CHAPTER 9

GENE FLOW IN CONIFERS

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Abstract. The question of gene flow from transgenic conifers will draw heavily upon population genetics theory for answers about gene flow to surrounding forests. For this, we provide a brief summary of the conifer mating system and present some population genetics basics. As reviewed, several DNA-based polymorphism systems are available for conifers, particularly for the Pinaceae, but have not yet been applied to the question of transgenic escape. Future gene flow studies will benefit from the biparental organelle inheritance in the Pinaceae which allows measuring gene flow distances independently for male and female lineages. If nuclear, codominant markers are needed, then biologists will have the choice of the stable and conservative allozymes or rapidly-evolving nuclear microsatellites. Because allozymes produce enzymes that regulate metabolism, they might occasionally be the targets of selection, while the microsatellites are mostly neutral markers. Mitochondrial (mt) DNA and chloroplast (cp) DNA polymorphisms provide the opportunity to identify and track maternal and paternal lineages, respectively. These organellar markers provide the best tools for dissecting gene flow with high precision. Because most conifer seeds tend to have shorter dispersal distances compared to pollen, mtDNA is useful for making inferences about numbers and geographic location of outliers in addition to tracing advection.

1. GENE FLOW

Gene flow has a major impact on the distribution of genotypes within and among populations. Gene flow is not a constant, but is dependent on the mating system and the dispersal distances of pollen and seeds, all of which vary with environmental factors and geographic localities.

Isolated populations will diverge genetically. Allelic frequencies drift over time in finite populations, due to stochastic variation in survival and reproduction. The incidental loss of alleles may differ between populations, increasing the genetic distance between them. In addition, mutations may introduce new alleles into

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populations, further distinguishing them. With sufficient time, perfectly isolated populations will become completely differentiated, so that they do not share any alleles.

Gene flow among populations retards or prevents divergence. Low levels of gene flow between populations will retard their divergence and make them more similar, while high gene flow will homogenize them. The impact of gene flow on population structure can be illustrated quantitatively by modeling genetic variation at a single gene. Consider gene flow into a population from populations that have different allelic frequencies. If the proportion of migrants into a population is *m*, and the frequency of *A* in the migrants is $\overline{p}$, then *p'*, the new frequency of *A* in the population, will be as follows:

$$p' = \overline{p}m + p(1-m).$$

If gene flow were unopposed by other forces, the populations connected by gene flow would ultimately share the same alleles, at the same frequencies.

1.1. The mating system in conifers

The importance of mating systems has long been apparent to theoreticians, field biologists, and plant breeders (WRIGHT 1931, 1969; ALLARD *et al.,* 1968; LANDE AND SCHEMSKE 1985; BROWN 1990). Mating systems determine the distribution of genotypes within populations, and influence the degree of differentiation among populations. While outcrossing promotes gene flow, and brings genotypic proportions to Hardy-Weinberg equilibrium, selfing reduces gene flow, and brings genotypic distributions to an equilibrium described by Wright's equilibrium law (WRIGHT 1931, 1969). Selfing reduces heterozygosity by half each generation, reducing the effective rate of recombination, and thereby promoting genetic organization within populations (ALLARD *et al.,* 1968).

Conifers have mixed mating systems (BROWN 1990). That is, some proportion of seed is produced by selfing (s), and the complementary proportion (t = 1-s) is produced by outcrossing. There is dramatic variation among species, with most species almost completely outcrossed (MULLER 1977; MITTON *et al.,* 1977, 1981; ADAMS AND JOLY 1980; MORAN *et al.,* 1980; SHAW AND ALLARD 1981, 1982; SHEN *et al.,* 1981; EL- KASSABY *et al.,* 1985, 1987; EPPERSON AND ALLARD 1984; FARRIS AND MITTON 1984; KING *et al.,* 1984; CHELIAK *et al.,* 1985; SNYDER *et al.,* 1985; FRIEDMAN AND ADams 1985a; NEALE AND ADAMS 1985a,b: FURNIER AND ADAMS 1986; SHEA 1987; DENTI AND SCHOEN 1988; ERICKSON AND ADAMS 1989, 1990; GIBSON AND HAMRICK 1991). A meta-analysis of 52 conifers from eight genera and three conifer families (O'CONNELL 2003) confirmed that conifers in the family Pinaceae were predominantly outcrossed. However, some species, such as tamarack, *Larix laricina*, eastern white cedar, *Thuja occidentalis*, and Chihuahua spruce, *Picea chihuahuana*, have population outcrossing rates as low as t = 0.53 (KNOWLES *et al.,* 1987), t = 0.51 (PERRY AND KNOWLES 1990), and t = 0.07 (LEDIG *et al.,* 1997), respectively. Some outcrossing rates in conifers are summarized in Table 1.

Table 1. Outcrossing rates for conifers. All taxa except Thuja plicata are members of the Pinaceae family. Picea chihuahuana, a species which occurs in small, isolated populations is included as the only conifer with predominantly selfing.

Species	t	Reference
Tamarack (*Larix laricina*)	.729	(KNOWLES *et al.,* 1987)
Douglas-fir (*Pseudotsuga menziesii*)	.752	(STAUFFER AND ADAMS 1993)
White spruce (*Picea glauca*)	.730	(INNES AND RINGIUS 1990)
Loblolly pine (*Pinus taeda*)	.994	(FRIEDMAN AND ADAMS 1985)
Norway spruce (*Picea abies*)	.956	(MORGANTE *et al.,* 1991)
Scots pine (*Pinus sylvestris*)	.940	(MUONA AND HARJU 1989)
Black spruce (*Picea mariana*)	.924	(BOYLE AND MORGENSTERN 1986)
Ponderosa pine (*Pinus ponderosa*)	.960	(MITTON *et al.,* 1977, 1981)
Limber pine (*Pinus flexilis*)	.980	(SCHUSTER AND MITTON 2000)
Noble fir (*Abies procera*)	.940	(SIEGISMUND AND KJAER 1997)
Radiata pine (*Pinus radiata*)	.900	(MORAN *et al.,* 1980)
Silver fir (*Abies alba*)	.890	(SCHOEDER 1989)
Serbian spruce (*Picea omorika*)	1.00	(KUITTINEN AND SAVOLAINEN 1992)
Western red cedar (*Thuja alicata*)	.770	(O'CONNELL 2003)
Chihuahua spruce (*Picea chihuahuana*)	.076	(LEDIG *et al.,* 1997)

In most conifers, each individual has separate male and female reproductive structures on one tree (monecy or 'one house'), although in some species, individuals are either male or female (dioecy or 'two houses'). If male and female strobili occur on the same plant then geitonomy or selfing via separate strobili on the same plant is possible (RICHARDS 1997). Autogamy, or selfing within the same flower, is not possible for conifers or other gymnosperms because male and female strobili are separate rather than combined into a single structure. For outcrossing monecious plants, selfing must be minimized at one or more stages despite the presence of male and female strobili on the same plant. In conifers, selfing is avoided at pollination or excluded after fertilization.

Mating systems, like other traits, are not constants, but are influenced by both environmental and genetic variation. Although there is still much variation that we do not yet understand, there are some patterns that we can predict. For example, the rate of outcrossing is expected to increase with stand density. Truly isolated trees, separated by many kilometers from conspecifics, can receive only their own pollen and set only selfed seed (FOWLER 1965). Similarly, in stands with low density, there will be low levels of outcrossing, for each tree stands in a mist of its own pollen, only partly diluted by the pollen of nearest neighbors. For this reason, lower levels of selfing are expected in years of low pollen production. In stands with normal or

high density, the abundance of pollen sources necessarily dilutes selfing pollen, and outcrossing climbs to nearly 100%. An example of the association between stand density and outcrossing was found in ponderosa pine at the forest-grassland ecotone in eastern Colorado (FARRIS AND MITTON 1984). In a stand of normal density within the continuous forest, the average of estimates of outcrossing from allozymes and from albino seedlings was 0.96, and none of the estimates different from 1.00 (MITTON *et al.,* 1981). Just a few kilometers to the east, a stand with nearest neighbor distances of 25-200 m had a level of outcrossing of only 0.80 (FARRIS AND MITTON 1984). However, the expected relationship between stand density and rate of outcrossing is not always observed (NEALE AND ADAMS 1985b).

The timing of pollen release and female receptivity will also affect variation in rates of outcrossing, and can impose limits to gene flow. In a study of Douglas- fir growing in a seed orchard, Erickson and Adams (1989, 1990) found considerable clone to clone variation in the timing of pollen release and female receptivity. Outcrossing was highest in clones whose pollen release followed female receptivity, and was lowest in clones in which male and female activities were coincident. A study of limber pine, *Pinus flexilis*, revealed that the timing of pollen release and female receptivity imposes limits to gene flow among populations at different elevations (SCHUSTER *et al.,* 1989). Limber pine occurs at elevations ranging from 1,500 m to 3475 m on the Great Plains and the Front Range of Colorado, where spring arrives first on the plains, and then proceeds to higher elevation. Strong winds and steep terrain guaranteed that pollen released from the highest elevation could reach trees at the lowest elevation. But pollination phenology responds to the seasons, so that pollen released at high elevations may reach lower elevations where female surfaces are no longer receptive. The timing of release and receptivity suggests that successful pollen dispersal might be limited to a mere 1,000 feet in elevation.

1.1.1. Maternal haploid gametophyte

Within a fertilized pine seed, the embryo is surrounded by female gametophyte which serves as storage tissue that provides the nutrition for early growth and development. This tissue is haploid, and of maternal origin, and it provides several opportunities for geneticists and population biologists. A sample of seeds from a single tree can be used to test for segregation at an allozyme or microsatellite locus. Similarly, a sample of tissue can be used to measure the rate of recombination among a set of nuclear markers. Thus, although conifers might live centuries or even millennia, and are somewhat difficult to cross, the haploid maternal tissue makes them handy for genetic analyses.

1.1.2. Paternity analyses

In each pine seed, a diploid embryo is enveloped in maternal haploid tissue that carries the maternal contribution to that embryo. Simple subtraction of the maternal

contribution from the offspring genotype yields the paternal contribution, or the set of genes carried by the pollen. The capability to clearly identify the paternal contribution provides more resolution to paternity analyses than can be obtained in angiosperms, which have triploid endosperm. With a sufficient number of loci, each with ample genetic variation, the paternal parent can be identified with moderate to high confidence.

A paternity analysis of limber pine, *Pinus flexilis*, examined gene flow within an isolated population on the eastern plains of Colorado (SCHUSTER AND MITTON 2000). This population is composed of 371 trees partially isolated among four small canyons and an adjacent hillside. All 277 trees producing pollen, the potential paternal parents, were scored for ten polymorphic allozyme loci. Ten polymorphic allozyme loci were scored in 474 embryos and their associated gametophytes. For 19 seeds, or 4% of the total, the paternal parent was identified with certainty. The average distance of pollen flow for these 19 seeds was 174 meters; similar estimates of pollen flow were obtained from most likely and fractional methods, which use probabilistic methods of identifying paternal parents and make fuller use of the data. These results indicated near panmixia within this population, which covers 15 hectares. Approximately 6.5% of the pollen came from outside the population; potential sources of pollen were 2-40 km away on the plains, and over 100 km away at lower elevations in the mountains.

1.2. Wind pollination

Because pollen is carried by the wind, the wind conditions at the time of pollen release will influence direction and distance of gene flow and levels of outcrossing. Total lack of wind would cause each tree to be enveloped in selfing pollen, and to receive little outcrossing pollen. At the other extreme, very turbulent air disperses pollen to exceptional distances and this promotes outcrossing.

1.3. Polyembryony

Gymnosperms have long been thought to control the genetic composition of their offspring by producing multiple embryos then selecting one to mature but there is little experimental support for this idea. The concept of polyembryony as a selective sieve was first proposed as developmental selection (BUCHHOLZ 1922). Developmental selection rests on a mistaken interpretation of *Pinus* embryology (BUCHHOLZ 1918) later extended to a comparative study of conifer genera (BUCHHOLZ 1920). The number of egg cells per archegonium was over-estimated by a factor of eight (BUCHHOLZ 1918). The rosette tier in the proembryo stage was mistakenly thought divided to form four rosette embryos which meant eight egg cells were present in one archegonium, not one (SKINNER 1992). If so, competition within an archegonium would have been far more intense with eight egg cells, naturally elevating the importance of developmental selection.

Later, based on correct morphology, simple polyembryony was re-considered as a potential selective force reducing fitness costs (SORENSEN 1982; KLEKOWSKI and KAZARINOVA-FUKSHANSKY 1984). Competition between selfed and outcrossed

embryos was hypothesized to arise from simple polyembryony (SORENSEN 1982). Only in the case of simple polyembryony would weaker selfed embryos will be less competitive against outcrossed embryos. Cleavage polyembryony was excluded from consideration as a selective sieve because embryos share a common genotype and are thus selectively equivalent to each other.

Simple polyembryony does not act as a selective sieve for many reasons. First, few archegonia are actually fertilized. Even fewer or even none of these fertilized archegonia arise from simple polyembryony or monozygotic embryos. Second, monozygotic embryos share a high degree of genetic similarity so the genetic differences among embryos within an ovule are slight. Perhaps the third and most important reason comes from the observation that a dead embryo, selfed or outcrossed, is rarely replaced by another embryo unless its death occurs in early stages of embryo development. Rather than a selective sieve, polyembryony is considered to be a vestigial character of the highly conserved female gametophyte.

1.4. Inbreeding depression

Inbreeding decreases heterozygosity, and relative to the benchmark of randomly mating populations, the fitness of inbred individuals is typically depressed. Inbreeding depression is manifest as a depression of growth rate, viability, developmental stability, fecundity, and fertility (LERNER 1954; WRIGHT 1977; CHARLESWORTH AND CHARLESWORTH 1987; WILLIAMS AND SAVOLAINEN 1996). Inbreeding depression can be severe in species which are typically outcrossing, (CRUMPACKER 1967; SIMMONS AND CROW 1977; FRANKLIN 1972; SORENSEN AND MILES 1982; CROW AND SIMMONS 1983), and it may be less intense (LANDE AND SCHEMSKE 1985) but not necessarily absent (CHARLESWORTH AND CHARLESWORTH 1987) in selfing species. Inbreeding depression and heterosis have been under investigation for decades (WRIGHT 1977; FRANKEL 1983), but there is still no consensus concerning the genetic mechanisms underlying these phenomena. Two contending hypotheses, the dominance hypothesis and the overdominance hypothesis, are compatible as the genetic explanation for inbreeding depression.

1.4.1. Partial dominance hypothesis

The partial dominance hypothesis focuses upon the phenotypic expression of recessive alleles which are either lethal or detrimental. Some groups of species, such as conifers and *Drosophila*, have relatively high frequencies of deleterious alleles, and the rate of mutation to deleterious alleles per gamete is surprisingly high (LYNCH 1988). Inbreeding causes rare recessive alleles to occur more frequently in the homozygous condition, increasing the frequency of aberrant phenotypes. From the perspective of the dominance hypothesis, inbreeding depression is the expression of deleterious alleles that had previously been masked in the heterozygous condition. Because completely inbred strains tend to be fixed for deleterious alleles at different

loci, crosses between strains produce genotypes bearing deleterious alleles in the heterozygous condition, masking the great majority of deleterious alleles, producing heterosis.

1.4.2. Overdominance hypothesis

The overdominance hypothesis supposes the performance of heterozygotes to be superior to that of homozygotes. From this perspective, inbreeding depression results when inbreeding decreases the frequency of heterozygous genotypes and increases the frequency of homozygous genotypes. The proponents of this hypothesis typically base their reasoning on the fact that the majority of enzyme kinetic studies have revealed that the genotypes at a locus have different biochemical properties (MITTON 1997; EANES 1999). Biochemical studies frequently reveal that the alternate homozygous genotypes at a locus reach their optimal performance under different conditions. Heterozygotes usually have intermediate optima, and perform efficiently over a broader range of conditions. This general (but not universal) pattern of kinetic variation provides a mechanism that might explain why higher heterozygosity is favored in fluctuating environments (MITTON 1997).

1.4.3. Interpretation of population surveys

Population-based surveys have been used to argue in favor of partial dominance. Molecular markers are assayed at the population level then correlated with phenotypic traits including fitness components. This approach constitutes a weak argument for partial dominance in conifers, aside from high embryo genetic loads. Positive correlations between a small number of heterozygous loci and fitness in conifers were interpreted as evidence for overdominance (BUSH AND SMOUSE 1991). A lack of positive correlation reported for other conifer species caused others to later conclude that partial dominance, not overdominance, is prevalent for conifer species (STRAUSS 1986; STRAUSS AND LIBBY 1987; SAVOLAINEN AND HEDRICK 1995). A third group reported negative correlations for conifers (Mitton and Grant 1984), perhaps because there is no genetic explanation (DENG AND FU 1998).

The weakness of the population heterozygosity argument lies in the untested assumptions about any given population under scrutiny. First, the population must be in gametic disequilibrium so that a positive correlation or functional overdominance can be detected. Second, random mating must be assumed by default yet deviations from random mating or panmixia is the major cause of incongruent results. Populations have undetected deviations from these idealized assumptions and these small deviations can generate any type of correlation (CHARLESWORTH *et al.,* 1991; DENG AND FU 1998). As a result, marker-based population surveys do not provide consistent findings even within a species because every local population has a different phylogeographic history (DENG AND FU 1998).

The majority of evidence is consistent with the partial dominance hypothesis, and the majority of forest biologists believe that the partial dominance hypothesis best describes the mechanism underlying inbreeding depression in conifers.

However, comparison of selected trees with a random sample yields data consistent with the overdominance hypothesis. When foresters established orchards of Engelmann spruce, *Picea engelmannii* (MITTON AND JEFFERS 1989), Norway spruce, *Picea abies*, (BERGMANN AND RUETZ 1991), Sitka spruce, *Picea sitchensis* (CHAISURISRI AND EL-KASSABY 1994), and Douglas-fir, *Pseudotsuga menziesii* (EL-KASSABY AND RITLAND 1996), they selected trees according to the normal criteria used by foresters, but they unknowingly chose trees with higher individual heterozygosities (Mitton and Pierce 1980) than would be found in a random sample of trees. It appears that foresters use the advantageous traits associated with highly heterozygous genotypes when they select trees for breeding.

1.5. Avoidance of inbreeding

For outcrossing monecious plants, selfing must be minimized at one or more stages despite the presence of male and female strobili on the same plant. In conifers, selfing is avoided at pollination or excluded after fertilization. Selfing avoidance is influenced by strobili position and morphology yet it is still highly subject to environmental vagaries. External factors such as wind speed, wind direction or even stand density determine how much pollen from non-self trees reach receptive female strobili (MITTON 1992; DYER AND SORK 2001). Proportions of selfed pollination vary widely from tree to tree, from year to year.

Separation between pollen release timing and female strobilus receptivity on the same tree (dichogamy) lowers self-pollination rates (ERICKSON AND ADAMS 1989). Selfing is avoided with spatial separation of female and male strobili in a single crown. Younger trees tend to have a prevalence of female or male strobili throughout the crown and older trees have female strobili clustered in the top of the crown and male strobili in the lower branches. Age of the tree also affects degree of selfing avoidance. Older *Pinus ponderosa* trees produce more cones and less pollen. Young *Pinus ponderosa* trees act as pollen donors, producing few cones yet providing pollen to older trees loaded with female strobili (MITTON 1992).

1.6. Selection against inbred genotypes

When self-pollination does occur, pre-zygotic barriers such as self-incompatibility systems do not eliminate self-pollinated ovules prior to fertilization. Self-exclusion in the *Pinaceae* and possibly other monoecious conifers occurs *after* fertilization. To date, there are no direct estimates of the proportion of archegonia pollinated or fertilized by a tree's own pollen. The proportion of self-fertilizations in terms of zygotes ranges from 10 to 25% in *Pinus sylvestris* (SARVAS 1962; KOSKI 1971). Self-pollination rates can be as high as 34% in the middle of the crown yet only 5% of viable seed is a product of self-fertilization in *Pinus taeda* (FRANKLIN 1969).

Similarly, the proportion of full seeds after self-fertilization compared to cross-fertilization in most conifers on average is only 39% (Griffin and Lindgren 1985). The viability gap between self-pollination and selfed seed maturity is the result of

post-fertilization exclusion. Self-exclusion is collectively defined as the embryo lethal system, a selective sieve against self-pollinated embryos soon after fertilization (BRAMLETT AND POPHAM 1971; KOSKI 1971).

Comparisons of seeds collected from field sites and the mature trees at the site typically provide evidence of selection against inbred genotypes. For seeds of Washoe pine, *Pinus washoensis*, the multilocus estimate of outcrossing was t = 0.86. The outcrossing rate, *t*, can be related to the equilibrium level of the inbreeding coefficient, F_{is}, as

$$F_{is} = (1-t)/(1+t),$$

so the outcrossing rate of t = 0.86 would produce an equilibrium value of $F_{is} = 0.11$. However, the value of F in the maternal seed trees was F = -.10 (MITTON, LATTA AND REHFELDT, 1997). These results indicate that the inbred genotypes detected in the seedlings are eliminated in natural populations. Similarly, the outcrossing rate in a stand of ponderosa pine was t = 0.96 (MITTON *et al.,* 1981; LINHART *et al.,* 1981), which would produce an equilibrium value of $F_{is} = 0.02$. However, the observed value in the mature trees at the site was $F_{is} = -011 \pm 0.02$. The shift from $F_{is} = 0.02$ to $F_{is} = -0.11$ indicates not only that inbred genotypes were eliminated, but also that selection had favored highly heterozygous trees.

2. MOLECULAR MARKERS

2.1. Nuclear markers

Numerous types of molecular markers might be used to study genetic variation in conifers. Large numbers of RAPDs and AFLPs can be developed quickly. However, these markers are most commonly presence/absence of bands, and without further analysis, it is not possible to determine whether presence of a band in an individual reflects heterozygosity or homozygosity of the dominant allele. Geneticists have usually favored codominant markers, historically allozyme loci, and more recently, the more variable microsatellite loci.

2.1.1. Allozymes

Surveys of electrophoretically detectable genetic variation of proteins, or allozyme variation, have been used to measure genetic variation and describe geographic variation of many species of conifers (HAMRICK *et al.,* 1979; HAMRICK AND GODT 1989; LOVELESS AND HAMRICK 1984; MITTON 1983; MITTON AND DURAN 2004). These surveys have identified conifers as the most genetically variable group of species (HAMRICK *et al.,* 1979; HAMRICK AND GODT 1989; HAMRICK *et al.,* 1992). For example, the proportion of loci polymorphic in gymnosperms, dicots and monocots was .71, .59, and .45, respectively ($P < 0.001$; Hamrick and Godt 1989).

Similarly, the proportion of loci heterozygous within populations in gymnosperms, dicots, and monocots was 0.16, 0.14, and 0.10, respectively ($P < 0.001$). A survey of genetic variation within populations of annuals, short-lived herbaceous species, short lived woody species, long-lived herbaceous species and long-lived woody plants reported expected heterozygosities of 0.101, 0.098, 0.096, 0.082, and 0.148, respectively ($P < 0.001$; HAMRICK AND GODT 1989).

Allozyme surveys of conifers have generally revealed only slight differentiation of allelic frequencies among populations. When values of F_{st} have been used to estimate the number of migrants among populations, Nm, or the average number of migrants moving among populations per generation, is typically in the range of 5-20, indicating very high gene flow via wind borne pollen (SCHUSTER AND MITTON 2000; SCHUSTER *et al.,* 1989; HAMRICK *et al.,* 1994; LATTA *et al.,* 1994).

2.1.2. Microsatellites

Microsatellites are tandem repeat motifs that occur in units of two to six nucleotides ($AAAA\ldots A_n$ or $CAGCAGCAGCAG\ldots CAG_n$). Because of these tandem repeat motifs, microsatellites are mutational hot spots, so they segregate for many alleles and have high heterozygosity. Little is known about the organization or function of microsatellites in the large conifer genome although they are abundant (SCOTTI *et al.,* 2000). Microsatellites are especially useful for outcrossing species because they are codominant, easy to score and have multiple alleles per marker locus. A set of 200+ nuclear microsatellites have been developed for *Pinus taeda* L. and many are polymorphic in other hard pines (AUCKLAND *et al.,* 2002).

Microsatellite markers are developed from short sequences of DNA enriched for the tandemly repeated units or repeat motifs. The short DNA sequences with repeat motifs are sequenced. If the sequences on either side of the repeat motif are long enough then a forward and a reverse primer can be designed for amplification via the polymerase chain reaction (PCR). In general a microsatellite marker is composed of five parts: 1) a region for binding the forward primer, 2) a flanking region, 3) the repeat motif, 4) another flanking region and 5) a region for binding the reverse primer. The DNA sequence is used to design a forward and reverse primer. Using the primers, each specific microsatellite sequence is amplified using the polymerase chain reaction and then run on a gel to observe the banding pattern. Scoring microsatellites is a measure of length polymorphisms.

Microsatellite alleles are measured by their band width using numbers of base pairs (bp). A homozygote (two copies of the same band) is viewed on a gel as a single band. A heterozygote is seen as two different bands, i.e. a difference in band length. What mutations cause changes in band width? A change in band width of an allele can be caused by a single base pair insertion or deletion or a change in the number of repeats. Single base pair changes can occur in the flanking or repeat regions. If a single base change occurs in the either of the two primer binding regions then null (absent) alleles occur. Primer binding is the essential step in obtaining a single PCR product.

Sequencing a sample of alleles is necessary to directly observe the type of nucleotide changes causing changes in allele band widths. Size homoplasy occurs when there are two alleles which have different sequence composition yet share the same band width (identical by state). For example, size homoplasy for one microsatellite allele has been observed between *P. palustris* (146 bp, repeat motif A_{14}) and *Picea rubens* (146 bp, motif $A_{14}CAA_6$) for locus PtTX3020. Similarly, size homoplasy has been observed between *P. radiata* (158 bp with a repeat motif CAG_7) and *P. strobus* (158 bp with repeat motif $CAG_2CAA_2CAG_2$) for locus PtTX2123 (KUTIL AND WILLIAMS 2001). In both cases, size homoplasy occurs at the phylogenetic extremes for microsatellite transfer so this does not appear to be a problem for intraspecific studies of mating systems. Because of size homoplasy, allele sequences should be checked when testing trans-specific markers and especially when microsatellites are used for determining phylogenetic relationships within a genus.

Polymorphism levels of these microsatellites vary within a species (ECHT *et al.,* 1996), especially if the microsatellites were developed from the low-copy regions of the conifer genome (ELSIK *et al.,* 2000). For the undermethylated (UM) microsatellites, allele numbers are lower and fewer are considered rare alleles (ZHOU *et al.,* 2001). Data collection for microsatellites has become automated thus reducing both cost and time to conduct large marker studies (ZHOU *et al.,* 2002). The high number of alleles per locus offers a method of measuring allelic diversity with higher resolution. For gene flow studies, the diagnostic or unique alleles within stands or subpopulations are useful.

To date, triplet repeat microsatellites developed in one species of hard pines can be polymorphic in other hard pines. DNA sequences appear highly orthologous and no ascertainment bias has been detected to date (SOKOL AND WILLIAMS 2005). Cross-amplification has been found in soft pines but not in hard pines. Contrary to earlier conjecture, use of microsatellites with a perfect repeat motif does not increase transfer rates within Pinaceae (KUTIL AND WILLIAMS 2001; SHEPHERD *et al.,* 2001).

2.2. Organellar markers

The organellar genomes of pines are ideal for measuring gene flow, for mtDNA has maternal inheritance and cpDNA has paternal inheritance in pines (NEALE *et al.,* 1986; STRAUSS *et al.,* 1989). These different modes of inheritance allow us to explicitly identify gene flow mediated by pollen and by seeds. In addition, pollen and seeds have disparate potentials for dispersal. The wind-borne pollen has the potential to travel great distances, but in contrast, the seeds of pines usually fall within a circle that has a radius equal to the height of the tree. Interesting exceptions to this general pattern of organellar inheritance are found in coast redwood, *Sequoia sempervirens* (NEALE *et al.,* 1989), and incense-cedar *Calocedrus decurrens*, (NEALE *et al.,* 1991) in which both organelles are paternally inherited.

Wright (1951) described the relationship between gene flow and the variance in allelic frequencies among populations at evolutionary equilibrium:

$$F_{st} = \frac{1}{4Nm+1}.$$

F_{st} is the standardized variance in allelic frequencies, N is the population size, and m is the migration rate. This relationship is slightly modified for haploid genomes in monecious species, such as the organellar genomes of pines:

$$F_{st} = \frac{1}{2Nm+1}$$

Gene flow of nuclear genes is influenced by both pollen and seed dispersal, which are the paternal and maternal components of gene flow. As shown below, Ennos (1994) described the contributions of maternal and paternal gene flow to biparental gene flow in terms of F_{st}:

$$(\frac{1}{F_{st(b)}}) = (\frac{1}{F_{st(m)}}) + (\frac{1}{F_{st(p)}}).$$

This relationship indicates that the gene flow and population structure of biparentally inherited genes will be most similar to the component with the highest gene flow. That is, the gene flow of nuclear genes in conifers will reflect predominantly the gene flow mediated by pollen. This expectation is intuitive and it is consistent with empirical data.

2.2.1. Chloroplast (Cp) DNA

CpDNA, like mtDNA in plants, has relatively low levels of genetic variation within populations. For example, only three minor variants were found in a study of 100 individuals of the lupine, *Lupinus texensis* (BANKS AND BIRKEY 1985). A survey of 384 trees from 19 populations of Monterey pine, Bishop Pine, and knobcone pine revealed little or no cpDNA variation within or among populations of knobcone pine and Monterey pine (HONG *et al.,* 1993). Bishop pine had some variation within populations, but more variation among populations. A total of 20 forms or haplotypes of cpDNA were detected in the 384 trees. A summary of the percent nucleotide changes per site in 46 studies of cpDNA yielded a mean of 0.07% at the intraspecific level, 0.8% at the interspecific level, and 3.4% at the intergeneric level (Schaal *et al.,* 1991). In contrast, values of 1% to 8% were reported within species for mtDNA sequences from a diverse set of animals studied in the southeastern U. S. (AVISE 1992). Although variation in cpDNA is relatively low, it is sufficient to support studies of geographic variation in some conifer species (MATOS 1992; WAGNER *et al.,* 1987; WAGNER 1992).

2.2.2. Mitochondrial (Mt) DNA

Low mutation rate is probably the reason for the paucity of useful variation in plant mtDNA. In most animals, the mutation rate of mtDNA is substantially higher than the mutation rate of nuclear DNA. However, in plants, the ranking of mutation rates is much different; the mutation rates are highest in nuclear DNA, intermediate in cpDNA, and lowest in mtDNA (PALMER 1985, 1990). For example, Lynch (1997) lists the synonymous substitution rates (substitutions/site/billion years) for plant nuclear DNA, cpDNA, and plant mtDNA, to be 7.31 ± 0.58, 1.70 ± 0.09 and 0.51 ± 0.05, respectively. That is, nuclear DNA and cpDNA evolve at 14 times and three times, respectively, the rate of plant mtDNA. For a reference, the evolutionary rate of mammalian mtDNA is 7.75 ± 0.43, or 15 times the rate of plant mtDNA.

To date, most studies of population structure of mtDNA in pines have been limited to RFLPs revealed by Southern blots (DONG AND WAGNER 1993, 1994; HONG *et al.,* 1993; STRAUSS *et al.,* 1993) or to size variants of introns amplified by the polymerase chain reaction and separated on sequencing gels (LATTA AND MITTON 1997, 1999; LATTA *et al*. 1998; MITTON *et al.,* 1999; SINCLAIR *et al.,* 1999; SPERISEN *et al.,* 2001; JARAMILLO-CORREA *et al.,* 2004). More recently, DNA sequence data have been used to detect nucleotide substitutions that either produce or erase palindromes. When polymorphic sequences are digested with the appropriate restriction enzymes, the cleaved amplified polymorphic sequences (CAPS) can be genotyped on agarose gels by simply recording which sequences cut (had palindromes) and which did not. This efficient technique for surveying organellar genetic variation has been used to describe geographic patterns in organellar genomes of whitebark pine, *Pinus albicaulis* (RICHARDSON *et al.,* 2002) and silver fir, *Abies alba* (LIEPELT *et al.,* 2003).

3. GENE FLOW ESTIMATED WITH MOLECULAR MARKERS

The *average* dispersal distances of wind-dispersed seed are typically small, usually a matter of meters or even a few kilometers but using averages provides a closer measure of local neighbourhood dispersal (LND) rather than long-distance dispersal (LDD) (WILLIAMS *et al.,* 2006). In contrast, pollen might be transported more than 100 km (see KATUL *et al.,* 2006) or more especially during turbulent storms. Regardless of the distance method, one would expect a disparity between seed and pollen dispersal distances in conifers with biparental organelle inheritance. In short, comparing seed-dispersed mtDNA versus pollen-dispersed cpDNA would give contrasting patterns of geographic variation, both within and among populations.

3.1. Variation within a stand

A long-term study of ponderosa pine provided the demographic and genetic data to study the distribution of genotypes within a stand. The population is on a south-facing slope at the lower entrance to Boulder Canyon, CO, at an elevation of 1,738. Within the population, which covers approximately 2 hectares, 217 trees were permanently marked, and their genotypes are known for seven polymorphic

allozyme loci (LINHART *et. al.,* 1981), one polymorphic mtDNA marker and one polymorphic cpDNA marker (LATTA *et al.,* 1998). Studies of old photos indicate that the site has filled in since the time since European settlers arrived. Analyses of mtDNA revealed a significant degree of patch structure at the site. Analyses of the ages of trees, their mtDNA haplotypes, and their allozyme genotypes revealed that the six clusters of trees on the site were half-sibs clustered around the maternal parent. In contrast the to pattern of mtDNA, no structure was detected in the pattern of cpDNA haplotypes.

3.2. Gene flow along an elevational transect

Potentials for dispersal of pollen and seed lead biologists to expect high gene flow in genes dispersed by pollen (nuclear genes, cpDNA) and low gene flow for genes dispersed solely by seed. This hypothesis was tested with a study of gene flow among populations of limber pine in the Front Range of Colorado (LATTA AND MITTON 1997). The populations were distributed from tree line at the Continental Divide to an isolated stand of trees 150 km to the east, on an escarpment on the Great Plains. Haplotype frequencies were used to calculate F_{st} for both cpDNA and mtDNA, and gene flow was inferred from $F_{st.}$ The F_{st} values were .02 and .68 for cpDNA and mtDNA, respectively, suggesting that the number of migrants among populations per year are 12.25 for pollen and 0.12 for seeds. The gene flow of cpDNA is high, and should tend to homogenize the frequencies of cpDNA haplotypes and nuclear genes among populations within distances of approximately 150 km. The data were consistent with this expectation; the value for nuclear allozymes was F_{st} = 0.02. In contrast, the gene flow of mtDNA is below the threshold at which the influence of genetic drift predominates. So mtDNA is expected to vary more among populations than nuclear genes and cpDNA, and genetic drift will cause populations to diverge with respect to mtDNA haplotypes.

3.3. Gene flow across the ponderosa pine transition zone

During the Wisconsin glaciation, ponderosa pine retracted to refugia in two general areas, northern Mexico and the Pacific Coast (BETANCOURT *et al.,* 1990; RICHARDSON 1998; BONNICKSEN 2000 and references therein). At the end of the ice age, ponderosa pines began to spread north from Mexico, reaching the San Andres Mountains of southern New Mexico 14,920 ya, the Santa Catalina Mountains of southern Arizona a few centuries later, Chaco Canyon, NM, 9,500 ya, and the Grand Canyon about 10,000 ya. They arrived in eastern Nevada 6,100 ya, and in northern Colorado 5,090 ya. Ponderosa pines reached northeastern Wyoming about 4,000 ya, and continued north around the northern edge of the Great Basin where they formed a narrow transition zone in eastern Montana with the ponderosa pines spreading east from their Pacific refugia. The western subspecies, *Pinus ponderosa ponderosa*, met the eastern subspecies, *P. p. scopulorum*, as recently as 1,000 years ago.

The subspecies are adapted to different moisture regimes, can be distinguished with morphological characters, monoterpene profiles and allozymes. Their

divergence is sufficient to be reflected in other members of their community. The western pine beetle, *Dendroctonus brevicomis*, utilizes ponderosa pine as one of its hosts. *D. brevicomis* contains cryptic species with mtDNA sequence divergence of 9% (KELLEY AND MITTON 1998). The cryptic species are segregated on the two subspecies of ponderosa pine.

A clear contrast between gene flow for mtDNA and cpDNA was reported in the transitional zone of ponderosa pine (LATTA AND MITTON 1999). Both cpDNA and mtDNA markers are diagnostic for these subspecies, allowing the tracking of gene flow by both pollen and seeds across the transition zone. The mtDNA markers reveal a sharp boundary between the subspecies, approximately 7 km wide, with no intermixing of eastern and western haplotypes (JOHANSEN AND LATTA 2003). The cpDNA had a gentle cline between the subspecies, with gene flow 100 km into the western subspecies and 150 km into the eastern subspecies.

3.4. Gene flow across the range of silver fir

A range-wide survey of 100 populations of silver fir, *Abies alba*, revealed sharply contrasting patterns of geographic variation for mtDNA and cpDNA markers (LIEPELT *et al.,* 2000). An 80 bp deletion in the fourth intron of mitochondrial NAD5 produced two haplotypes that could be scored by amplifying the intron and running the PCR products on an agarose gel. A substitution in the chloroplast psbC gene produced a presence/absence polymorphism for a palindrome. Digestion of the psbC PCR products with HAEIII produced either three or four fragments that could be distinguished in agarose gels. More than 1000 trees from 100 populations were typed by these efficient methods.

The mitochondrial haplotypes showed two major regions in the range of silver fir, with relatively little mixing or introgression. Populations in the eastern portion of the range were fixed for one allele, and populations in the western portion of the range were fixed for the other allele. This pattern was consistent with earlier allozyme studies that postulated an eastern Mediterranean refugium in the Balkan Peninsula and a western Mediterranean refugium in the Apennines (KONNERT AND BERGMANN 1995). Mixed populations were found in Croatia, Slovenia, and northeastern Italy, but the majority of populations were fixed for a single allele, and therefore clearly descendant from either the eastern or western refugium.

The cpDNA haplotypes revealed a steep, continuous cline that spanned the entire range of the species. The refugia were probably fixed for alternate alleles during one of the last several glacial maxima, but gene flow among populations during the interglacials, when populations were in close proximity, produced a cline. This cline formed in the present interglacial, it would have formed about 7,500 years ago in southern Europe and between 1,000 and 4,000 years ago in the Carpathians (LIEPELT *et al.,* 2000). However, it is likely that the cline was generated several interglacials ago (HEWITT 2000), and has been smoothed in the last several interglacials.

4. CONCLUSIONS

To date, DNA-based polymorphisms for tracking gene flow are plentiful for conifers but these methods have not yet been applied to the question of transgenic escape in forest trees. Knowledge of population genetics and of the conifer mating system in particular will be important to determining the degree of gene flow between transgenic conifer plantations and surrounding forests. The potential for using these methods to track transgenic escapes is great for three reasons: 1) genomics resources is providing better molecular marker systems, 2) better analytical and computational methods are coming available which are quite powerful for studying gene flow on either contemporary or evolutionary time scales and 3) conifer species mostly likely to be developed for transgenic plantations have biparental organelle inheritance. The latter point means that mitochondrial (mt) DNA and chloroplast (cp) DNA polymorphisms will provide two independent methods for tracking maternal and paternal genetic contributions, respectively. These markers can dissect gene flow and hybrid zones with high precision.

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