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Paternal leakage of mitochondrial DNA in *Pinus*

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Summary. We studied mitochondrial DNA restriction fragment length polymorphism in 11 parents and 125 seedlings of 23 controlled matings within and between jack pine (*Pinus banksiana* Lamb.) and lodgepole pine (*P. contorta* Dougl.). A potential mitochondrial distinction between these two conifers was evident in the parental samples. Only maternal mitochondrial restriction fragments were observed in a majority of the seedlings, which is consistent with results from angiosperms and other members of the genus *Pinus* L. However, we detected exclusively paternal mitochondrial DNA in six of the seedlings. These unusual seedlings were not attributable to heteroplasmy or contamination of the experimental material, indicating that mitochondrial inheritance was not strictly maternal. Paternal mitochondrial leakage in *Pinus* may permit novel insights into the transmission genetics and evolution of organellar polymorphisms.

Key words: RFLPs – Organelles – Maternal inheritance – Cytonuclear disequilibrium

Introduction

Organellar and nuclear genetic factors differ in many ways, perhaps most notably in their modes of inheritance. For example, both mitochondria and chloroplasts are inherited maternally in many angiosperms (e.g., Correns 1909; Smith 1988a, b; Chiu et al. 1988; Tilney-Bassett and Almouslem 1989).

As in angiosperms, cytoplasmic and nuclear loci are differentially inherited in coniferous gymnosperms. However, the predominance of maternal organellar inheritance is not apparent in at least three taxonomic families of conifers. Maternal inheritance of mitochondrial DNA (mtDNA) restriction fragments has been observed in a hybrid of *Pinus strobus* L. × *P. griffithii* McClelland (Neale and Sederoff 1988) and in controlled matings of *P. taeda* L. (Neale and Sederoff 1989), but paternal mtDNA inheritance has been inferred in *Sequoia sempervirens* D. Don Endl. (Neale et al. 1989) and in *Calocedrus decurrens* [Torr.] Florin (Neale et al. 1991). Moreover, predominantly paternal chloroplast inheritance has been reported in *Cryptomeria japonica* D. Don. (Ohba et al. 1971), *Pseudotsuga menziesii* (Mirb.) Franco (Neale et al. 1986), *Larix* Mill. hybrids (Szmidt et al. 1987), *Pinus* L. hybrids (Wagner et al. 1987; Neale and Sederoff 1989), *Picea* A. Dietr. hybrids (Szmidt et al. 1988; Stine et al. 1989; Stine and Keathley 1990), *S. sempervirens* (Neale et al. 1989), *P. banksiana* Lamb. (Wagner et al. 1989), and *C. decurrens* (Neale et al. 1991).

The observed inheritance patterns in controlled matings of conifers (cited above) are not inconsistent with ultrastructural observations of organellar fates during reproduction (e.g., Chesnoy 1987; Bruns and Owens 1989), because chloroplast and mitochondrial segregation ratios are "net" outcomes of gametogenesis, fertilization, and development. However, ultrastructural evidence has led Bruns and Owens (1989) to suggest that paternal leakage of mitochondria might occur in *Pinus*. Here we present mitochondrial inheritance data from controlled crosses of *P. banksiana* and *P. contorta* Dougl., which provide the first genetic confirmation of paternal mtDNA leakage in Pinaceae.

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Materials and methods

Parents and crosses

We studied 125 seedlings from 23 controlled matings of seven *P. banksiana* individuals and four *P. contorta* var. *latifolia* Engelm. individuals. Parental mtDNA genotypes were not known a priori, because the crosses were not originally designed to test mtDNA inheritance. *P. banksiana* progenies are growing in a field trial planted in May 1986 at Chalk River, Ontario (Wagner et al. 1989). The *P. contorta* and interspecific pollinations were made in 1985 and 1986, and their seedlings are maintained in a greenhouse at the University of Kentucky.

Mitochondrial DNA analyses

Parental and progeny DNA samples were prepared by a modification (Wagner et al. 1987) of the method of Murray and Thompson (1980). Each DNA sample was isolated from foliage of a single branch tip and was cut to completion with the restriction endonuclease SstI. Pine restriction fragments were separated electrophoretically in agarose gels, transferred to filter membranes, blot-hybridized (Southern 1975) with a ^{32}P-labeled (Rigby et al. 1977) *COXII* sequence from maize (*Zea mays* L.) mitochondria (Fox and Leaver 1981; Dawson et al. 1986), and visualized by autoradiography.

P. banksiana and *P. contorta* chloroplast DNA (cpDNA) can be variable within single trees (Govindaraju et al. 1988), and mtDNA variation occurs within individuals of maize (Newton et al. 1989). Therefore, in the present study we prepared multiple DNA samples from individuals with sufficient available foliage samples (Tables 1, 2). DNA was isolated independently from at least two branch tips of each multiply sampled tree, and multiple samples were spaced as widely as possible within each individual.

Results and discussion

Our investigation detected several variable restriction fragments (Fig. 1, Table 1). These fragments appear to distinguish *P. banksiana* from *P. contorta* and may have utility as genetic markers in regions of natural hybridization between the two species. Restriction fragment length polymorphism (RFLP) was evident within *P. contorta*, despite the very limited sample from only two sources in British Columbia. In contrast, intraspecific polymorphism was undetectable in samples that nearly spanned the wide longitudinal range of *P. banksiana* (Table 1). These data are intriguing, in view of the environmental and genetic heterogeneity associated with the mountainous natural range of *P. contorta* in western North America (e.g., Critchfield 1957). Population surveys could test the reliability of the apparent taxonomic distinction and determine whether or not this RFLP is truly less variable in *P. banksiana* than it is in *P. contorta*.

The mode of inheritance could be inferred from 12 of the controlled crosses, involving four *P. banksiana* and three *P. contorta* individuals, because restriction fragment sizes differed between the maternal and paternal parents in each of these matings (Tables 1, 2). We detected strictly maternal restriction fragments in approximately 92% of the seedlings from these 12 informative crosses (Fig. 1, Table 2). Maternal inheritance was predominant, regardless of whether matings were interspecific, intraspecific, or reciprocal.

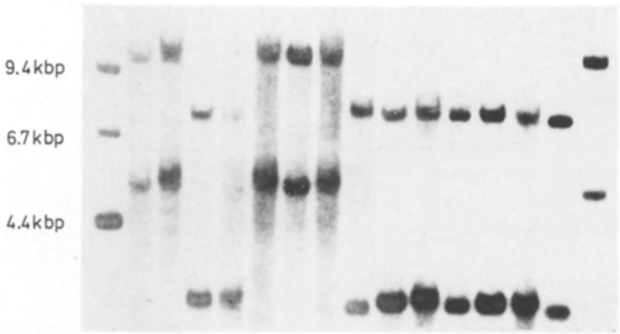

Fig. 1. Mitochondrial DNA inheritance in a reciprocal cross of parent trees 14-13-6 and 55-9-6: phage lambda HindIII fragments, as size markers (*lane 1*); three seedlings from the cross 14-13-6 × 55-9-6 (*lanes 2–7*); parent 14-13-6 (*lane 8*); parent 55-9-6 (*lane 9*); three seedlings from the cross 55-9-6 × 14-13-6 (*lanes 10–14*); parent 55-9-6 (*lane 15*); parent 14-13-6 (*lane 16*). For five of the seedlings, restriction fragments from two independent DNA purifications are shown, with each of the following five pairs of samples originating from a single seedling: *lanes 2* and *3*; *4* and *5*; *6* and *7*; *11* and *12*; *13* and *14*. Gel lanes are numbered *left-to-right*; *lanes 1–9* were not run on the same gel with *lanes 10–16*

Table 1. Parental sources and mtDNA genotypes

Parent[a]	Species	Geographic origin	Genotype[b]
61	*P. banksiana*	Unknown	2.9/7.6(6)
65	*P. banksiana*	Somerset County, Maine	2.9/7.6(1)
101	*P. banksiana*	Smoky Lake, Alberta, Canada	2.9/7.6(4)
104	*P. banksiana*	Smoky Lake, Alberta, Canada	2.9/7.6(1)
14-6-3	*P. contorta*	Kamloops, British Columbia, Canada	5.2/10.2(1)
14-13-6	*P. contorta*	Kamloops, British Columbia, Canada	5.2/10.2(12)
55-9-6	*P. contorta*	Smithers, British Columbia, Canada	3.1/7.6(8)

[a] Locations of trees are as follows: 61 (provided by the Massachusetts Department of Conservation) and 65 are in arboreta of the Institute of Forest Genetics, Placerville/CA; 101 and 104 occur in a natural stand at the Alberta Forest Service Pine Ridge Forest Nursery, Smoky Lake, Alberta; and *P. contorta* individuals are in a provenance test plantation of the British Columbia Ministry of Forests, Prince George, BC. Parents are listed only if involved in informative crosses (see text for details)

[b] Genotypes are denoted by sizes, in kilobase pairs, of polymorphic restriction fragments. The numbers of DNA samples analyzed per parent tree are indicated in parentheses

The observed predominance of maternal inheritance permits us to infer that the restriction fragments identi-

Table 2. Mitochondrial DNA segregation ratios[a]

Cross	Maternal progeny	Paternal progeny	Non-parental progeny	Total progeny
Interspecific crosses:				
104 × 14-13-6	9(4)	1(0)	0	10(4)
14-13-6 × 104	8(8)	0	0	8(8)
104 × 14-6-3	7(2)	2(0)	1(0)[b]	10(2)
14-6-3 × 104	3(3)	0	0	3(3)
61 × 55-9-6	2(2)	0	0	2(2)
14-13-6 × 61	2(2)	0	0	2(2)
14-13-6 × 65	5(1)	1(0)	0	6(1)
14-13-6 × 101	5(5)	0	0	5(5)
Crosses within *P. contorta*:				
55-9-6 × 14-13-6	19(19)	0	0	19(19)
14-13-6 × 55-9-6	5(2)	1(1)	0	6(3)
55-9-6 × 14-6-3	5(1)	1(1)	0	6(2)
14-6-3 × 55-9-6	7(7)	0	0	7(7)
Total	77(56)	6(2)	1(0)	84(58)

[a] Data is for crosses in which the two parents differed in mtDNA genotype; female parent of each cross is listed first. Numbers of seedlings, for which two independent DNA samples were analyzed per seedling, are indicated in parentheses

[b] The single nonparental individual's genotype was 2.4

fied here by *COXII* are indeed mitochondrial. Although cross-homologies among the three major plant genomes (e.g., Timmis and Scott 1983; Stern and Palmer 1984; Sederoff et al. 1986) could cause misinterpretation of restriction fragments identified by molecular hybridizations, chloroplast or nuclear restriction fragments would not be predominantly maternally inherited in *Pinus* (Wagner et al. 1987; Neale and Sederoff 1988, 1989; Wagner et al. 1989).

Six unusual seedlings had mitochondrial restriction fragments exclusively like those of their paternal parents, and one individual's mtDNA genotype was unlike either of its parents. In principle, these nonmaternal progeny may have resulted from contamination of the experimental materials, from mtDNA polymorphism within maternal parents, or from paternal mtDNA transmission.

Pollen contamination could not have influenced seedling genotypes of a strictly maternally inherited polymorphism. Therefore, if germ plasm contamination alone was responsible for the nonmaternal progeny, this contamination must have occurred through seeds. However, seed contamination would be unexpected in the material of this study, because seeds were harvested, cleaned, and planted by hand.

The following two lines of reasoning also indicate that the unusual progeny did not result from seed contamination. First, seed contamination should not be restricted to informative crosses (Table 2), but should also occur in crosses between parents with identical mtDNA genotypes. However, we failed to detect any unexpected mtDNA genotypes in 41 seedlings from the 11 matings of the latter type, even though one of these 11 matings had a female parent (55-9-6) that mothered an unexpected seedling in one of the 12 informative crosses. Second, cpDNA analyses (e.g., Wagner et al. 1987) indicated that all seven unusual seedlings had the same cpDNA genotype as their pollen parents, exactly as would be expected if the crosses were uncontaminated.

Within-individual mtDNA variability (Newton et al. 1989) of maternal parents is also a potential explanation for the unusual seedlings. If female parents contain undetected mtDNA genotypes, occasional individuals could receive strictly maternal mtDNA and yet appear nonmaternal in our assay. Under this hypothesis, however, unanticipated progeny should not have occurred in only the 12 informative crosses. Moreover, we were unsuccessful in all direct attempts to detect within-tree mtDNA variation, despite the fact that these attempts included two maternal parents (14-13-6 and 55-9-6) of unexpected progeny (Tables 1, 2).

The single nonparental seedling was a species hybrid and possessed a unique mtDNA genotype (Table 2). This individual could have resulted from paternal mtDNA leakage alone, if mtDNA was variable within its pollen parent (14-6-3) or if contaminant pollen was responsible. As mentioned above, however, within-individual mtDNA variation seems infrequent in *P. banksiana* and *P. contorta*. Also, the cpDNA genotype (Wagner et al. 1987) of this unusual seedling was identical with that of its pollen parent, which is consistent with a lack of germ plasm contamination. Alternatively, rearrangement or recombination of the mitochondrial genome (e.g., Belliard et al. 1979; Rothenberg et al. 1985; Robertson et al. 1987) may account for the nonparental seedling. Further study is clearly indicated to resolve the origin of this unusual individual.

We conclude that paternal mtDNA was inherited by six of the unexpected progeny in the 12 informative matings (Table 2), confirming the prediction from ultrastructural data that paternal mtDNA is incompletely excluded in Pinaceae (Bruns and Owens 1989). This conclusion does not contradict other RFLP evidence for maternal mitochondrial inheritance in *Pinus* (Neale and Sederoff 1988, 1989), because the previous data was based on different species and on sample sizes that may have been insufficient to detect occasional paternal leakage. Although paternal mtDNA inheritance has already been reported in two families of conifers (Cupressaceae, Taxodiaceae) (Neale et al. 1989, 1991), the present data represent the first exception to strict uniparental mtDNA inheritance.

Paternal mtDNA leakage in *P. banksiana* and *P. contorta* is not due merely to instabilities in species hybrids, because paternal progeny arose from both intraspecific and interspecific crosses. Neither was paternal leakage restricted to certain parental genotypes: four pollen donors (representing all three mtDNA genotypes) and three mothers (again representing all three mtDNA genotypes) parented paternal progeny (Tables 1, 2). Larger experimental designs may elucidate genetic mechanisms controlling the frequency of paternal mtDNA leakage (e.g., organellar versus nuclear genotype, maternal versus paternal genotype).

In principle, incomplete exclusion of paternal mtDNA may produce zygotes with varying mixtures of maternal, paternal, and recombinant mtDNA (e.g., Birky et al. 1989). Within-individual mtDNA variation then could be maintained within single cells or be distributed into genotypically distinct plant sectors. Alternatively, parental or recombinant mtDNA genotypes could evolve to fixation within individuals during development. Although our results are consistent with mtDNA genotypic fixation within individuals, detailed investigations will be necessary to understand the within-individual dynamics and ultimate consequences of paternal mtDNA leakage in *P. banksiana* and *P. contorta*.

Nuclear-encoded allozyme markers and paternally inherited cpDNA markers have been identified previously in *P. banksiana* and *P. contorta* (e.g., Wheeler and Guries 1987; Wagner et al. 1987). Therefore, these two species now represent an unusual model system in which differentially inherited polymorphisms of the three major plant genomes are available. This differential inheritance may confer substantial power on cytonuclear population investigations (Schnabel and Asmussen 1989). However, the actual utility of *P. banksiana* and *P. contorta*, as a cytonuclear model, depends on the extent of paternal mtDNA leakage and on the associated implications for cytonuclear disequilibria, both of which are unknown at present.

The unusual coniferous modes of organellar inheritance have been recognized as potentially significant for enhanced evolutionary understanding of mechanisms that control chloroplast and mitochondrial inheritance. Harrison and Doyle (1990) recently stated, "Although natural instances of organelle genome recombination remain unproven, it will surely not be too much of a surprise to find that organelle lineages anastomose ..." In view of the apparent (though possibly transient) simultaneous presence of maternal and paternal mtDNA early in the *P. banksiana* and *P. contorta* life cycle, these two species may be an especially useful experimental system for evolutionary studies of mtDNA inheritance, as well as for tests of mitochondrial recombination.

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