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Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids

(organelle DNA/paternal inheritance/introgression)

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ABSTRACT **Samples taken from throughout the ranges of distribution of lodgepole pine (*Pinus contorta* Dougl. ex. Loud.) and jack pine (*Pinus banksiana* Lamb.) were assayed for *Sal* I and *Sst* I chloroplast DNA restriction fragment variation. Although the chloroplast genome is often regarded as highly conserved, at least 2 distinct *Sal* I and 13 distinct *Sst* I restriction fragment banding patterns occur in these closely related species. None of the chloroplast DNA restriction fragment banding patterns observed in allopatric lodgepole pine was observed in allopatric populations of jack pine, and vice versa, even though the two species share an extensive zone of sympatry, and gene flow between the species has been reported for nuclear genes. However, several atypical *Sst* I restriction fragment banding patterns occur only in or near the zone of sympatry. Chloroplasts have been reported to be inherited maternally in the great majority of species studied; however, restriction fragment analyses indicated that chloroplasts are inherited paternally in controlled matings between lodgepole pine (♀) and jack pine (♂).**

The chloroplast genome, which averages about 150 kilobase pairs (kbp) in size, encodes gene products that are involved in photosynthesis (1). It is inherited maternally in the great majority of species that have been studied, but biparental inheritance has been reported in at least 20 species (2, 3). Although studies of chloroplast DNA (cpDNA) have usually been based on one or a few samples per species, they have demonstrated that diversity among species is common and they have been informative phylogenetically (4–6). There have been few studies of intraspecific cpDNA polymorphisms. Although examples of intraspecific cpDNA polymorphisms have been reported (7–11), small sample sizes have been employed or the variants observed have been at low frequency; consequently, little is known of the extent of intraspecific cpDNA diversity. In this paper we present evidence, from large samples, that lodgepole pine (*Pinus contorta* Dougl. ex. Loud.) and jack pine (*Pinus banksiana* Lamb.) differ in their *Sal* I and *Sst* I cpDNA phenotypes and that there are a large number of distinct *Sst* I restriction fragment variants throughout the wide allopatric distributional ranges of both species, as well as within a zone of sympatry in which the ranges of the two species overlap. We also present evidence that introgression of *Sal* I and *Sst* I variants is rare between the species and that cpDNA is inherited paternally in matings of *P. contorta* (♀) with *P. banksiana* (♂).

MATERIALS AND METHODS

Plant Materials. In total, 371 individuals were sampled, including 153 individuals of lodgepole pine from 63 populations in the allopatric (nonoverlapping) region, 115 individuals of jack pine from 68 populations in the allopatric region, and 95 individuals from 16 populations in the zone of sympatry (Fig. 1); all four subspecies of *P. contorta* were represented in the sample of lodgepole pine. To maximize the number of populations represented, only a single individual was sampled in most allopatric populations. We also examined eight F_1 interspecific hybrid trees from controlled matings between lodgepole pine (♀) and jack pine (♂).

DNA Preparation. Total cellular DNA was prepared from each individual by a modification of the method of Murray and Thompson (14). Needle tissue (10 g, fresh weight) was homogenized with a Brinkmann homogenizer at 4°C in 180 ml of extraction buffer [50 mM Tris, pH 8.0/5 mM EDTA/0.35 M sorbitol/10% (wt/vol) polyethylene glycol (M_r 3350)/0.5% spermine/0.5% spermidine/0.1% 2-mercaptoethanol]. The homogenate was filtered through several layers of cheesecloth and one layer of Miracloth (Calbiochem); a pellet was collected from the homogenate by centrifugation (13,000 × *g*, 4°C, 15 min). The pellet was suspended in 5 ml of wash buffer (50 mM Tris, pH 8.0/25 mM EDTA/0.35 M sorbitol/0.5% spermine/0.5% spermidine/0.1% 2-mercaptoethanol) and brought to room temperature; *N*-lauroylsarcosine was added to a concentration of 1% (wt/vol) and, after 15 min at room temperature, the suspension was brought to a final concentration of 1% (wt/vol) hexadecyltrimethylammonium bromide/0.7 M NaCl and incubated at 60°C for 10 min. After one chloroform/octanol (24:1) extraction, the remainder of the procedure followed the method of Saghai-Maroof *et al.* (15).

Detection of cpDNA Restriction Fragments. One microgram of total cellular DNA from each sample was digested singly to completion with *Sal* I and with *Sst* I. Restriction fragments were separated by electrophoresis in 0.7% agarose and 100 mM Tris acetate/12.5 mM sodium acetate/1 mM EDTA, pH 8.1, at 2 V/cm for 24 hr. DNA was transferred from gels to Biodyne blotting matrix. Two nonoverlapping cloned fragments from the large single-copy region of *Petunia hybrida* cpDNA (6) were nick-translated and hybridized to pine DNA (16–18). An 11.7-kbp *P. hybrida Sal* I fragment was hybridized to pine *Sal* I fragments, and a 9.0-kbp *P. hybrida Pst* I fragment was hybridized to pine *Sst* I fragments. Pine cpDNA was visualized by autoradiography. These clone–enzyme combinations were chosen after preliminary work

Abbreviation: cpDNA, chloroplast DNA.

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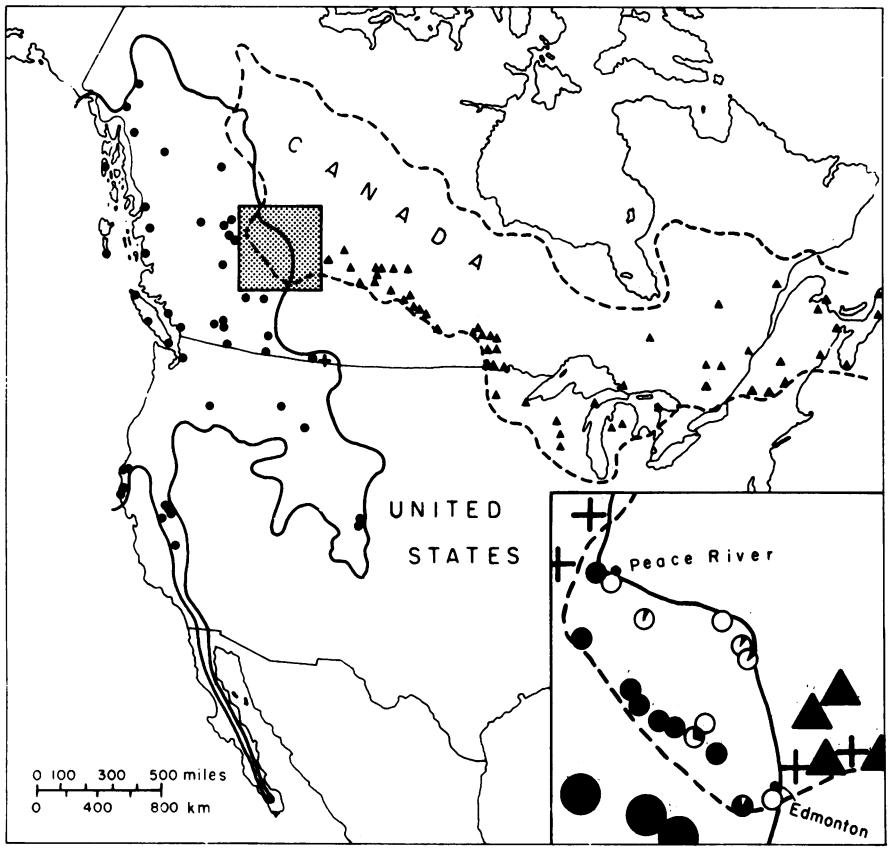

FIG. 1. Range maps showing the distribution of lodgepole pine (solid line) and jack pine (broken line) in North America (adapted from refs. 12 and 13). Circles and triangles represent populations sampled in the allopatric ranges of lodgepole pine and jack pine, respectively; a single symbol sometimes represents two populations located closely adjacent to each other. Pie diagrams in the *Inset* give the proportions of lodgepole pine (black) and jack pine (white) cpDNA in the 16 populations sampled in the zone of sympatry. Crosses mark allopatric populations in which atypical cpDNA phenotypes were observed (details in text).

identified the petunia chloroplast fragments required to visualize variable pine cpDNA fragments.

RESULTS

Populations from the Allopatric Regions. Digestion with *Sal* I produced two distinct phenotypes (detected by the 11.7-kbp *Sal* I fragment), one that included 5.4- and 9.5-kbp fragments and one that included 5.9- and 9.5-kbp fragments (Fig. 2; additional, invariant fragments are not shown). *Sal* I cpDNA banding pattern 5.9/9.5 was found in all of the 153 allopatric-region individuals of *P. contorta* examined, whereas all 115 allopatric-region individuals of *P. banksiana* examined had the 5.4/9.5 banding-pattern. Thus our samples indicate that these two species are completely differentiated for *Sal* I fragments (5.4 kbp in *P. banksiana* and 5.9 kbp in *P. contorta*).

Digestion with *Sst* I revealed nine variable restriction fragment size classes (detected by the 9.0-kbp *Pst* I fragment) ranging in size from 4.3 kbp to 5.7 kbp in the two species. Combinations of these nine fragments produced 13 different banding patterns, including one found only in the region of sympatry; we designate these 13 banding patterns *Sst* I cpDNA phenotypes (Fig. 3, Table 1). Among the six *Sst* I phenotypes found in allopatric *P. contorta*, three (4.4/5.0, 4.5/5.0, and 4.7/5.0) were found throughout the allopatric region, whereas the other three (4.3/5.0, 4.5/4.8, and 4.3/4.5/5.0) were rare and found only in or near the zone of sympatry. Similarly, among the six *Sst* I phenotypes found in allopatric *P. banksiana*, four (4.4/5.7, 4.7/5.7, 4.8/5.7, and 5.0/5.7) were found throughout the allopatric region and two (4.7/5.5 and 4.3/4.8/5.7) were rare and found only in or near the zone of sympatry. None of the six *Sst* I cpDNA phenotypes found in allopatric *P. contorta* was found in allopatric *P. banksiana*, nor were any of the six phenotypes found in allopatric *P. banksiana* found in allopatric *P. contorta*; thus, despite substantial intraspecific polymorphism, the two species are completely differentiated with respect to the *Sst* I cpDNA restriction phenotypes.

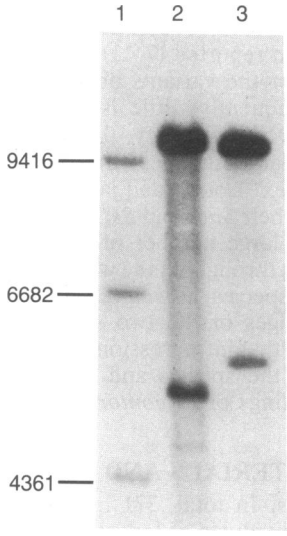

FIG. 2. *Sal* I cpDNA banding patterns (phenotypes) observed in jack pine (lane 2) and lodgepole pine (lane 3). *Hin*dIII-digested bacteriophage λ DNA is in lane 1 (fragment sizes in base pairs at left). Origin is at the top.

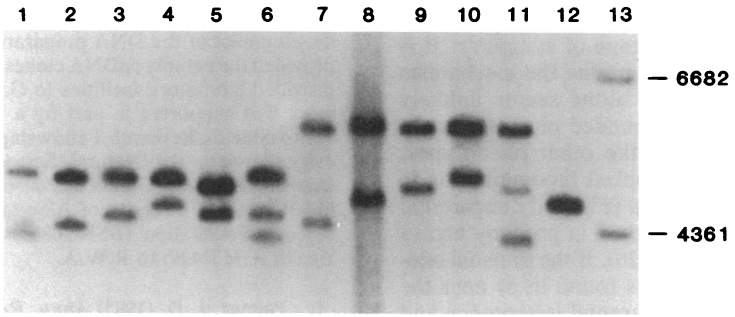

FIG. 3. *Sst* I cpDNA banding patterns (phenotypes) observed in lodgepole pine (lanes 1–6) and jack pine (lanes 7–12). cpDNA phenotypes are in the same order (left to right) as in Table 1 (top to bottom), except that cpDNA phenotype 4.7/5.5 is not included in the figure. *Hin*dIII-digested λ DNA is in lane 13 (fragment sizes in base pairs at right). Origin is at the top.

Our sampling scheme was not designed to detect intrapopulation variation. However, we sampled two or more individuals in 43 allopatric populations, and polymorphism was detected in 28 of these 43 populations.

Populations from the Zone of Sympatry. Ninety-five individuals were studied from 16 different populations within the zone of sympatry (Fig. 1). Forty-two individuals had the typical allopatric lodgepole pine *Sal* I cpDNA phenotype (5.9/9.5); among these 42 individuals, 40 had typical allopatric *Sst* I cpDNA phenotypes. Phenotypic frequencies of the individuals with typical phenotypes in the region of sympatry were similar to frequencies found in the allopatric region of lodgepole pine. The two remaining individuals with lodgepole pine *Sal* I cpDNA both had the three-banded 4.3/4.5/5.0 *Sst* I cpDNA phenotype.

Fifty-three individuals in the sample from the zone of sympatry had the typical jack pine *Sal* I phenotype (5.4/9.5); among these 53 individuals, 51 had typical allopatric jack pine *Sst* I phenotypes. Phenotypic frequencies in the region of sympatry were similar to those in the allopatric region of jack pine. The two remaining individuals with jack pine *Sal* I cpDNA in the zone of sympatry had *Sst* I phenotypes 4.3/4.8/5.7 and 4.7. The 4.3/4.8/5.7 phenotype is atypical in that it is three-banded and also includes restriction fragment 4.3, which was otherwise found only in *P. contorta*. The 4.7 phenotype is unique in two respects: (*i*) it has only one instead of two of the variable *Sst* I restriction fragment size classes and (*ii*) it was not found in the allopatric regions of either species. The data thus indicate that *Sst* I cpDNA phenotypes are more diverse in the zone of sympatry than in the much larger and more ecologically diverse allopatric regions of the two species.

Table 1. *Sst* I cpDNA phenotypes observed within taxa in the allopatric regions and in the region of sympatry

Phenotype	No. of individuals (frequency)		
	Allopatric *P. contorta* (n = 153)	Allopatric *P. banksiana* (n = 115)	Sympatric region (n = 95)
4.3/5.0	2 (0.01)		2 (0.02)
4.4/5.0	22 (0.14)		8 (0.08)
4.5/5.0	104 (0.68)		26 (0.27)
4.7/5.0	23 (0.15)		4 (0.04)
4.5/4.8	1 (0.01)		
4.3/4.5/5.0	1 (0.01)		2 (0.02)
4.4/5.7		5 (0.04)	3 (0.03)
4.7/5.7		17 (0.15)	10 (0.11)
4.8/5.7		85 (0.74)	36 (0.38)
5.0/5.7		6 (0.05)	2 (0.02)
4.7/5.5		1 (0.01)	
4.3/4.8/5.7		1 (0.01)	1 (0.01)
4.7			1 (0.01)

n, Number of individuals sampled.

Inheritance. *Sal* I and *Sst* I cpDNA restriction fragment phenotypes were determined for eight F_1 interspecific hybrid individuals obtained from four controlled pollinations between lodgepole pine (♀) and jack pine (♂), and for the single maternal lodgepole pine parent of four of these F_1 hybrids (19). Neither the maternal nor the paternal parents of the four remaining interspecific F_1 hybrids were available for study. Three of the four subspecies of *P. contorta* were represented among the female parents of the F_1 hybrid individuals; *P. banksiana* pollen used in making the hybrids came from several sources. The female parent available for study had typical *P. contorta Sal* I (5.9/9.5) and *Sst* I (4.7/5.0) cpDNA phenotypes. We deduce from our survey of allopatric *P. contorta* and *P. banksiana* that the unavailable female lodgepole pine parents had typical lodgepole pine cpDNA phenotypes and that the male jack pine parents had typical jack pine cpDNA phenotypes. All eight of the F_1 hybrid individuals examined had typical *P. banksiana Sal* I (5.4/9.5) and *Sst* I (4.8/5.7) cpDNA phenotypes, suggesting that each F_1 hybrid received its chloroplast genome from its paternal parent. Thus chloroplast inheritance appears to be predominantly paternal in F_1 interspecific hybrids between *P. contorta* (♀) and *P. banksiana* (♂). Paternal inheritance of cpDNA has recently been discovered in another member of the Pinaceae, *Pseudotsuga menziesii* (Mirb.) Franco (20).

DISCUSSION

Interspecific introgression of morphological, terpene, and alloenzyme characters (reviewed in ref. 12) has been reported in allopatric populations of lodgepole pine. A few trees were found in the zone of sympatry that, although classified on the basis of cone and needle morphology (21) as belonging to one species, carried cpDNA fragments that were otherwise unique to the other species. However, there was no indication of interspecific cpDNA flow in the allopatric populations of either species. Some of the same allopatric populations on which previous reports of introgression were based were included in our study, and they showed no sign of cpDNA introgression. This apparent contradiction may result from differences in sample size or may indicate that studies of morphological, terpene, and alloenzyme characters detected intraspecific polymorphism rather than interspecific introgression. Alternatively, it may indicate that nuclear genes may sometimes be able to cross species borders that cpDNA is unable to cross. The reverse situation—that organelle DNA may sometimes cross species borders that nuclear genes do not appear to cross—has been reported for mitochondrial DNA (22, 23).

The unusual cpDNA phenotypes that were observed in and adjacent to the zone of sympatry might be due to heteroplas-

my, recombination, or an elevated rate of establishment of insertion/deletion mutants near the zone of sympatry. It is not possible from present data to determine the mechanism responsible; however, heteroplasmy alone seems unlikely because not all of the one- or three-banded phenotypes are obtainable from simple overlays of the other phenotypes. Evidence exists, however, for chloroplast recombination in *Chlamydomonas* (24) and in *Nicotiana* (25), and nonparental cpDNA phenotypes have been observed in progeny arrays from *Pseudotsuga menziesii* hybrids (20). If the unusual one- and three-banded cpDNA phenotypes found in or near the sympatric zone are recombinant, biparental inheritance and its attendant transient heteroplasmy might be expected to occur, at least rarely. In such a case, the apparently paternal inheritance in the eight hybrids we studied may have resulted from a paternally biased contribution of chloroplasts to zygotes, from developmental selection, or from genotype-dependent inheritance.

We note that the restriction fragments of this study may possibly reside in either the nuclear or the mitochondrial genome (e.g., see ref. 26). However, if the fragments belong to the nuclear genome, they would be expected to follow the rules of Mendelian inheritance, which was not the case in this study. Homology of the fragments with the mitochondrial genome cannot be excluded until purified pine cpDNA and mitochondrial DNA are available, or until tissue-specific stoichiometries are examined. The clones we used were, however, not from the cpDNA inverted repeat, the region of greatest homology between the chloroplast and mitochondrial genomes (26); thus it seems likely that the restriction fragments studied were chloroplast rather than mitochondrial DNA fragments.

Our sampling strategy was designed to explore geographic patterns of two cpDNA polymorphisms, and not to determine whether the detected variants resulted from point mutations or from insertion/deletion events. Evidence from other studies (9, 10) suggests that insertion/deletion events sometimes occur in cpDNA and thus that they may have been a cause of the polymorphisms we observed.

Finally, we note that the utility of cpDNA in addressing different issues varies with the level of observed genetic diversity. Intraspecifically monomorphic markers, such as the *Sal* I phenotypes, appear to be useful phylogenetic tools. More variable markers, such as the *Sst* I variants, appear to be potentially powerful tools for studies of plant population genetic structure, particularly under circumstances when uniparentally inherited markers are advantageous.

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