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# Paternal chloroplast inheritance patterns in pine hybrids detected with *trnL–trnF* intergenic region polymorphism

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**Abstract** The inheritance patterns of the chloroplast genomes of shortleaf pine (*Pinus echinata* Mill.), loblolly pine (*Pinus taeda* L.) and slash pine (*Pinus elliottii* Engelm.) were investigated through the *trnL–trnF* intergenic spacer polymorphism analysis. The DNA sequences of this spacer differ among these three closely related *Pinus* species. A modified 'cold' PCR-SSCP (single-strand conformation polymorphism) analysis of this spacer shows that the artificial hybrids (F1) from the shortleaf pine (seed parent) × loblolly pine (pollen parent) cross, exhibit the loblolly pine profile. Additionally, nine putative hybrids between shortleaf pine and loblolly pine, previously identified by the IDH (Isocitrate dehydrogenase) allozyme marker, presented the shortleaf pine profile indicating that shortleaf pine, not loblolly pine, sired all of the putative hybrids. Nondenatured polyacrylamide-gel electrophoresis of the *trnL–trnF* intergenic spacer demonstrated that the artificial hybrids (F1) from the cross, slash pine (seed parent) × shortleaf pine (pollen parent), present the shortleaf pine profile. Those results confirmed that the chloroplast genome is paternally inherited in these three species of the genus *Pinus*. The significance of the *trnL–trnF* intergenic region polymorphism and our modified 'cold' SSCP protocol for population genetic studies is discussed.

**Keywords** *trnL–trnF* intergenic spacer · Single-strand conformation polymorphism · Chloroplast inheritance pattern · *Pinus taeda* L. · *Pinus echinata* Mill. · *Pinus elliottii* Engelm

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## Introduction

Chloroplast genomes are paternally inherited in *Pinus* (Brent and David 1989). Most of the previous studies (Wagner et al. 1987; Neale and Sederoff 1989; Dong et al. 1992; Sunnucks et al. 2000) used traditional RFLP with isotope-labeled probes to determine the inheritance mode of the chloroplast genome, which is time-consuming and cumbersome (Tadashi et al. 1993). Recently, PCR-RFLP analysis of the *rbc*L gene has been successfully employed to study chloroplast inheritance (Cipriani et al. 1995; Edwards et al. 1997), but the rareness of mutation of the *rbc*L gene between closely related species limits the utilization of this method for paternal analysis. In this article, we established a simple screening method to reveal the chloroplast haplotypes in two controlled crosses of three closely related *Pinus* species using chloroplast *trnL–trnF* intergenic spacer polymorphism.

The plant *trnL–trnF* intergenic spacer is less than 500-bp long. The two universal primers designed by Taberlet et al. (1991) can be used to amplify this spacer in various plant species. In addition, high polymorphism generally exists in the *trnL–trnF* intergenic spacer among species. For example, the *trnL–trnF* intergenic spacer sequences of *Acer pseudoplatanus* and *Acer platanoides*, two closely related species, are different (Taberlet et al. 1991). The sequence difference between different species within this spacer region can be detected using polyacrylamide-gel electrophoresis, PCR-SSCP or even agarose-gel electrophoresis. Therefore, the existence of the two universal primers and the high polymorphism in the *trnL–trnF* intergenic spacer make it a good marker for paternal analysis of many plant species and its use should facilitate population genetic studies in plants.

In this paper, a modified 'cold' PCR-SSCP analysis and nondenatured acrylamide-gel electrophoresis of the *trnL–trnF* intergenic spacer have been successfully applied to track chloroplast inheritance in hybrids between shortleaf pine (*Pinus echinata* Mill.) and loblolly pine (*Pinus taeda* L.), and in hybrids between slash pine

(*Pinus elliottii* Engelm.) and shortleaf pine. Shortleaf, loblolly and slash pine all belong to subsection *Australes* Loudon, section *Trifoliis* within the subgenus *Pinus* (Price et al. 1998). Our results also track paternity in putative hybrids in the genus *Pinus*. The significance of *trnL–trnF* intergenic region polymorphism and the modified 'cold' SSCP for population genetic studies is discussed.

## Materials and methods

### Plant materials

Needles of parents of two controlled crosses, shortleaf pine (Z15, seed parent) × loblolly pine (#631, pollen parent) and slash pine (#1204, seed parent) × shortleaf pine (#1351, pollen parent), and 20 F1 hybrids from each cross, were kindly supplied by the USDA-Forest service, Southern Institute of Forest Genetics, USA, and by Dr. Bruce Bongarten in the Warnell School of Forest Resources, The University of Georgia. The identity of the individuals Z15 as shortleaf pine and #631 as loblolly pine was confirmed by a codominant DNA marker from nuclear ribosomal DNA internal transcribed spacer 1 (ITS1) (Chen 2001).

Nine putative hybrids between shortleaf pine and loblolly pine, previously identified by Raja et al. (1997) were also analyzed. Raja et al. (1997), identified these putative hybrids based on the heterozygosity of one allozyme marker (IDH: Isocitrate dehydrogenase) reported by Huneycutt and Askew (1989) to be indicative of a hybrid between shortleaf and loblolly pine.

Needles from the parent trees, the artificial hybrids and the nine putative hybrids were stored at – 80 °C before use.

### DNA extraction

Total DNA was extracted from needles using the CTAB protocol (Doyle and Doyle 1988).

### PCR amplification, SSCP analysis and polyacrylamide-gel electrophoresis

The *trnL–trnF* intergenic region of the chloroplast DNA of the above materials was amplified by PCR in a DNA thermocycler (PTC100, MJ Research Inc) with two universal primers e and f, which were designed by Taberlet et al. (1991). Conditions for PCR amplification were: 10 mM of Tris-HCl (pH 9.0 at 25 °C), 50 mM of KCl, 0.1% Triton X-100, 1.8 mM of $MgCl_2$, 0.16 mM of dNTP mix, 1.6 µM of each primer and 1 unit of DNA *Taq* polymerase, with 20 ng of DNA in a final reaction volume of 25 µl. Cycling conditions were 70 °C 3 min → 94 °C 2 min, 50 °C 40 s, 72 °C 2 min, two cycles → 94 °C 30 s, 50 °C 30 s, 72 °C 1 min 30 s, 35 cycles → 72 °C 8 min. Agarose-gel electrophoresis (1.5%) and ethidum bromide staining were used to check the PCR products.

Initially, we used the SSCP method to detect *trnL–trnF* intergenic spacer polymorphisms among these three *Pinus* species; it only detected polymorphism between shortleaf and loblolly pine. Nondenatured polyacrylamide gel-electrophoresis, however, detected polymorphism between shortleaf and slash pine but not between shortleaf and loblolly pine. The two detection methods were as follows:

(1) SSCP analysis of the amplified *trnL–trnF* intergenic spacer was conducted based on the protocol of Tadashi et al. (1993) with a minor modification. Approximately 12 µl of PCR product was mixed with 0.4 µl of methylmercury hydroxide (Johnson Matthey Electronics, Inc., War Hill, Mass), 2.5 µl of 5 × TBE, 8 µl of loading buffer (7 µl of 15% Ficoll dye and 1 µl of 95% Formamide dye) and 2.1 µl of $H_2O$. The 15% Ficoll (MWt 400,000) dye includes 0.25% bromophenol blue and 0.25% xylene cyanol; the 95% Formamide dye includes 20 mM of EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The samples were denatured for 5 min at 95 °C and then loaded on a pre-cooled and nondenatured sequencing gel consisting of 8% polyacrylamide (49:1 acrylamide:bis), 5% (v/v) glycerol with a 0.5 × TBE running buffer. Five percent glycerol was added to the acrylamide gel to increase DNA mobility. The electrophoresis was performed in a 4 °C cold room for 10 h at 190 V in a vertical chamber (BioRad). A thermostatically controlled circulator was not required.

(2) Nondenaturing polyacrylamide-gel electrophoresis was employed for the analysis of *trnL–trnF* intergenic segments of shortleaf pine, slash pine and their artificial hybrids (F1). The 15% (w/v) Ficoll loading buffer (2 µl) was mixed with a 8 µl PCR product and loaded on a 6% nondenatured polyacrylamide gel (49:1 acrylamide:bis) then electrophoresized for 5 h at 120 V in 0.5 × TBE running buffer in the same vertical chamber (BioRad).

PCR-SSCP bands and double-stranded DNA (dsDNA) separated by polyacrylamide-gel electrophoresis were stained in 0.5 µg/ml of ethidum bromide solution for 15 min and then destained in distilled water for 5 min. The stained bands were visualized under UV light and photographed.

### DNA sequencing

PCR products for the *trnL–trnF* intergenic region were cut from an agarose gel (1.5%) and gel-purified with Qiaquick columns (Qiagen, Chatsworth, Calif.). The purified PCR products were sequenced by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Primers e or f were used as sequencing primers. The resulting sequences were aligned with the Clustalw (fast) program available at http://bionavigator.com and then deposited in the Genbank database (Accession numbers AF343576 for loblolly pine, AF343577 for shortleaf pine and AF343578 for slash pine).

## Results

### The *trnL–trnF* intergenic spacer sequences

Sequencing showed a length of 471 bp for the loblolly pine *trnL–trnF* PCR fragment, 468 bp for shortleaf pine, and 467 bp for slash pine. Excluding the two regions 5′ and 3′ corresponding to the exons, the actual sizes of the intergenic regions in loblolly, shortleaf and slash pine were 430 bp, 427 bp and 426 bp, respectively. Agarose-gel electrophoresis (1.5%) of these *trnL–trnF* intergenic spacers can not distinguish the three *Pinus* species (see Fig. 3A).

Alignment between the sequences of the loblolly parent (631#) and the shortleaf parent (Z15) in Fig. 1 reveals two substitutions and one insertion (TTT) occurring in loblolly pine. Restriction-site analysis shows that loblolly pine has one *Mse*I restriction site, but shortleaf pine has none. PCR-RFLP analysis of the *trnL–trnF* intergenic spacer using *Mse*I restriction (data not shown) confirmed the sequence analysis and PCR-SSCP results. Alignment between the sequences of slash pine and shortleaf pine (Fig. 1) revealed four continuous base substitutions (TACC in shortleaf pine replaced by GGTA in slash pine) and one deletion (T) in slash pine.

**Fig. 1** Comparison of complete *trnL–trnF* intergenic spacer sequences of loblolly pine, shortleaf pine and slash pine. The base insertions, deletions and substitutions are shown in *boldface*. The amplification primers (*underlined*) span from bases 1 to 21 of the *trnL* (UAA) 3′ exon (primer e) and from bases 452 to 471 of *trnF* (GAA) (primer f)

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consensus     1 GGTTCAAGTCCCTCTATCCCCACCCAGGTTCGTTCCCGAACGGATTGATCTATCTTCTCC 60
P.taeda         ............................................................
P.echinata      ............................................................
P.elliotti      ............................................................

consensus    61 AATTCCATTGGTTCGAATCCATTCTAATTTCTCGATTCTTTTACCTCGCTATTTTTTTTT 120
P.taeda         ............................................................
P.echinata      ............................................................
P.elliotti      ............................................................

consensus   121 T---TCATGAAGAGAAGAAATTAGAACATGAATCTTTTCATCCATCTTATGACAAGTTGA 180
P.taeda         .TTT.A......................................................
P.echinata      ............................................................
P.elliotti      ....-.......................................................

consensus 181   GTTGATCTGTTAATAAGTTGATCATATGATCAATTTATTTTGTGATATATGATCTACATA 240
P.taeda         ..............................C.............................
P.echinata      ............................................................
P.elliotti      ............................................................

                241       251       261       271       281       291
consensus 241   GAATAGATTAGATCATTTTTAAATTATTCAATTGCAGTCCATTTTTATCATATTAGTGAC 300
P.taeda         ............................................................
P.echinata      ............................................................
P.elliotti      ............................................................

                301       311       321       331       341       351
consensus 301   TTCCAGATCGAAAATAATAAAGATCATTCTAAAAACTAGTAAAAATACCTTTTTACTTCT 360
P.taeda         ............................................................
P.echinata      ............................................................
P.elliotti      ..........................................GGTA..............

consensus 361   TTTTAGTTGACACAAGTTAAAACCCTGTACCAGGATGATCCACAGGGAAGAGCCGGGATA 420
P.taeda         ............................................................
P.echinata      ............................................................
P.elliotti      ............................................................

consensus 421   GCTCAGTTGGTAGAGCAGAGGACTGAAAATCCTCGTGTCACCAGTTCAAAT 471
P.taeda         ...................................................
P.echinata      ...................................................
P.elliotti      ...................................................
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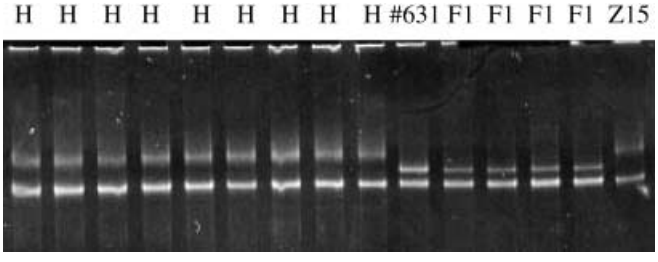


**Fig. 2** PCR-SSCP analysis of the *trnL–trnF* intergenic region of loblolly pine, shortleaf pine and their hybrids. *H*: the putative hybrids identified based on the allozyme marker; *#631*: loblolly pine (pollen parent); *Z15*: shortleaf pine (seed parent); *F1*: artificial hybrids between loblolly pine (#631) and shortleaf pine (Z15)

### Chloroplast inheritance

PCR-SSCP analysis (Fig. 2) of the *trnL–trnF* intergenic spacer region detected two different haplotypes corresponding to shortleaf pine and loblolly pine. Their artificial hybrids all had the same haplotype as their pollen parent, loblolly pine. These results confirmed that the chloroplast was paternally inherited in these pine species. The nine putative hybrids showed the shortleaf pine pattern.

Nondenatured polyacrylamide-gel electrophoresis was successfully used to distinguish shortleaf pine and slash pine (Fig. 3B). Their artificial hybrids (F1) show the same pattern as shortleaf pine (pollen parent). These results also confirmed that the chloroplast genome was paternally inherited in these pine species.

## Discussion

### Significance of *trnL–trnF* intergenic spacer polymorphism in pine population genetics

Our studies took advantage of the *trnL–trnF* intergenic spacer polymorphisms to confirm the paternal inheritance of the chloroplast genome in two artificial crosses of three closely related *Pinus* species. The two universal primers and the high polymorphism of the *trnL–trnF* intergenic spacer make it very useful to distinguish closely related species. The *trnL–trnF* polymorphism should facilitate population genetic studies in other plant species. In this study, all of the nine putative hybrids identified

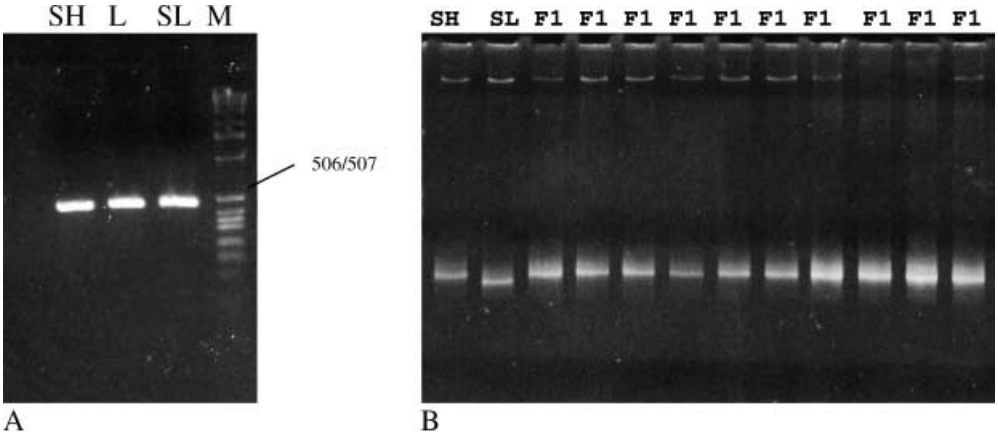


**Fig. 3A, B** Analysis of the amplified *trnL–trnF* intergenic region of shortleaf pine, loblolly pine, slash pine and their hybrids. **A** A 1.5% agarose gel does not detect polymorphism among loblolly, shortleaf and slash pine. **B** Nondenatured acrylamide gel-electrophoresis of the amplified *trnL–trnF* intergenic region of shortleaf pine, slash pine and their hybrids. *L*: loblolly pine (#631); *SH*: shortleaf pine (#1204, pollen parent); *SL*: slash pine (#1351, seed parent); *F1*: artificial hybrids (F1) between slash pine (#1351) and shortleaf pine (#1204); *M*: 1-kb DNA extension ladder (LIFE TECHNOLOGIES)

by Raja et al. (1997) from eight shortleaf pine populations have the shortleaf pine chloroplast profile. This was consistent with Edwards et al. (1997) who reported paternal inheritance in natural loblolly × shortleaf pine hybrids. These results can not explain why the putative hybrids are morphologically similar to shortleaf pine (Edwards et al. 1997). AFLP or microsatellite analyses of a larger population, combined with maternally inherited mitochondrial markers, may give a more-clear answer.

## Significance of our modified 'cold' PCR-SSCP protocol in population genetics

Single-strand conformation polymorphism (SSCP), first developed by Orita et al. (1989), is becoming widely used to detect DNA polymorphisms and point mutations when combined with PCR amplification techniques. It is based on the principle that a small change in a single-stranded DNA (ssDNA) sequence can cause a conformation change which affects ssDNA mobility in nondenaturing gel electrophoresis. The size of the DNA fragment for SSCP analysis is commonly less than 400 bp; however, Orti et al. (1997) reported that DNA fragments of 775-bp length may be analyzed successfully. Tadashi et al. (1993) found that DNA of 1.35 kb in length can give sharp resolution after denaturation. Most previous SSCP studies used radioactive SSCP (Sunnucks et al. 2000), which is time-consuming and increases hazardous waste-management concerns (Tadashi et al. 1993). Later-developed silver-staining (Calvert et al. 1995) and multiple fluorescent-based PCR-SSCP (MF-PCR-SSCP) protocols (Iwahana et al. 1994) for SSCP analysis seemed to be good solutions, but their requirement for expensive equipment and careful operation limits broad use. Tadashi et al. (1993) developed a simple, fast and nonradioactive SSCP method, in which a denaturant, methylmercury hydroxide, was used in conjunction with ethidum bromide staining and UV light to visualize ssDNA, since ethidum bromide alone does not stain ssDNA efficiently. This modification is very important but few studies have reported using this protocol, possibly because a thermostatically controlled circulator is required to maintain a constant gel temperature. Such a circulator may be unavailable in most laboratories. We modified this 'cold' SSCP protocol. Our loading buffer was 15% Ficoll dye and 95% Formamide dye, at a volume ratio of 7:1. Five percent glycerol was added to the 8% nondenatured acrylamide gel to increase the DNA mobility, and 0.5 × TBE was utilized as the running buffer. Electrophoresis was performed in a cold room; thus a thermostatically controlled circulator was not required. This modified PCR-SSCP protocol is very simple, should be feasible in most laboratories, and should facilitate both animal and plant population genetic studies. For animals, numerous polymorphisms were found within the mitochondrial introns or intergenic regions. For example, the hypervariable D-loop region of *mt*DNA has been used for population genetic analyses because its size is only 200 to 300 bp and many polymorphisms exist in this region (Marklund et al. 1995). For plants, the *trnL–trnF* intergenic spacer is very short, less than 500 bp. The two universal primers (Taberlet et al. 1991) are suitable for most plants. Thus, PCR-SSCP analysis of the *trnL–trnF* intergenic spacer should be feasible to address population questions for many plant species. We tried our modified 'cold' PCR-SSCP protocol to study the *trnL–trnF* intergenic spacer of shortleaf pine, slash pine and their artificial hybrids, but it did not work. This is because PCR-SSCP is mainly sequence-dependent and, although four continuous base substitutions occur between shortleaf pine and slash pine, the substitutions are the reverse complement (TACC for shortleaf pine was replaced by GGTA for slash pine), and there is only one deletion (T) in slash pine. Our modified 'cold' PCR-SSCP protocol did work for loblolly pine, shortleaf pine and their hybrids because there are two substitutions (C→A, T→C)

and one insertion (TTT) in loblolly pine not present in the shortleaf pine *trnL–trnF* intergenic spacer sequence.

In addition, PCR-SSCP analysis of nuclear DNA genes can be employed to identify hybrids. Quijada et al. (1997) used PCR to amplify the nuclear rDNA internal spacer-2 (ITS2), which is about 240-bp long. SSCP analysis showed that parental species exhibited two different strands while the hybrids showed four strands, which were a combination of their parent strands. However, PCR-SSCP analysis of ITS2 can not be used to identify hybrids between shortleaf and loblolly pine because these two species have the same nuclear rDNA ITS2 nucleotide sequences, based on our unpublished data. Recently, PCR-SSCP has been employed to study microsatellite polymorphism (Habano et al. 2000; Park et al. 2000).

In summary, this study establishes a simple screening method to detect plant *cp*DNA haplotypes using *trnL–trnF* intergenic spacer polymorphisms. Our modified 'cold' SSCP protocol can be utilized for other species to detect *cp*DNA polymorphisms simply and quickly.

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## References

Brent HM, David DE (1989) Unusual inheritance patterns of organelle DNA in conifers. Tibtech 7:257–258

Calvert RJ, Weghorst CM, Buzard GS (1995) PCR amplification of silver-stained SSCP bands from cold SSCP gels. Biotechniques 18:782–784

Chen JW (2001) Introgression between shortleaf pine (*Pinus echinata* Mill.) and loblolly pine *Pinus taeda* L.), PhD thesis, Oklahoma State University, Stillwater, Oklahoma

Cipriani G, Testolin R, Morgante M (1995) Paternal inheritance of plastids in interspecific hybrids of the genus *Actinidia* revealed by PCR-amplification of chloroplast DNA fragments. Mol Gen Genet 247:693–697

Dong J, Wagner DB, Yanchuk AD, Carlson MR, Magnussen S, Wang X, Szmidt AE (1992) Paternal chloroplast DNA inheritance in *Pinus contorta* and *Pinus banksiana*: independence of parental species or cross direction. J Hered 83:419–422

Doyle JJ, Doyle JL (1988) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Edwards MA, Hamrick JL, Price RA (1997) Frequency and direction of hybridization in sympatric populations of *Pinus taeda* and *P. echinata* (*Pinaceae*). Am J Bot 84:979–886

Habano W, Sugai T, Nakamura SI, Uesugi N, Yoshida T, Sasou S (2000) Microsatellite instability and mutation of mitochondrial and nuclear DNA in gastric carcinoma. Gastroenterology 118:835–841

Huneycutt M, Askew GR (1989) Electrophoretic identification of loblolly pine-shortleaf pine hybrids. Silvae Genet 38:3–4

Iwahana H, Yoshimoto K, Mizusawa N, Kudo E, Itakura M (1994) Multiple fluorescence-based PCR-SSCP analysis. Biotechniques 16:296–297

Marklund S, Chaudhary R, Marklund L, Sandberg K, Andersson L (1995) Extensive *mt*DNA diversity in horses revealed by PCR-SSCP analysis. Anim Genet 26:193–196

Neale DB, Sederoff RR (1989) Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. Theor Appl Genet 77:212–216

Orita M, Suzuki Y, Sekiya T, Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5:874–879

Orti G, Hare MP, Avise JC (1997) Detection and isolation of nuclear haplotypes by PCR-SSCP. Mol Ecol 6:575–580

Park HY, Nabika T, Jang Y, Kim D, Kim HS, Masuda J (2000) Identification of new single-nucleotide polymorphisms in the thrombin receptor gene and their effects on coronary artery diseases in Koreans. Clin Exp Pharmacol Physiol 27:690–693

Price RA, Liston A, Strauss SH (1998) Phylogeny and systematics of *Pinus*. In: Richardson DM (ed) Ecology and biogeography of *Pinus*. Cambridge University Press, pp 49–68

Quijada A, Liston A, Robinson WA, Alvarez-buylla ER (1997) The ribosomal ITS region as a marker to detect hybridization in pines. Mol Ecol 6:995–996

Raja RG, Tauer CG, Wittwer R, Huang Y (1997) An isoenzymatic study of the genetic structure in natural populations of shortleaf pine, *Pinus echinata* Mill. Can J Forest Res 27:740–749

Sunnucks P, Wilson AC, Beheregaray LB, Zenger K, French J, Taylor AC (2000) SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. Mol Ecol 9:1699–1710

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17:1105–1109

Tadashi H, Buzard GS, Calvert RJ, Weghorst CM (1993) 'cold SSCP': a simple, rapid and non-radioactive method for optimized single-strand conformation polymorphism analyses. Nucleic Acids Res 21:3637–3642

Wagner DB, Furnier GR, Saghai-Maroof MA, Williams SM, Dancik BP, Allard RW (1987) Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc Natl Acad Sci USA 84:2097–2100