ORGANELLE GENOMES IN CONIFERS: STRUCTURE, EVOLUTION, AND DIVERSITY

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ABSTRACT

The two organelle genomes of conifers, as in other land plants, display marked differences in their structure and evolution. Mitochondrial genomes vary widely in size, structure, and gene organization, whereas chloroplast genomes only infrequently undergo major structural changes. Mitochondrial DNA size and structure remain relatively unstudied in conifers and other gymnosperms, however, results to date indicate that the genomes are large, contain dispersed repetitive DNA, exhibit a low nucleotide substitution rate, and undergo rapid structural evolution. Chloroplast genome inversions and length mutations often occur in localized areas of the genome containing repeated sequences and tRNA genes. These may be subject to frequent convergence and should thus be used cautiously as population and phylogenetic markers. The chloroplast genomes of Pinaceous conifers are distinguished from most other taxa by being significantly rearranged, lacking the large inverted repeat, and containing substantial dispersed repetitive DNA. Knowledge of the conifer chloroplast genome has recently been greatly extended through the sequencing of the entire chloroplast genome of *Pinus thunbergii* (Japanese black pine). Genes not identified in land plant chloroplast DNA (cpDNA) were found in black pine, including light-independent chlorophyll synthesis genes, while other genes common to land plant cpDNA have apparently been lost, including genes involved in the chlororespiratory pathway.

Gene sequencing, primarily of *rbc*L, has been useful for dating ancient phylogenetic events such as the split between angiosperms and gymnosperms, reconstructing phylogenies, and studying variation in evolutionary rates. Based on relative rate tests, conifers and other perennial plant species tend to have substantially slower rates of evolution than annual species. Chloroplast DNA phylogenies using DNA sequence and restriction site data have significantly expanded concepts of phylogeny, especially in *Pinus*, as well as contradicted significant aspects of traditional taxonomies. Care must be taken when studying closely related species, however, because past hybridization and associated "chloroplast capture" can bias organelle phylogenies of conifers and other plant taxa compared to their nuclear genome phylogenies.

Restriction fragment analyses have shown that although total diversity is limited compared to nuclear genes, high levels of population differentiation can accumulate, particularly when compared to the commonly studied allozymes, making conifer organelle DNA diversity potentially useful as markers for genetic conservation and breeding. Species- specific organelle restriction fragments have been useful for seedlot classification and studies of introgression and hybridization. Because of contrasting modes of organelle inheritance in Pinaceae (paternal cpDNA, maternal mtDNA, Mendelian nuclear loci), opportunities exist for studies of population subdivision and cytonuclear evolution. Research needs include studies of mitochondrial genome structure in conifers, PCR-based methods for assessing structural genome diversity, additional studies of interspecific chloroplast genome phylogeny, and critical assessment of mutation hotspots as organelle population genetic markers.

Key words: chloroplast DNA, mitochondrial DNA, Coniferales, phylogeny

INTRODUCTION

Because of their ease of study compared to nuclear genomes and lack of basic knowledge of their genetic biology, a great deal of effort has been applied to the study of diversity, inheritance, and phylogeny of organelle genomes in conifers in the last 10 years. The purpose of this paper is to provide a brief review and critique of that work, and suggest some areas where additional work is needed.

We first discuss basic genome structure, including gene content, gene evolution, and structural diversity; we then examine a variety of other topics, including mode of inheritance, diversity within and among

populations, and use of organelles as phylogenetic markers. We conclude by nominating four areas where there are substantial needs and opportunities for additional research.

REVIEW

GENOME STRUCTURE AND EVOLUTION

Structure

Although both chloroplast and mitochondrial genomes of land plants evolved from bacteria-like progenitors that were incorporated into nucleated host cells over one billion year ago, chloroplasts appear to be the reduced remnant of a cyanobacterium-like endosymbiont, while the closest contemporary to the mitochondrion is purple photosynthetic bacteria (GRAY 1989; PALMER 1990). Its large size, high degree of dispersed repetitive sequences, and variable organizations have made studies of the mitochondrial genome difficult. The chloroplast genome has therefore received far more study to date.

Chloroplast genome structure has been characterized by physical and genetic mapping in a variety of species. Restriction endonuclease cleavage site maps are available for over 350 species of land plants (DOWNIE & PALMER 1992), and the chloroplast genomes of tobacco (SHINOZAKI *et al.* 1986), rice (HIRATSUKA *et al.* 1989), *Marchantia polymorpha* (liverwort) (OHYAMA *et al.* 1986), *Euglena gracilis* (HALLICK *et al.* 1993), *Epifagus virginiana* (WOLFE *et al.* 1992), and the conifer, black pine (*Pinus thunbergii*) (WAKASUGI *et al.* 1994), have been completely sequenced. Typical cpDNAs of land plants and green algae vary in size between 140 and 160 kb, have a highly conserved gene order, and exist as two circular, double-stranded genome isomers (PALMER 1990). A single, large inverted repeat (IR) of 10–76 kb in length is present in these cpDNAs and undergoes recombination to create equimolar proportions of the two inversion isomers (PALMER 1991). Conifer cpDNA lacks one copy of the repeat, resulting in one master chromosome of correspondingly smaller size (about 120 kb) (STRAUSS *et al.* 1988; RAUBESON & JANSEN 1992). The IR loss has also occurred in other lineages of vascular plants, most notably some legumes (DOWNIE & PALMER 1992). An incomplete loss of the IR has been proposed in black pine as its genome retains a 495 bp IR (TSUDZUKI *et al.* 1992).

Mitochondrial DNA (mtDNA) structure has only been examined in several dozen angiosperms, a few green algae, and two nonflowering land plants (a fern and a horsetail) (PALMER *et al.* 1992). A complete mtDNA sequence has been determined for liverwort (ODA *et al.* 1992). We are aware of no published data on mtDNA genome organization for gymnosperms. Land plant mtDNA varies widely in size (200 to 2500 kb), is usually several times larger than cpDNA, and can contain relatively large repeated sequences that recombine to generate multiple interconverting forms of master chromosomes and subgenomes. Species-specific assortments of master and subgenomes result from recombination between varied sizes and orientations of repeats (reviewed in PALMER 1990; LONSDALE 1989).

Structural Evolution

MtDNA contains large and small dispersed repeats that serve as sites of homologous recombination (LONSDALE 1989). Unlike cpDNA, the plant mtDNA genome is likely tolerant of the high frequency of structural rearrangements observed because large intergenic spacers exist that can accept mutations, and mtDNA does not contain a structural element like the cpDNA IR that generally prohibits inversions in the chloroplast genome (PALMER 1990; STRAUSS *et al.* 1988).

In contrast to most angiosperms, conifer cpDNA not only lacks the IR, but also contains dispersed repetitive DNA that is associated with structural rearrangements. Six large dispersed repeat families several hundred basepairs in length found in Douglas-fir (*Pseudotsuga menziesii*) and Monterey pine (*Pinus radiata*) cpDNA cluster in four regions of the genome (TSAI & STRAUSS 1989). Members of one family are located at the endpoints of a large 40–50 kb inversion that distinguishes Douglas-fir from Monterey pine, two closely related genera of Pinaceae. These inversion border endpoints map near *atp*A and *trn*G (UCC) (STRAUSS *et al.* 1988), sites of inversion endpoints in some angiosperm cpDNAs (*e.g.*, grasses (*Poaceae*) (DOYLE *et al.* 1992)).

In addition to large dispersed repeated sequences, conifer chloroplast DNA also possess a number of small repeats. *Pinus contorta* and *P. banksiana* cpDNA contain variable numbers of tandem repeats of 124 and 150 bp in size which map to a polymorphic rearranged region near *trn*K–*psb*A where the *psb*A gene has been duplicated (LIDHOLM & GUSTAFSSON 1991b). cpDNA restriction maps of *P. radiata* and *P. contorta* are essentially collinear with the exception of the *psb*A duplication in *P. contorta* (LIDHOLM & GUSTAFSSON 1991c). *P. thunbergii* cpDNA does not contain a duplicated *psb*A gene but does contain duplicated *psa*M, *trn*H (GUG), *trn*T (GGU), and *trn*S (GCU) genes (WAKASUGI *et al.* 1994). *psa*M is a green algal

Abbreviations: cpDNA, chloroplast DNA; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RAPD, random amplified polymorphic DNA; kb, kilobases; IR, inverted repeat

photosynthetic apparatus gene not identified in angiosperms. In Douglas-fir and related species, a polymorphic locus (ALI *et al.* 1991) is characterized by hundreds of base pairs of imperfect tandem direct repeats derived from a flanking *trn*Y (GUA) gene (HIPKINS *et al.* 1995). This length mutation hotspot varied by as much as 280 bp among trees within *P. menziesii*, and up to 1,000 bp among congeneric species. The tandem repeats are less than 30 bp in length and their sequences resemble a region of the flanking *trn*Y gene (Figure 1). This polymorphism has been used to identify paternity in Douglas-fir seed orchards (NEWTON *et al.* 1994). The majority of cpDNA variation, in any one species, resides in such localized mutation hotspots that are restricted to one or a few genomic locations (STRAUSS *et al.* 1988; TSAI & STRAUSS 1989; ALI *et al.* 1991; HIPKINS *et al.* 1995; LIDHOLM *et al.* 1988; GOVINDARAJU *et al.* 1989a,b; WHITE 1990a,b).

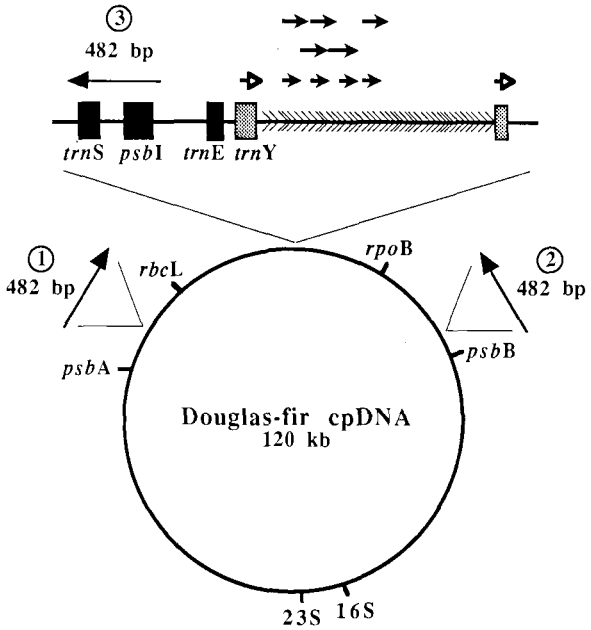

Figure 1 Conifer chloroplast genomes are characterized by a high degree of repeated sequences. Large 482 bp dispersed repeats, duplicating the *trn*S and *psb*I genes, are found in three locations throughout the chloroplast genome of Douglas-fir (indicated by numbered, long arrows with filled heads). A length mutation hotspot is composed of dozens of small direct tandem repeats (herringbone arrows) located between a partial *trn*Y gene duplication (unfilled closed arrows) (described in HIPKINS *et al.* 1995). These short, tandem repeats are themselves grouped into a variety of hierarchical, nested configurations (short arrows, solid heads).

Gene Content

The completely sequenced cpDNA of black pine was shown to contain 32 tRNA genes (one of which has not been found in any other cpDNA), 4 rRNA genes, 61 protein coding genes, and 11 open reading frames (WAKASUGI *et al.* 1994). Although a chlororespiratory pathway appears to exist in land plants based on the homology of chloroplast *ndh* genes to the mitochondrial respiratory-chain NADH dehydrogenase complex, 4 functional *ndh* cpDNA genes in black pine have been lost while 7 others remain as pseudogenes. It is possible that either chlororespiration is not an essential function in conifers, or that the *ndh* genes have been transferred to the nucleus. A function that appears to be present in conifers and absent in other land plants is light-independent chlorophyll synthesis (ALOSI & NEALE 1992; YAMAMOTO *et al.* 1991). Homologues of genes required for dark synthesis of chlorophyll in algae were identified in cpDNA of black pine (*Chlamydomonas* homologues *chl*L, *chl*N, and *chl*B) (WAKASUGI *et al.* 1994), *Pinus contorta,* and *Picea abies* (green algal *gid*A and liverwort *frx*C homologues) (LIDHOLM & GUSTAFSSON 1991a). The presence of these genes in conifer cpDNA indicate that light- independent chlorophyll synthesis may be occurring by the same or related mechanism in which it occurs in green algae.

Though usually several times larger than cpDNA, land plant mtDNA encodes several times fewer proteins. The complete sequence of liverwort mtDNA contained 94 possible genes including those for rRNA, tRNA, and proteins (ODA *et al.* 1992). In a study of mtDNA inheritance in the conifer *Larix,* DEVERNO *et al.* (1993) confirmed the presence of sequences homologous to the wheat mtDNA genes *atp*A, *atp*9, *nad3/rps* 12, *nad*5, *cox*I, *cob*, and *orf*25. Additionally, mtDNA contains an abundance of nuclear and cpDNA derived genes and pseudogenes.

Gene Evolution

Although plant mtDNA evolves faster than cpDNA in size and structure, the reverse is true with respect to synonymous nucleotide substitution rates. Chloroplast genes generally evolve more quickly than plant mtDNA, yet more slowly than mammalian mitochondrial and nuclear genes (WOLFE *et al.* 1987; BIRKY 1988; PALMER 1987). Silent substitution rates in protein genes are lowest in plant mtDNA, 4-fold higher in cpDNA, and 4-fold higher still in the plant and mammalian nucleus (BIRKY 1989). Substitution rates differ among chloroplast genes (PALMER *et al.* 1988), and most substitutions are silent changes at the third codon position (PALMER 1987).

Comparisons of plant families suggest that perennial species have a lower cpDNA substitution rate than annual species (SMITH & DOYLE 1986; WILSON *et al.* 1990), and the relative rate of sequence change may vary drastically in different, even closely related, plant lineages (GAUT *et al.* 1993; BOUSQUET *et al.* 1992).

*rbc*L sequences show a general slowdown of evolutionary rates in conifers and perennial angiosperms (BOUSQUET *et al.* 1992).

The phenomenon of RNA editing has been observed in plant mitochondria and chloroplasts. In both organelles, the editing mainly results in the substitution or altering of specific cytidines of the primary transcripts to uridines in the mature mRNAs to preserve the conservative amino acid sequences encoded. Numerous editing events have been observed in mitochondria of land plants, including the gymnosperms *Thuja plicata* (GLAUBITZ & CARLSON 1992), *Ginkgo biloba, Picea abies*, and *Cycas revoluta* (HIESEL *et al.* 1994). Mitochondrial mRNA editing supposedly occurs in all major groups of land plants except the Bryophyta. Based on the comparative analyses of the *cox*III region, RNA editing in the mitochondrial genome may generally be more frequent in gymnosperms than in angiosperms (see HIESEL *et al.* 1994). There have been relatively few observations of editing reported in angiosperm chloroplasts (HOCH *et al.* 1991; MAIER *et al.* 1992; KUDLA *et al.* 1992). We are aware of no report of editing in the chloroplast of a gymnosperm.

INHERITANCE

In contrast to nuclear genomes, plant organelle genomes are haploid, uniparentally inherited, and do not undergo sexual recombination. In angiosperms, cpDNA is inherited maternally in over 70% of the plant genera and biparentally in about 25% of genera (HARRIS & INGRAM 1991). In dicotyledonous tree species, only a maternal mode of plastid transmission has been found (MEJNARTOWICZ 1991; RAJORA & DANCIK 1992).

However, cpDNA shows predominant paternal inheritance in those conifers studied, which include species in the Cupressaceae and Pinaceae: *Pseudotsuga* (NEALE *et al.* 1986), *Picea* (NEALE & SEDEROFF 1988; STINE *et al.* 1989; SUTTON *et al.* 1991b; SZMIDT *et al.* 1988; STINE & KEATHLEY 1990), *Pinus* (WAGNER *et al.* 1987; WAGNER *et al.* 1989; WAGNER *et al.* 1992; NEALE & SEDEROFF 1989; DONG *et al.* 1992; BOSCHERINI *et al.* 1994), *Larix* (SZMIDT *et al.* 1987), *Sequoia* (NEALE *et al.* 1989), and *Calocedrus* (NEALE *et al.* 1991). Genetic data indicating paternal cpDNA inheritance in conifers is consistant with ultrastructural findings (OWENS & MORRIS 1990, 1991; see WAGNER 1992). Although it is generally assumed that an individual plant is homoplastic for a unique chloroplast DNA molecule, unusual genotypes have been observed in some conifers (WAGNER *et al.* 1987; SZMIDT *et al.* 1987; GOVINDARAJU *et al.* 1988; GOVINDARAJU *et al.* 1989a; WAGNER *et al.* 1988; DONG *et al.* 1992; WHITE 1990b). In some cases, heteroplasmy was suggested as a potential cause of the observation. Progeny with non-paternal cpDNA genotypes were observed in *Pseudotsuga menziesii* (NEALE *et al.* 1986), some hybrids in *Larix* Mill. (SZMIDT *et al.* 1987), *Pinus contorta, P. banksiana* (WAGNER *et al.* 1989; DONG *et al.* 1992), and *Calocedrus decurrens* (NEALE *et al.* 1991).

Dicotyledonous forest tree species have maternal inheritance of mitochondrial genomes as do most animals and plants (RAJORA *et al.* 1992). In Pinaceous conifers, predominant maternal inheritance has been found in *Pseudotsuga menziesii* (MARSHALL & NEALE 1992), *Larix* (DEVERNO *et al.* 1993), *Pinus* (NEALE & SEDEROFF 1988; WAGNER *et al.* 1991; NEALE & SEDEROFF 1989), and *Picea* (SUTTON *et al.* 1991b). However, in the Cupressaceae, mtDNA displays paternal inheritance in those species examined (*Sequoia sempervirens* (NEALE *et al.* 1989) and *Calocedrus decurrens* (NEALE *et al.* 1991)). These studies are consistent with ultrastructural evidence (see WAGNER 1992; OWENS & MORRIS 1990, 1991).

Because of the contrasting inheritance of organelle genomes in Pinaceous conifers, a unique opportunity exists to make evolutionary inferences from studies of cytonuclear disequilibria among all three genomes (ASMUSSEN *et al.* 1987; SCHNABEL & ASMUSSEN 1989). Paternal inheritance of chloroplasts and maternal inheritance of mitochondria in *Pinus* species can lead to unusual cytonuclear associations as well as to patterns of population subdivision that differ between mitochondrial and chloroplast polymorphisms (DONG & WAGNER 1994; PETIT *et al.* 1993).

DIVERSITY

Intraspecific Variation

Most cpDNA variants are uncharacterized length or fragment changes, rather than site mutations. As discussed above, such polymorphisms are often associated with localized hotspots that contain repetitive DNA (HIPKINS 1993; HIPKINS *et al.* 1995). Relative to the genome as a whole, their rates of mutation are therefore likely to be variable and mutant alleles subject to reversion and homoplasy (cf. KIM *et al.* 1992). Because of their mutational complexity and lack of representativeness of the genome, they can provide biased estimates of nucleotide diversity and thus may also give rise to incorrect estimates of genetic subdivision. HONG *et al.* (1993a) identified regions of the genome subjected to frequent length mutations and found that estimates of differentiation based on length variant frequencies differed strikingly from those based on site mutations or allozymes. These data are in a good accordance with results from GOLENBERG *et al.* (1993), who reported that a phylogenetic tree based on nucleo-

tide substitutions was consistent with expectations from study of other characters, but a tree based on length mutations was not.

Thus, population genetic estimates from such variants need to be treated cautiously. Although repetitive nuclear loci such as microsatellites suffer from the same problem, the study of multiple independent loci afforded by nuclear genes allows averaging over the homoplasies occurring at individual loci. In contrast, with cpDNA there is in effect only a single genetic locus, and usually only one or a very few repetitive, polymorphic regions in the genome.

Chloroplast DNA variants have been detected in *Picea glauca* (SZMIDT *et al.* 1988; STINE *et al.* 1989), *P. engelmannii* (STINE & KEATHLEY 1990), *P. sitchensis* (SZMIDT *et al.* 1988), *Tsuga canadensis* (WANG 1990), *Pseudotsuga menziesii* (ALI *et al.* 1991; PONOY *et al.* 1994), *Calocedrus decurrens, Sequoia sempervirens* (ALI *et al.* 1991), *Juniperus scopulorum, J. virginiana, Pinus hartwegii, P. edulis* (see WAGNER 1992), *P. taeda* (WAGNER *et al.* 1992), *P. elliottii* (WAGNER *et al.* 1992; NELSON *et al.* 1994), *P. caribaea* (NELSON *et al.* 1994), and *P. monticola* (WHITE 1990b). In the last study, frequencies of two cpDNA variants were significantly different in interior versus coastal populations of *P. monticola*. TSUMURA *et al.* (1994) found a north-south cline of cpDNA variants in *Abies mariesii*.

Chloroplast DNA diversity has been intensively studied in *Pinus banksiana* and *P. contorta* by WAGNER and coworkers. Thirteen cpDNA variants were detected among 363 trees sampled from sympatric and allopatric zones (WAGNER *et al.* 1987). Many of these variants plus some novel types were detected among 902 trees from the sympatric zone (WAGNER *et al.* 1988; GOVINDARAJU *et al.* 1989a). Additional variants were detected using a *Pinus contorta* cpDNA probe containing *psb*A (DONG & WAGNER 1994). These variants likely resulted from insertions/deletions in what has been characterized as a hotspot (LIDHOLM & GUSTAFSSON 1991b). As a part of a large survey of nearly 2,300 trees from 152 populations of *Pinus banksiana* and *P. contorta* (WAGNER 1992; DONG & WAGNER 1994), 14 cpDNA RFLP variants located near the duplicated *psb*A gene were found among 745 individuals from 16 allopatric natural populations (DONG & WAGNER 1994). Within population variabilities were substantial and similar in both *P. contorta* and *P. banksiana*; unbiased gene diversity was 0.44 and 0.43, respectively.

In some cases, no intraspecific cpDNA variation in coniferous species was found: *Larix decidua, L. leptolepis* (SZMIDT *et al.* 1987); *Picea pungens* (STINE *et al.* 1989); *Pinus echinata* (46 trees from 7 populations, WAGNER *et al.* 1992); *P. michoacana* (see WAGNER 1992); *P. palustris* (44 trees from 6 populations, WAGNER *et al.* 1992); *P. taeda* (NEALE & SEDEROFF 1989; 30 trees, ALI *et al.* 1991); *P. tabulaeformis, P. yunnanensis, P. massoniana* (WANG 1992; WANG & SZMIDT 1990, 1993), and *P. torreyana* (WATERS & SCHALL 1991). An apparent contradiction is the lack of intraspecific cpDNA variation in *P. taeda* found by ALI *et al.* (1991), and the detection of cpDNA variants in this species by WAGNER *et al.* (1992). Whereas WAGNER and others found one tree with a different cpDNA variant among the 78 trees collected from different regions, ALI and others sampled 30 *P. taeda* trees collected from only one part of its area. Additional study of many of the above species would be useful to correct for small sample sizes (either in numbers of cpDNA base pairs or in numbers of trees). No variation was found at two cpDNA spacer regions between tRNAs in *Pinus leucodermis* (BOSCHERINI *et al.* 1994). In this study, DNA was amplified from 80 germinated embryos from each of seven populations by PCR and analyzed by 11 four-base restriction endonucleases.

When length mutations and uncharacterized RFLP variation is ignored, intrapopulation cpDNA diversity appears very low. HONG *et al.* (1993a) studied 384 trees from 19 populations of the California Closed-Cone Pines (*P. attenuata, P. muricata*, and *P. radiata*). Mean nucleotide diversity within populations was only 0.003 (±0.002)% (approximately four variable nucleotides per 120 kb genome).

Intraspecific mtDNA variation in conifers appears to be generated by recombination among repeated sequences resulting in complex insertions/deletions or rearrangements. RFLP surveys of several *Pinus* species using mtDNA gene probes revealed variable levels of mtDNA diversity. STRAUSS *et al.* (1993) surveyed 268 trees from 19 populations of the California Closed-Cone Pines for variation associated with the *cox*I gene and found limited intrapopulation variation (H_{ep} = 0.07); this was less than for allozymes (15%) and cpDNA length mutations (17%), but comparable with cpDNA site mutations (6%). RFLP associated variation of *cox*I and *cox*II in 741 individuals from 16 populations of *P. banksiana* and *P. contorta* (DONG & WAGNER 1993) showed greater levels of *cox*II-associated variation in *P. contorta* (H_{es} = 0.68) than in *P. banksiana* (H_{es} equaled less than 0.17). These estimates of diversity (H_{es}) may exceed those reported in the Closed-Cone Pines because they pool intrapopulation and interpopulation variation, whereas those reported by STRAUSS *et al.* are intrapopulation diversity (H_{ep}) derived from study of isolated populations.

Population and Species Differentiation

Because of the uniparental inheritance and smaller effective population size of organelle genomes, they are

expected to show different evolutionary dynamics, and may be more sensitive to population subdivision than nuclear genes (BIRKY *et al.* 1989). HONG *et al.* (1993a) found that cpDNA differentiation among three groups of populations of *P. muricata* was strong (G_{st} = 0.87 ± 0.8), and several-fold higher than allozyme differentiation in the species (G_{st} = 0.22) (Figure 2). In contrast, population subdivision was weak (F_{st} < 0.05) within and among *P. contorta* subspecies and in *P. banksiana*, consistent with theoretical predictions for paternally--inherited markers in wind-pollinated outcrossers. Possible causes for this discrepancy are thoroughly discussed by DONG & WAGNER (1994) and include high levels of gene flow in *P. banksiana* and *P. contorta* as compared to the California Closed-Cone Pines (HONG *et al.* 1993a), and the use of a highly variable length mutation by DONG and WAGNER compared to the restriction site mutations studied by HONG and others. Species- or race-specific cpDNA restriction fragment patterns can be used to detect interspecific hybridization and identify hybrid seedlots (WAGNER *et al.* 1987; SZMIDT *et al.* 1988; WANG & SZMIDT 1990; SUTTON *et al.* 1991a, 1991b, 1994).

Strong population differentiation has been observed for mtDNA in conifers. In STRAUSS *et al.*'s (1993) study of *cox*I sequences in the California Closed-Cone Pines, strong differentiation among populations was observed in all three species (G_{st} = 0.75 – 0.96) that far exceeded estimates for allozymes (G_{st} = 0.12 – 0.22), but was similar to that for cpDNA in the one species where there was sufficient restriction site diversity for an estimate (*P. muricata*: G_{st} > 0.87) (Figure 2). Strong differentiation was also observed for mtDNA in range-wide studies of *P. contorta* and *P. banksiana* (DONG & WAGNER 1993). Two mtDNA *cox*I- and *cox*II-associated RFLPS (10 variants in total) were found among 741 individuals from 16 allopatric natural populations. The mtDNA variants distinguished the two species well, and population differentiation was substantial in *P. contorta* (F_{st} = 0.31 among subspecies; F_{st} = 0.56 – 0.82 within subspecies) and *P. banksiana* (F_{st} = 0.50 between populations, but only 0.04 if an atypical Saskatchewan population is excluded). Based on maternal inheritance, AAGARD *et al.* (1995) identified a large number of mtDNA-derived bands in RAPD profiles of Douglas-fir. Genetic differentiation among the coastal (var. *menziesii*) and interior (var. *glauca*) varieties for haplotype frequencies based on these bands was very high (G_{st} = 0.62) compared to estimates from allozyme frequencies (G_{st} = 0.26). The high differentiation of mtDNA has allowed it to be used for studies of introgression and hybridization (DONG & WAGNER 1993; SUTTON *et al.* 1991a, 1991b, 1994).

Patterns of population subdivision in Pinaceous conifers may be influenced by the contrasting mode of cpDNA and mtDNA inheritance. Gene flow of organelle genes distributed only through seed (*e.g.* maternal inheritance of mtDNA in pines), can be significantly less among wind pollinated tree species compared to organelle genes distributed through pollen (e.g. paternal inheritance of cpDNA) (DONG & WAGNER 1994).

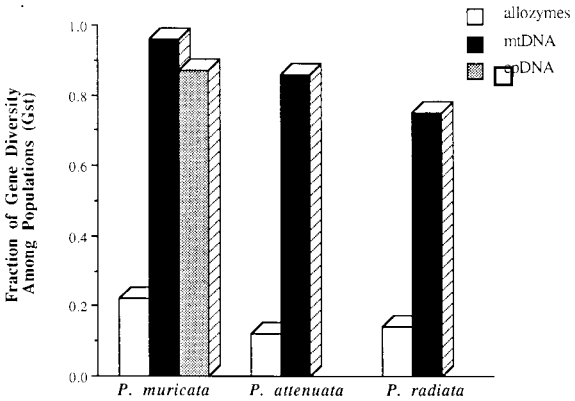

Figure 2 Comparison of organelle DNA and allozyme differentiation in the California Closed-Cone Pines as measured by G_{st}. mtDNA and allozyme estimates were reported in STRAUSS *et al.* 1993; cpDNA estimate from HONG *et al.* 1993a. Only *P. muricata* contained sufficient cpDNA restriction site diversity for an estimate of population differentiation.

PHYLOGENY

The conservative rate of structural change and nucleotide substitution in conifer cpDNA makes it suitable for determining interspecific and intergeneric relationships. Restriction fragment analysis of cpDNA has been used extensively when determining phylogenies of conifers at the interspecific level and above (reviewed in STRAUSS *et al.* 1992; e.g., KORMUŤÁK *et al.* 1993; WANG *et al.* 1991; WAGNER *et al.* 1992; NELSON *et al.* 1994; WANG & SZMIDT 1993; KRUTOVSKII *et al.* 1994). KRUPKIN *et al.* (in press) recently generated a robust cpDNA phylogeny for the hard pines (subgenus *Pinus*) from restriction site mutations that challenge major aspects of traditional phylogenetic and taxonomic hypotheses for the group. Point mutations are strongly preferred for phylogenetic analysis; as discussed above, length mutations tend to cluster in hotspots characterized by repetitive DNA, and may therefore contain high degrees of intrapopulation diversity and homoplasy (HONG *et al.* 1993a). However, hotspots may have utility at the intra- or interpopulation level, particularly when there are a large number of alleles, or multiple hotspot loci, on which to base inferences.

Intrapopulation restriction site variation is usually extremely low and can be ignored in most interspecific phylogenetic studies (see HONG *et al.* 1993a). However, population and racial diversity within species can be

substantial, and therefore can bias phylogenetic results when studying relationships among closely related species. HONG *et al.* (1993b) found that the southern race of *Pinus muricata* was considerably more differentiated from the northern races than it was from a related species, *P. radiata*. If only the southern race of *Pinus muricata* had been sampled a very different picture of chloroplast phylogeny would have resulted. This study also showed that "chloroplast capture" (RIESEBERG & BRUNSFELD 1992) and related phenomena occur in conifers, cautioning against phylogenetic inferences among closely related species based solely on organelle genomes. RFLP analyses of conifer mtDNA has not been found suitable for phylogenetic reconstructions. As a consequence of the high levels of intragenomic recombination, RFLP patterns are rarely affected by single point mutations and show frequent convergence (STRAUSS *et al.* 1993).

At higher taxonomic levels, where restriction fragment differentiation is often too large for interpreting site mutations, DNA sequencing of the conserved gene *rbc*L has been used successfully to assess phylogenetic relationships (BOUSQUET *et al.* 1992; GIELLY & TABERLET 1994). Using sequence data from *rbc*L and the nuclear ribosomal gene *Rrn*18, SAVARD *et al.* (1994) calibrated five molecular clocks and estimated the divergence time of extant gymnosperms and angiosperms to be 285 million years. Although *rbc*L sequence data is usually too conserved to generate well supported phylogenies among closely related genera of conifers, it has been used with some success to infer relationships in the *Cupressaceae* (including *Taxodiaceae*) (GADEK & QUINN 1993), as well as placing families of conifers and other gymnosperms in relation to the families and orders of angiosperms (CHASE *et al.* 1993).

DIRECTIONS FOR FUTURE WORK

There has been tremendous progress over the last ten years in understanding the structure, inheritance, diversity, and phylogeny of organelle genomes in conifers and other plant species. Significant challenges for the future include:

1. mtDNA structure: Mitochondrial genomes vary widely in size and structure among plant species. Conifers, with their large, complex nuclear genomes and unusual chloroplast genomes, may also have distinctive mitochondrial genomes. A high frequency of mtDNA bands were observed in RAPD profiles of Douglas-fir (AAGARD *et al.*, 1995) suggesting that the genome may be very large and complex.

2. Rapid means to study mtDNA diversity: The high cost of RFLPs has precluded all but a few large scale studies of organelle diversity. It has also prevented many possible uses for genetic conservation surveys. PCR based methods, to be useful for mtDNA, will need to survey large portions of the genome for structural polymorphism (hotspots, rearrangements), as gene sequences evolve extremely slowly. The advent of "long distance PCR" (amplifying 10–20 kb fragments), coupled with an abundance of conserved priming sites identifiable from the large number of sequenced genes now in databases, should make PCR approaches feasible.

3. Phylogenetic studies: There remains an abundance of phylogenetic problems amenable to chloroplast DNA analysis. Our recent study of cpDNA restriction site phylogeny among the hard pines (subgenus *Pinus*) has revealed many new insights into pine origins and biogeography, several of which substantially conflict with, or greatly elaborate upon, published taxonomies (KRUPKIN *et al.*, in press). With large datasets, comparable insights are available in many other conifer taxa.

4. Reliability of organelle hotspot markers: Because of the rarity of polymorphisms in organelle DNA, many workers have relied on variants generated by mutation hotspots. More comparisons to other markers are needed to test whether they are reliable, or give biased estimates of genomic diversity, differentiation, and phylogeny.

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