

Our knowledge of seed and pollen dispersal in C. nootkatensis is mostly based on comparison of morphology of these structures in related species. Because seed and pollen are the primary vectors responsible for gene dispersal (movement due to clonality appears to be spatially limited), their movement in the landscape will directly influence genetic structure and genetic variation. Patterns in these parameters observed in this thesis are confounded by age structure and selection at the zygotic and seedling stages. Consequently, inferred levels of gene flow from patterns of genetic structure and genetic variation are often highly distorted. A better knowledge of pollen dispersal may confirm that the size of clonal patches limits most mating events to selfing (effective geitonogamy) while a knowledge of seed dispersal could

confirm that limited dispersal is an important cause of the high genetic structure observed in this species.

The types of habitats and/or biotic and abiotic factors, which are conducive to reproduction through clonal spread in *C. nootkatensis* have only been hinted at in this thesis. This knowledge, however, could be useful in using clonality as a means to naturally regenerate harvested sites where the conditions required for maximum clonal reproduction are met. An experimental design where numerous sites would be chosen randomly and then described for a given set of characteristics could be more useful than the design presented in this study. Characterizing clonal extent in numerous localities, however, would require an extensive amount of work and could potentially produce uncertain results. For instance, clonality may be more extensive in sites of very low productivity, sites which are unlikely to be harvested. The data presented in this thesis, however, support the existence of clonal reproduction in mature and highly productive sites suggesting the practical utility of understanding clonality.

Aging of trees and more importantly, ramets within clonal genets, could provide greater insight into the age of clones and therefore the role clonal growth plays in a population of *C. nootkatensis* trees. This information may shed light on the past role of clonal growth in the establishment of this species and possibly inform us of the potential for this mode of reproduction in natural reforestation in this species. Furthermore, a classification by age of all trees in a stand and a subsequent investigation of genetic structure in these different age classes may provide us with a clearer picture of when and how selection operates in *C. nootkatensis*. Finally, the aging of clones may reveal extremely ancient clones which work, in a similar manner as a seedbank, as "holders" of ancient alleles and genotypes, and "providers" of ancient genetic variation. This ancient genetic variation could prove useful because it may contain genes that have been exposed to past stresses such as climate changes, pests, and diseases. These genes may have been instrumental in the perpetuation of the species under these adverse conditions at the time and may become useful again under similar modern threats.

Controlled crosses between individuals of varying genetic distances may provide crucial clues to breaking the enigma behind extremely low frequencies of sexual recruitment in *C. nootkatensis*. Germination trials, however, may not fully reveal what is at play. Tests examining selection at the gametic, fertilization, zygotic, seedling, and various adult stages, in association with a strong understanding of the level of inbreeding or "outbreeding" of the offspring, could allow a dissection of the selection process and establish if selection due to environmental conditions are responsible for poor sexual recruitment or if a strong genetic component may be involved. Also, controlled crosses between highly clonal genets in comparison with crosses between aclonal genets may also show clear differences in germination because of accumulated genetic load in clonal genets. In association with this experiment, an estimation and comparison of the resources allocated to sexual reproduction between highly clonal genets and aclonal genets may prove interesting. For instance, this kind of experiment could reveal a trend towards a decrease in energy and resources allocated to sex in highly clonal plants and, therefore, demonstrate a shift from a sexual to an asexual mode of reproduction.

In essence, it is clear that much further research is needed; both in order to better understand the ecology and evolution of *C. nootkatensis* so that better management of this species may be possible, but also to understand the role of clonality in the maintenance of genetic variation and genetic structure and in the evolution of clonal plants.

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