Theor Appl Genet (1996) 93: 587–592

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Inter- and intraspecific polymorphism at chloroplast SSR loci and the inheritance of plastids in *Pinus radiata* D. Don

Received: 19 October 1995 / Accepted: 2 February 1996

Abstract DNA sequence analysis of chloroplast genomes has revealed many short nucleotide repeats analogous to nuclear microsatellites, or simple sequence repeats (SSRs). We designed PCR primers flanking five of these regions identified in the chloroplast sequence from *Pinus thunbergii* and tested them for amplification in *Pinus radiata*, *P. elliotii*, *P. taeda*, *P. strobus*, *Pseudotsuga menziesii*, *Cupressus macrocarpa*, four New Zealand native conifer species (*Podocarpus totara*, *Podocarpus hallii*, *Podocarpus nivalis*, *Agathis australis*), and four angiosperms (*Vitex lucens*, *Nestegis cunninghamii*, *Actinidia chinensis*, and *Arabidopsis thaliana*). A PCR product in the expected size range was amplified from all species and interspecific polymorphism was detected at all five loci. Intraspecific polymorphism was detected in *P. radiata* with four of the five primer pairs. One of these polymorphic chloroplast SSR (cpSSR) was then used to determine the inheritance of chloroplasts in 206 progeny from four control-pollinated, full-sibling *P. radiata* families. Approximately 99% of the progeny had the cpSSR variant of the pollen parent indicating that in *Pinus radiata*, like most other conifers, chloroplasts are typically inherited from the paternal parent. These results suggest that polymorphic chloroplast SSRs will be a valuable tool for studying chloroplast diversity, cyto-nuclear disequilibrium, and plastid inheritance in a range of species, and for the analysis of gene flow via pollen and paternity in species with paternal transmission of chloroplasts.

Key words Chloroplast DNA · SSRs · *Pinus radiata* · Plastid inheritance

Communicated by D. R. Pring

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Introduction

In most angiosperms, cytoplasmic organelles are inherited from the maternal parent (Sears 1980; Whatley 1982; Corriveau and Coleman 1988). In gymnosperms, however, chloroplast inheritance is predominantly paternal, while mitochondria are typically maternally transmitted (Wagner 1992 and references therein). Cytological studies in conifers have suggested that paternal chloroplast transmission results from the transformation of maternal plastids into large inclusions in the egg cell, which are excluded from the cytoplasm of the developing proembryo (Chesnoy and Thomas 1971; Owens and Morris 1990; 1991). The occasional incorporation of these inclusions into the cytoplasm of the proembryo may give rise to the maternal transmission of chloroplasts, or "maternal leakage" (Owens and Morris 1990; 1991).

Various genetic methods have been used to investigate the inheritance of chloroplast (cp) DNA in conifers. Plastid mutants (either natural or induced) have been used to follow chloroplast inheritance in reciprocal crosses. Using this approach, Ohba et al. (1971) demonstrated that in *Cryptomeria japonica* plastids were paternally inherited in 90% of the progeny, while approximately 7% of the trees expressed both normal and mutant phenotypes (perhaps as a result of biparental inheritance) and 3% of the progeny had maternal phenotypes. More recently, restriction fragment length polymorphisms (RFLPs) have been used to follow the transmission of cpDNA. These studies have confirmed a largely paternal mode of cpDNA inheritance in many conifer species, including *Pseudotsuga menziesii* (Neale et al. 1986), *Larix* hybrids (Szmidt et al. 1987), *Pinus banksiana*, *P. contorta*, and their hybrids (Wagner et al. 1987; Govindaraju et al. 1988; Wagner et al. 1989; Dong et al. 1992), *Pinus rigida*×*Pinus taeda* hybrids (Neale and Sederoff 1989), *Sequoia sempervirens* (Neale et al. 1989), *Picea* hybrids (Szmidt et al. 1988; Stine et al. 1989; Stine and Keathley 1990), *Calocedrus decurrens* (Neale et al. 1991), and *Pinus monticola* (White 1990). However, occasional progeny with maternal, non-parental or bi-parental cpDNA genotypes were observed in most of these studies (e.g. Neale et al. 1986; Szmidt et al. 1987; Wagner et al. 1989; Dong et al. 1992; Neale et al. 1991; White 1990; Govindaraju et al. 1988).

Although chloroplast RFLPs are more abundant than phenotypic mutants, RFLPs are time-consuming, and even with improved DNA extraction methods several grams of tissue are typically required to extract sufficient quantities of cpDNA for analysis using RFLPs. Conversely, genetic analysis using the polymerase chain reaction (PCR) is fast, technically simple and requires very small amounts of DNA, and hence starting tissue. To capitalise on these PCR attributes, some workers have successfully converted informative RFLPs to PCR-based markers to study cpDNA inheritance (Cruzan et al. 1993). Others have used "universal chloroplast primers" designed from highly conserved regions within the cp-genome to amplify potentially polymorphic regions across a range of species (Taberlet et al. 1991; Boscherini et al. 1994; Vicario et al. 1995). Unfortunately, these "universal chloroplast primers" frequently fail to detect polymorphism even with subsequent restriction digestion of the PCR products (e.g. Boscherini et al. 1994; Vicario et al. 1995), presumably due to the relatively low mutation rate of cpDNA (Wolf et al. 1987).

Simple sequence repeats (SSRs, microsatellites) assayed by PCR amplification have become the marker of choice for many genetic studies because they are abundant and highly polymorphic in most species studied (Tautz 1989; Moore et al. 1991; Lagercrantz et al. 1993; Morgante and Olivieri 1993). Recently the complete sequence of the *P. thunbergii* chloroplast genome was published (Wakasugi et al. 1994). Computer searches of this sequence revealed single nucleotide repeats analogous to nuclear SSRs. The relative conservation of the chloroplast genome suggests that these repeats (and their flanking sequences) may be conserved in other coniferous species. Powell et al. (1995) recently demonstrated that primers designed around one of these chloroplast simple sequence repeats (cpSSRs) revealed interspecific polymorphism in several *Pinus* species and intraspecific polymorphism in *P. leucodermis* arising from variation in the length of the repeat.

In the study presented here we designed PCR primers based on the *P. thunbergii* sequence that flank five cpSSRs and tested them for amplification in ten gymnosperm and four angiosperm species from diverse families. One of the polymorphic loci was then used to carry out the first study of plastid inheritance in *Pinus radiata*.

Materials and methods

Sequence analysis and primer design

Searches of the published *P. thunbergii* chloroplast genome sequence in GenBank were carried out using Genetics Computer Group Inc software (Madison, WI). These searches revealed 19 single nucleotide repeats greater than 10 nucleotides in length. PCR primers flanking 5 of the longest repeats (>14 nucleotides) were designed using PRIMER 0.5 software (Lincoln et al. 1991). Where possible PCR primers were positioned in coding sequence to increase the likelihood of amplification from heterologous species. Subsequently, PCR primers for one of these loci (cpSSR 2) were re-designed to match those tested by Powell et al. (1995).

Plant material

Tissue samples were collected from representatives of ten gymnosperm and four angiosperm species including five species of Pinaceae (*Pinus radiata*, *P. elliotii* , *P. taeda*, *P. strobus*, and *Pseudotsuga menziesii*), three Podocarpaceae (*Podocarpus totara*, *P. hallii*, and *P. nivalis*), and one species each from the Cupressaceae (*Cupressus macrocarpa*), Araucariaceae (*Agathis australis*), Verbenaceae (*Vitex lucens*), Oleaceae (*Nestegis cunninghamii*), Actinidiaceae (*Actinidia chinensis*), and Brassicaceae (*Arabidopsis thaliana*). In addition, tissue samples were collected from 23 *Pinus radiata* clones to test for intraspecific cpSSR polymorphism and from a total of 206 progeny and the parents from four control-pollinated, full-sibling *P. radiata* families to determine the transmission of cpDNA in this species.

DNA extraction, PCR and electrophoresis

Genomic DNA from all species was extracted using a modified CTAB protocol (Doyle and Doyle 1987). Briefly, an Eppendorf tube was cooled with liquid nitrogen, and a small amount of fresh or frozen tissue was ground to a fine powder using a glass pestle. Six hundred microliters of CTAB buffer (2% CTAB, 1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* TRIS-HCl (pH 8.0), 1% polyvinylpyrollindone) and 1.2 µl β-mercaptoethanol were added and the tissue incubated at 65°C for 30 min followed by two chloroform-isoamyl alcohol extractions. DNA was then precipitated with cold isopropanol and the pellet redissolved in 500 µl of 1 *M* NaCl. Following centrifugation at 13 000 g for 5 min, the supernatant was removed to a fresh Eppendorf tube and incubated at 95°C for 30 min. DNA was reprecipitated with cold isopropanol and resuspended in 100 µl of distilled water. One microliter of RNaseA (1 gl^{-1}) was then added and the samples incubated at 37°C for 30 min. A GeneQuant DNA calculator (Pharmacia) was used to quantify the DNA for PCR.

PCR amplifications were carried out in a total volume of 10 µl containing 12 ng of template DNA, 0.4 µ*M* of each primer, 2 m*M* $MgCl_2$, 0.1 m*M* each of dATP, dGTP, dTTP, 0.01 m*M* dCTP, 0.06 µl of [^{32}P]dCTP (Amersham), 0.5 units of *Taq* polymerase (Boehringer Mannheim), 50 m*M* KCl and 10 m*M* TRIS-HCl (pH 8.3). Samples were overlayed with paraffin oil and amplified in 96-well polycarbonate plates on a Techne PHC-3 thermal cycler with 1 cycle at 93°C for 3 min and 30 cycles of 30 s at 94°C, 30 s at 60°C (63°C for cpSSR 1), and 30 s at 72°C. Following amplification, PCR products were electrophoresed in a 10% (6% for cpSSR1) denaturing polyacrylamide gel (Sambrook et al. 1989) in 1×TBE at 35 W for 115 min to permit resolution of 1-base pair (bp) allelic differences. [^{32}P]-labelled ssDNA from the bacteriophage M13mp18 (T7 sequencing Kit, Pharmacia) was used as a marker-weight-size standard. Gels were fixed in a 10% methanol, 10% acetic acid solution, dried, and autoradiographed with Kodak X-OMAT XK-1 film for 1 h at –70°C with one intensifying screen.

Results and discussion

Heterologous amplification

GenBank searches of the published *P. thunbergii* chloroplast sequence revealed 19 single nucleotide repeats of greater than 10 nucleotides in length. PCR primers flanking the 5 longest repeats were designed and tested on 14 species from four conifer and four angiosperm families.

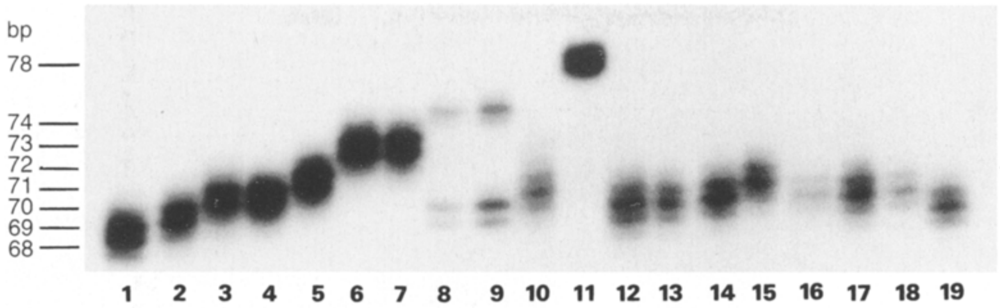

Fig. 1 Inter- and intraspecific polymorphism detected with cpSSR 2. *Lanes 1–3 Pinus radiata, 4–5 P. elliotii, 6–7 P. taeda, 8–9 P. strobus, 10 Cupressus macrocarpa, 11 Pseudotsuga menziesii, 12 Podocarpus totara, 13 P. hallii, 14 P. nivalis, 15 Agathis australis, 16 Vitex lucens, 17 Nestegis cunninghamii, 18 Actinidia chinensis, 19 Arabidopsis thaliana*. The additional bands present in the two *P. strobus* samples may result from multiple priming sites in the chloroplast genome

For 12 of the 14 species a PCR product in the predicted size range was amplified with all five primer pairs (Table 1, Fig. 1). The exceptions were *Nestegis cunninghamii* with cpSSR 1 and *Actinidia chinensis* with cpSSR 1, 3 and 4 (data not shown). These results corroborate previous studies in which PCR primers designed from consensus chloroplast sequence successfully amplified cpDNA in heterologous species (Taberlet et al. 1991; Powell et al. 1995). Our results suggest that primer pairs flanking cpSSRs may amplify potentially polymorphic regions in the chloroplast genome in a broader range of taxa than has been previously reported.

Inter- and intraspecific polymorphisms

Interspecific polymorphism was detected at all five loci across the ten conifer and four angiosperm species tested (Table 1, Fig. 1). This observation extends the results of Powell et al. (1995) who reported a total of eight variants at cpSSR 2 in ten *Pinus* species different from those tested here and suggested that interspecific polymorphism at cpSSRs may be common. In several previous studies using "universal chloroplast primers" flanking the intergenic spacers between four *trn* genes, inter- or intraspecific polymorphism was not detected by PCR or with subsequent restriction digests (Vicario et al. 1995; Boscherini et al. 1994). However, in these studies the PCR amplification products were electrophoresed on agarose gels incapable of resolving single base pair differences in fragment length. Examination of the partial nucleotide sequence of the intergenic spacer between two of these genes, *trnL* and *trnF* (Taberlet et al. 1991), reveals a thymidine SSR differing in length by 1 bp (10 and 11 bases) in the two *Acer* species reported. Similar variants may have been detected in other studies using "universal primers" had the PCR products been electrophoresed on more sensitive gel systems.

Four of the five cpSSR loci that we tested also detected intraspecific single base pair variants in the 23 *P. radiata* individuals tested (Table 1, Fig. 2). The estimate of gene diversity (Weir, 1990) for this sample based on cpSSR 2 variant frequencies is 0.53. This is only slightly lower (0.69–0.71) than the estimate obtained for the same 23 individuals using the two polymorphic nuclear microsatellites reported in Smith and Devey (1994) and much higher than previous estimates using isozymes in *P. radiata* (Moran et al. 1988). Powell et al. (1995) also reported greater within-population genetic diversity for cpSSR 2 (0.0–0.629) than for nuclear isozymes in a sample of 305

Table 1 Summary of cpSSR loci tested

Locus	Micro-satellite repeat	PCR Primers[a]	Fragment sizes in *P. radiata* (bp)	Number of inter-specific variants
cpSSR 1	$(A)_{12}(G)_{10}$	5′ CAACAGAAGCCCAAGCTTATGG 3′ 5′ TGTATTGTATGCGGAATCAACTGG 3′	217, 218	5
cpSSR 2[b]	$(A)_{17}$	5′ TGCGTTGCACTTCGTTATAG 3′ 5′ CAATTGGAATGAGAACAGATAGG 3′	68, 69, 70	9
cpSSR 3	$(T)_{16}$	5′ AGAATCTTTGCAAGAAGGATGG 3′ 5′ TAATATGGGGAGTAATCCGTGG 3′	120, 121	5
cpSSR 4	$(T)_{15}$	5′ TGATCGACCCAATATGGAAC 3′ 5′ GAAACCCCAAGAACAAGAGG 3′	123, 124	2
cpSSR 5	$(T)_{14}$	5′ TCCAGGATAGCCCAGCTG 3′ 5′ TATATCCCCCGTACTTGGACC 3′	112	4

[a] The underlined bases are located in identified genes or ORFs (Wakasugi et al. 1994)
[b] The primers used for this locus are the same as those used by Powell et al. (1995)

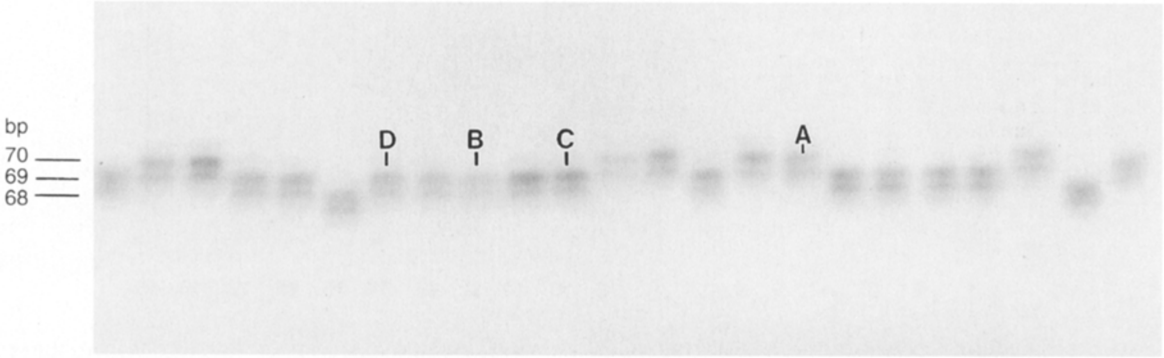

Fig. 2 Intraspecific polymorphism at cpSSR 2 in 23 *P. radiata* individuals. Parents used for the chloroplast transmission study are indicated as *A*, *B*, *C*, and *D*. Parent *E* has the same haplotype as parents *B*, *C*, and *D*

individuals from seven *Pinus leucodermis* populations in Greece and southern Italy. These results suggest that in spite of the overall lower mutation rate of cpDNA relative to nuclear DNA (Wolf et al. 1987), cpSSRs may share the hypervariable nature of nuclear SSRs that has led to their use for genetic analyses in many plant and animal species (Tautz 1989; Moore et al. 1991; Lagercrantz et al. 1993; Morgante and Olivieri 1993).

Chloroplast inheritance in *P. radiata*

We used cpSSR 2 to investigate chloroplast inheritance in four *P. radiata* families where the parents had different cpSSR 2 variants (Fig. 2). A total of 206 progeny from four control pollinated, full-sib families were tested. Of these progeny, 199 had the paternal haplotype and 7 had the haplotype of the maternal parent (Table 2, Fig. 3). Subsequent analysis of the progeny exhibiting non-paternal haplotypes with two nuclear SSR loci for which the parental genotypes and population allele frequencies are known revealed that 5 of these progeny were sired by contaminating pollen, and as a result paternal transmission of cpDNA could not be ruled out. Two progeny from cross A×E exhibiting the maternal cpSSR 2 variant were found to be consistent with the expected parentage based on their nuclear SSR genotypes, and hence are possible examples of maternal chloroplast leakage. An additional 58 progeny with paternal cpSSR 2 haplotypes were also tested with nuclear SSRs, and no further pollen contamination was detected.

Table 2 Inheritance of chloroplasts in four *P. radiata* families

Maternal parent	Paternal parent	Number of progeny tested	Number of maternal haplotypes observed
B	A	50	0
C	A	50	0
D	A	49	5[a]
A	E	57	2
Total		206	7

[a] Subsequent analysis of these 5 progeny with two nuclear SSR loci revealed that all were sired by contaminating pollen for which the cpSSR 2 haplotype is unknown. Paternal transmission of the observed cpSSR 2 variant therefore could not be ruled out for these 5 progeny

This low level of maternal chloroplast leakage (1%) across genotypes and cross directions in *P. radiata* is similar to that found in a study of chlorophyll mutants in *Cryptomeria japonica* where 3% of the progeny demonstrated maternal phenotypes and 7% were chimeric (Ohba et al. 1971), and in many RFLP studies of cpDNA inheritance in other conifers. For example, Neale et al. (1989) detected no maternal chloroplast leakage in 22 progeny from two *Sequoia sempervirens* families; Wagner et al. (1989) reported at least 96% paternal haplotypes in 146 *Pinus banksiana* progeny from a complete diallel; Dong et al. (1992) found one maternal RFLP haplotype in 221 *Pinus contorta*×*P. banksiana* hybrids; and Neale et al. (1991) detected one maternal RFLP type in ten *Calocedrus decurrens* progeny. Evidence for possible biparental inheritance has been reported in *Pinus monticola* (White 1990) and interspecific hybrids of *P. contorta*×*P. banksiana* (Govindarju et al. 1988).

Our detection of multiple paternity among control-pollinated progeny formerly assumed to be full-siblings suggests that verifying parentage is critical in studies of organelle transmission where detecting low levels of leakage is an aim. This is supported by the results of Wagner et al. (1989) who found germplasm contamination to be at least partially responsible for non-paternal haplotypes in *Pinus banksiana*. Moreover, most other conifer studies report finding significant numbers of non-*parental* cpDNA haplotypes in progeny from controlled crosses, though few suggest that pollen contamination could be the cause (but see Dong et al. 1992) and parentage is typically not verified. Since commercial seed orchards are often the source of materials for organelle transmission (and other genetic) studies, and ramet mislabelling is present to some degree in most conifer seed orchards tested to date (Wheeler and Jech 1992 and references therein; Richardson unpublished data) verification of proper parentage should be established prior to further genetic analysis.

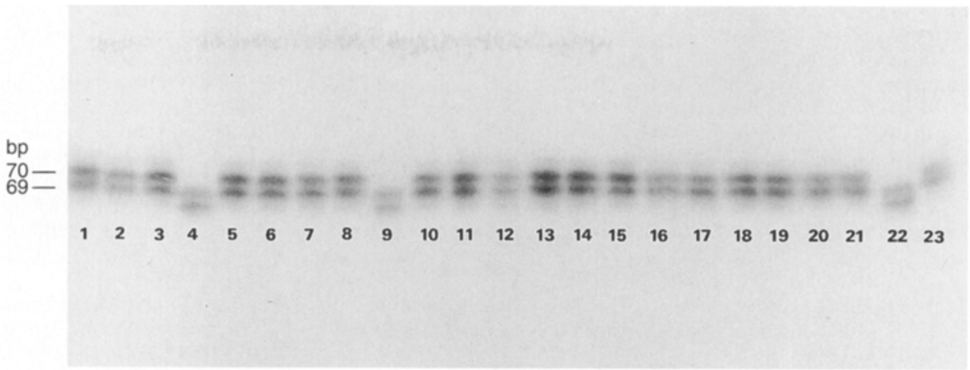

Fig. 3 cpSSR 2 haplotypes from control-pollinated family D×A demonstrating possible maternal chloroplast leakage. *Lanes 1–21* progeny, *22* maternal parent, *23* paternal parent. Progeny in *lanes 4* and *9* have maternal haplotypes

This work is the first application of chloroplast SSRs to study plastid inheritance and the first demonstration that PCR primers designed around these loci will amplify DNA and detect interspecific polymorphisms in a wide range of plant families. There are additional single nucleotide repeats in the *Pinus thungergii* chloroplast genome (and others, see Powell et al. 1995) that may also be informative in other species. Careful design of PCR primers should allow several loci to be amplified in a single PCR reaction (i.e., multiplex PCR) enabling multiple loci to be assayed for each individual in a single lane on an electrophoretic gel. Our results suggest that polymorphic chloroplast SSRs will be a valuable tool for studying chloroplast diversity, plastid inheritance and cytonuclear disequilibrium in a range of species, and for establishing paternity and estimating gene flow via pollen in species with a paternal transmission of chloroplasts.

Acknowledgements We thank R. Gardner and P. Barrell for providing DNA from *Actinidia* and *Vitex*, J. A. Rafalski of DuPont for providing manuscripts prior to publication, and S. Carson and R. Gardner for comments on the manuscript. Funding for this project was provided by the FRI-GEENZ joint venture, the New Zealand Public Good Science Fund (FRST 94-FRI-09-033), and the New Zealand Seed Orchard Research Group.

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