INTROGRESSION BETWEEN SHORTLEAF PINE (*PINUS ECHINATA* MILL.) AND LOBLOLLY PINE (*PINUS TAEDA* L.)

By

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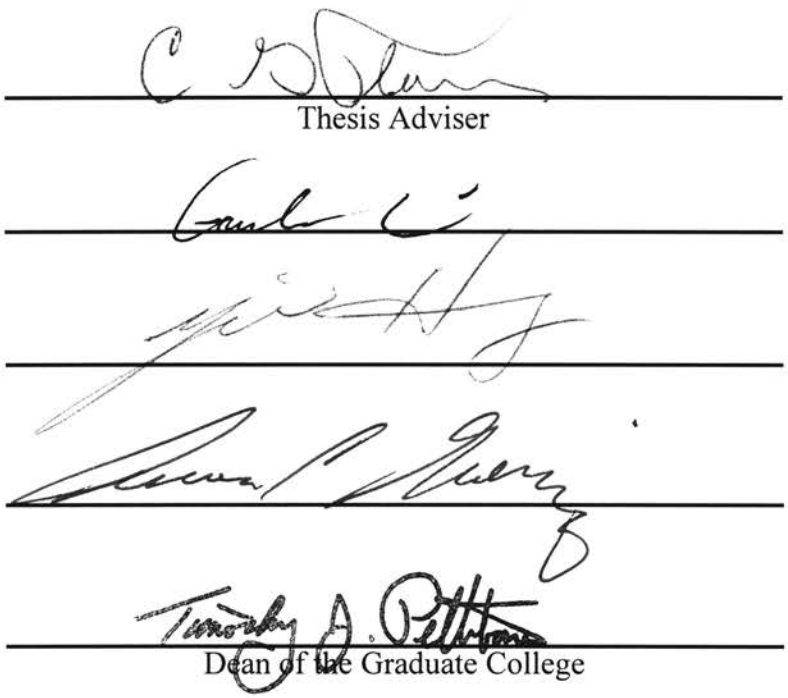
Thesis Approved:



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INTRODUCTION

Loblolly pine (*Pinus taeda* L.) and shortleaf pine (*Pinus echinata* Mill.) are economically important species in the United States, and they have widely overlapping geographic ranges. Hybridization between the two species has interested tree breeders for long time because the hybrids are more resistant to both littleleaf disease and fusiform rust, and also often grow faster, however the extent of occurrence of natural hybrids is still unresolved. Morphological characters, which were used to characterize species and identify hybrids in the past, offer limited help when the genotypes of the parents and their probable hybrids are compounded by environmental factors such as disease or drought stress, resulting in a wide range of phenotype variability. This confusion is aggravated by the fact that subsequent backcrossing of hybrids to either parent species results in morphological characters exhibiting progressively greater similarity to the recurrent parent species. The limits of morphological characters resulted in the identification of the allozyme marker IDH (*Isocitrate dehydrogenase*) to identify hybrids (Huneycutt and Askew, 1989). The high frequency of IDH variation seen in natural shortleaf pine populations outside the natural range of loblolly pine (Raja *et al.*, 1997) suggests either profuse hybridization between the two species or that IDH is an unreliable marker. These data required us to look for new markers to confirm the identity of putative hybrids between the two pine species. Highly polymorphic markers such as SSRs (simple sequence repeats) should reveal the relationship between the putative hybrids and the two species.

In addition, the direction of natural hybridization between the two species is still unclear. Artificial hybridizations in both directions have been successful between the two species. Generally, it is most convenient to use shortleaf pine or shortleaf X loblolly pine trees as the female parents because fresh pollen is available from the earlier flowering loblolly pine trees (Schultz, 1997). Our controlled crosses with shortleaf pine as pollen parent resulted in no seed while hybridization with loblolly pine as pollen parent provided many fertile seeds. However, Edwards *et al.* (1997) found that shortleaf pine sired the putative hybrids they found in the two natural shortleaf-loblolly pine sympatric populations.

Several approaches were used in this study to address the following questions: (1) how to reliably identify shortleaf pine, loblolly pine and their hybrids; (2) whether the IDH marker is still a reliable marker to identify hybrids between the two species; (3) what is the frequency and direction of natural hybridization between shortleaf pine and loblolly pine; (4) what is the genetic relationship between the putative hybrids and the two species; (5) can we develop stable mitochondrial DNA markers to help confirm the status of putative hybrids?

This work consists of four separate articles, Chapters 1, 2, 3 and 4. In Chapter 1, the 615-bp nucleotide sequences of the first partial internal transcribed spacer (ITS-1), 5.8S rDNA and ITS-2 region from loblolly pine and shortleaf pine were reported. We corrected an error in the 5.8S rDNA region for shortleaf pine previously reported (accession number: AF037016). Our data also showed that the two species share the same nucleotide sequences in this region, and Thus, PCR-SSCP of ITS-2 region could not be used for hybrid identification. In Chapter 2, a simple and fast method has been developed

to distinguish the two closely related pine species using chloroplast *trn*L-*trn*F intergenic region polymorphisms. We found that this intergenic region could be used to examine inheritance of pine hybrids. Twelve putative hybrids from eight shortleaf pine populations were examined using this marker, and all showed the shortleaf pine pattern. In Chapter 3, the maternal inheritance of mitochondrial DNA markers in Pine was confirmed in an artificial hybrid between slash pine and shortleaf pine. One mitochondrial DNA marker varied among both shortleaf pine and loblolly pine individuals from widely separate populations, but no variation for this *mt*DNA marker was observed from all eighty individuals within one Arkansas shortleaf–loblolly pine sympatric population. These results suggest variation of *mt*DNA markers within pine species should be examined before application to maternal analysis or natural genetic introgression studies. Also, the data in this article show that *mt*DNA variation among the three *Pinus* species is from gene rearrangement or microsatellite length differences and not from sequence substitutions. In Chapter 4, a biparentally-inherited nuclear DNA marker and a paternally-inherited chloroplast DNA marker have been combined with morphological data to show bi-directional genetic introgression between shortleaf pine and loblolly pine. Microsatellite analysis of a transect of stands sampled across one shortleaf-loblolly pine population was used to examine the genetic relationship between putative hybrids and the two species.

This study will not only settle controversies regarding the occurrence and direction of natural hybridization between the two species but also provide insight into the effect of natural hybridization on genetic diversity. It will help us understand the extent and nature of hybridization between shortleaf and loblolly pine.

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CHAPTER 1

NUCLEOTIDE SEQUENCES OF THE INTERNAL TRANSCRIBED SPACERS AND 5.8S REGION OF NUCLEAR RIBOSOMAL DNA IN *PINUS TAEDA* L. AND *PINUS ECHINATA* MILL.

Abstract

The 615-bp nucleotide sequences of the first partial internal transcribed spacer (ITS-1), 5.8S rDNA and ITS-2 region from loblolly pine (*Pinus taeda* L.) (accession number: AF367379) and shortleaf pine (*Pinus echinata* Mill.) (AF367378) are reported. The two pine species show the same nucleotide sequences in this region, which indicates their close phylogenetic relationship. However, our reported nucleotide sequence in this region from shortleaf pine is different from the previous report for shortleaf pine in the GenBank (AF037016). Our PCR-RFLP analysis of this region confirms our sequencing data. This correction is important for pine phylogenetic studies because it is located in the conserved 5.8S rDNA region.

Keywords: ITS-1, ITS-2, 5.8S rDNA, *Pinus taeda* L. and *Pinus echinata* Mill., nuclear ribosomal DNA

Introduction

The pine nuclear ribosomal DNA internal transcribed spacer (ITS) region includes two internal transcribed spacers (ITS-1 and ITS-2) and 5.8S rDNA. The size of this region in pine is approximately 3000bp (Liston *et al.* 1996). Liston *et al.* (1999) sequenced a 650-bp portion of the nuclear ribosomal DNA internal transcribed spacer region (Partial ITS-1, 5.8S rDNA and ITS-2) from 47 species of the genus *Pinus*. The published results show that in pine the 5.8S rDNA was consistantly 162bp and the ITS-2 region varied from 241-243bp (Liston *et al.* 1999). High polymorphism exists in ITS-1 and ITS-2 regions among different species in the genus *Pinus*. PCR-RFLP analysis of

ITS-1 and PCR-SSCP (Single Strand Conformation Polymorphism) analysis of ITS-2 were successfully applied to study the hybridization events in the genus *Pinus* (Quijada *et al*. 1997).

The objective of this study was to look for the sequence polymorphisms in this partial ITS-1, 5.8S rDNA and ITS-2 region between shortleaf and loblolly pine. We sequenced a 615-bp portion of the nuclear ribosomal DNA internal transcribed spacer region of loblolly pine. This region for shortleaf pine has been sequenced and its GenBank number is AF037016. The nucleotide sequence differences in this region between shortleaf and loblolly pine would be useful for their hybrid identification.

Materials and Methods

Plant materials

Loblolly pine (#631) was used for sequencing partial ITS-1, 5.8S rDNA and ITS-2 nucleotide sequence of loblolly pine; the sequencing sample (Strauss 80/24) for AF037016 (Liston *et al*. 1999) was also collected for PCR-RFLP analysis and re-sequencing. Two other shortleaf pine samples (Z15 and #2009) and one artificial hybrid (F1) between shortleaf pine (Z15) and loblolly pine (#631) were used for comparative analyses. The needles of Z15, #631, #2009 and one hybrid between Z15 and #631 were kindly supplied by the USDA-Forest service, Southern Institute of Forest Genetics, USA and Dr. Bruce Bongarten in Warnell School of Forest Resources, The University of Georgia. The identities of the individuals Z15, #2009 and Strauss 80/24 as shortleaf pine and #631 as loblolly pine were confirmed by a codominant DNA marker from nuclear ribosomal DNA internal transcribed spacer 1 (ITS-1) (Chen 2001).

DNA extraction

Needles from every sample were stored at –80°C before use. Total DNA was extracted from needles using the CTAB protocol (Doyle and Doyle 1988).

PCR amplification and DNA sequencing

The primers PIN2451 (Liston *et al.* 1999) and 26S-25R (Nickrent *et al.* 1994) were used to amplify this region from all samples collected. Polymerase chain reaction (PCR) amplifications of the ITS region followed the protocol of Liston *et al.* (1992, 1996). PCR products for this region were cut from a low-melting agarose gel (1.5%) and gel-purified with Qiaquick columns (Qiagen, Chatsworth, CA). The Oklahoma State University Recombinant DNA/Protein Resource Facility sequenced the purified PCR products. Primers PIN2451 or 26S-25R 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 AF367379 for loblolly pine and AF367378 for shortleaf pine).

Results and Discussion

In this paper, we first report a 615-bp portion of the nuclear ribosomal DNA internal transcribed spacer region in loblolly pine (Figure 1). It includes a partial ITS-1 sequence (211-bp) and complete DNA sequences for 5.8S rDNA (162-bp) and ITS-2 (243-bp).

We compared its sequence with the one from shortleaf pine previously reported in the GenBank (accession number: AF037016), only one nucleotide difference exists in the 5.8S rDNA (Figure 1 in position 264, G for loblolly pine and C for shortleaf pine (AF037016)). If it were C in this position, there should be one *RsaI* restriction site for

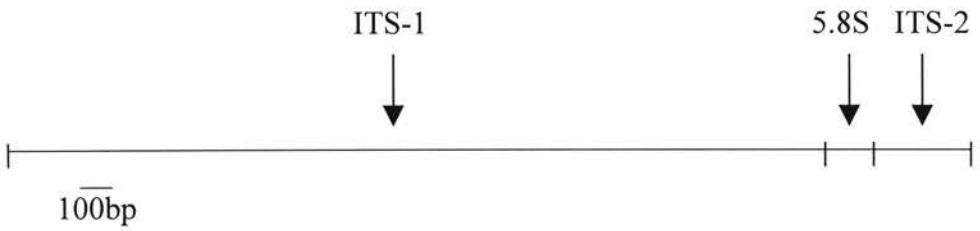
shortleaf pine and no *RsaI* restriction site for loblolly pine. The sequencing sample for AF037016 is Strauss 80/24 (Liston *et al.* 1999). The amplified PCR products of these shortleaf and loblolly pine samples were digested by the restriction enzyme, *RsaI*. The restriction result (Figure 2) shows that there is no *RsaI* restriction site for shortleaf pine (Strauss 80/24 and Z15), loblolly pine or their hybrids (F1). Then, we re-sequenced this region from the sample Strauss 80/24 using the primer PIN2451 or 26S-25R, and our sequencing result (Figure 1) shows G not C in position 264. Based on our phylogenetic analysis, this correction does not change the phylogenetic tree reported by Liston *et al.* (1999), but this correction is important because it is located in the 5.8S rDNA region which is conserved.

The nucleotide sequence similarity in this region between the two species indicates their close phylogenetic relationship. In addition, because shortleaf pine and loblolly pine have the same nucleotide sequences in the region, the only possible nucleotide sequence difference could be in the rest of ITS-1 region, which was not sequenced.

Figure 1. a. Schematic diagram of the *Pinus* nuclear ribosomal DNA internal transcribed region including two internal transcribed spacers (ITS-1 and ITS-2) and 5.8S rRNA gene; b. The nucleotide sequences of shortleaf pine (*Pinus echinata* L.) and loblolly pine (*Pinus taeda* Mill.) internal transcribed spacer 1 (partial sequence), 5.8S ribosomal RNA gene (in bold) and internal transcribed spacer 2 (complete sequence). The sequences labeled *Pinus taeda* and *Pinus echinata* are our reported nucleotide sequences, AF037016 is the shortleaf pine sequence from the GenBank.

Figure 1.

a.



b.

```
                1         11        21        31        41        51
consensus       GATCGCTGTTCCATGCATTTCCCCCCCATTTTGCGAGGGCGTGGGGCTCGGGGATATTGT partial ITS-1
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ..................................................
                61        71        81        91        101       111
consensus       TCTCTGTTGGAGCCTCCTCCTTTGAGGGGGGTAGGCACCTTTGTTTCTCTTTTCTCCCAT
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ..................................................
                121       131       141       151       161       171
consensus       GCGTTTTC-CCTCCCTTTGGCCCTTGGCCTTGCATTTGTTGTTGGGGGGTGCCCCTATTC
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ..................................................
                181       191       201       211       221       231
consensus       AGTGTTGTGGCCGATATTTCGGTAATACGTGGAAACGACTCTCGGCAACGGATATCTCGG
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ..................................................
                241       251       261       271       281       291
consensus       CTCTTGTTACGATGAAGAACGTAGCGAAATGCGATACTTAGTGTGAATTGCAGAATCCCG 5.8S
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ......................c...........................
                301       311       321       331       341       351
consensus       TGAATCATCGAGTTTTTGAACGCAATTTGCGCCCGAGGCCTCGGTCGAGGGCACGTCTGT
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ..................................................
                361       371       381       391       401       411
consensus       CTGGGCGTCGCATCCCATTCCAACGCGCTCCCTGCAATATGCTAGGGAGCAGCGGAGCTG ITS-2
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ..................................................

                421       431       441       451       461       471
consensus       GTCGTCCGTGCCCAACGCGGTGCGGTTGGCTGAAATACCTCAAGCGATGTTTCGTGGCGT
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ..................................................
                481       491       501       511       521       531
consensus       GCGTCGGCGAGCGGTGATCTTGTCCCCTTGGATGGGCAGTCGGCGTTAGCTGATGCGGGC
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ..................................................

                541       551       561       571       581       591
consensus       TTTGTGTGGCATCGCTCGAACTTGCTTTGCTCTCTCTTGCCCTCCCATCGGGTAGGGCGG
Pinus echinata  ..................................................
Pinus taeda     ..................................................
AF037016        ..................................................

                601       611
consensus       ATTTAGCTCCAACTTT
Pinus echinata  ................
Pinus taeda     ................
AF037016        ................
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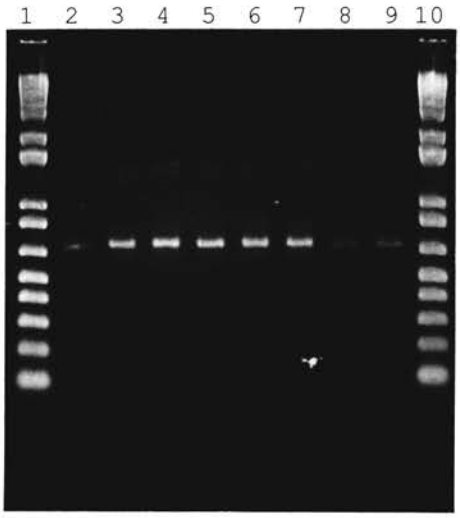
Figure 2.

The partial ITS-1, 5.8S rDNA, ITS-2 restriction fragment patterns generated by *RsaI.*



1, 10-1kb plus DNA marker; 2, 3, 4, 5-Strauss 80/24; 6-shortleaf pine (Z15); 7- shortleaf pine (#2009); 8-artificial hybrids (F1) of shortleaf pine (seed parent) and loblolly pine(pollen parent); 9-loblolly pine (#631).

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CHAPTER 2

USING *TRN*L-*TRN*F INTERGENIC REGION POLYMORPHISMS TO EXAMINE INHERITANCE OF PINE HYBRIDS

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) x 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) x 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.

Introduction

Chloroplast genomes are paternally inherited in *Pinus* (Brent and David, 1989). Most of the previous studies (Wagner *et al.* 1987; Dong *et al.* 1992; Neale and Sederoff, 1989; 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 coding for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) 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 500bp 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 *A. 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 (Single Strand Conformation Polymorphism) 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 for 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 subgenus *Pinus* (Price *et al.* 1998). Our results also track paternity in putative hybrids in *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) x loblolly pine (#631, pollen parent) and slash pine (#1204, seed parent) x 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 Dr. Bruce Bongarten in 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 (ITS-1) (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 Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, 1.8 mM $MgCl_2$, 0.16 mM dNTP mix, 1.6 μM of each primer and 1 unit DNA *Taq* polymerase, with 20 ng of DNA in a final reaction volume of 25 μl. The PCR amplification profile was as follows: 3 min at 70 °C, two cycles of 2 min at 94 °C, 40 sec at 50 °C, 2 min at 72 °C. Then 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C, 1 min 30 sec at 72 °C, finally followed by 8 min at 72 °C and a 4 °C soak. Agarose gel electrophoresis (1.5%) and ethidium 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:

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 PCR product was mixed with 0.4 µl methylmercury hydroxide (Johnson Matthey Electronics, Inc., War Hill, MA), 2.5 µl 5 X TBE, 8 µl loading buffer (7 µl 15% Ficoll dye and 1 µl 95% Formamide dye) and 2.1 µl H_2O. The 15% Ficoll (M.W. 400,000) dye includes 0.25% bromophenol blue and 0.25% xylene cyanol; the 95% Formamide dye includes 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The samples were denatured for 5 min at 95 ^{o}C and then loaded on a precooled and nondenatured sequencing gel consisting of 8% polyacrylamide (49:1 acrylamide:bis), 5%(v/v) glycerol with a 0.5 x TBE running buffer. Five percent glycerol was added to the acrylamide gel to increase DNA mobility. The electrophoresis was performed in a 4 ^{o}C cold room for 10 hours at 190v in a vertical chamber (BioRad). A thermostatically controlled circulator was not required.

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 (2µl) was mixed with 8µl PCR product and loaded on 6% nondenatured polyacrylamide gel (49:1 acrylamide:bis) then electrophoresized for 5 hours at 120v in 0.5 x 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 ethidium bromide solution for 15 minutes and then destained in distilled water for 5 minutes. 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, CA). The Oklahoma State University Recombinant DNA/Protein Resource Facility sequenced the purified PCR products. Primers e or f was 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 471bp for the loblolly pine *trnL-trnF* PCR fragment, 468bp for shortleaf pine, and 467bp 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 430bp, 427bp, 426bp, respectively. Agarose gel electrophoresis (1.5%), of these *trnL-trnF* intergenic spacers could not distinguish the three *Pinus* species (Figure 3A).

Alignment between the sequences of the loblolly parent (631#) and the shortleaf parent (Z15) in Figure 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 (Figure 1.)

revealed four continuous base substitutions (TACC in shortleaf pine replaced by GGTA in slash pine) and one deletion (T) in slash pine.

Chloroplast inheritance

PCR-SSCP analysis (Figure 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 (Figure 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 *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 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 x 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.* in 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 single stranded DNA (ssDNA) sequence can cause conformation changes which affect 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 in length could 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. In 1993, Tadashi *et al.* developed a simple, fast and nonradioactive SSCP method, in which a denaturant, methylmercury hydroxide, was used in conjunction with ethidium bromide staining and UV light to visualize ssDNA, since ethidium 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 the volume ratio of 7:1. Five percent glycerol was added to the 8% nondenatured acrylamide gel to increase the DNA mobility, and 0.5 x 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 300bp and many polymorphisms exist in this region (Marklund *et al.* 1995). For plants, the *trnL-trnF* intergenic spacer is very short, less than 500bp. 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 were 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 used to identify hybrids. Quijada *et al.* (1997) used PCR to amplify the nuclear rDNA internal spacer-2 (ITS-2), which is about 240bp 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 ITS-2 can not be used to identify hybrids between shortleaf and loblolly pine because these two species have the same nuclear rDNA ITS-2 nucleotide sequences based on our unpublished data. Recently, PCR-SSCP has been employed to study microsatellite polymorphism (Park *et al*. 2000; Habano *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.

Acknowledgment

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Figure 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 *trnL* (UAA) 3' exon (primer e) and from bases 452 to 471 of *trnF* (GAA) (primer f).

```
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      ............................................................

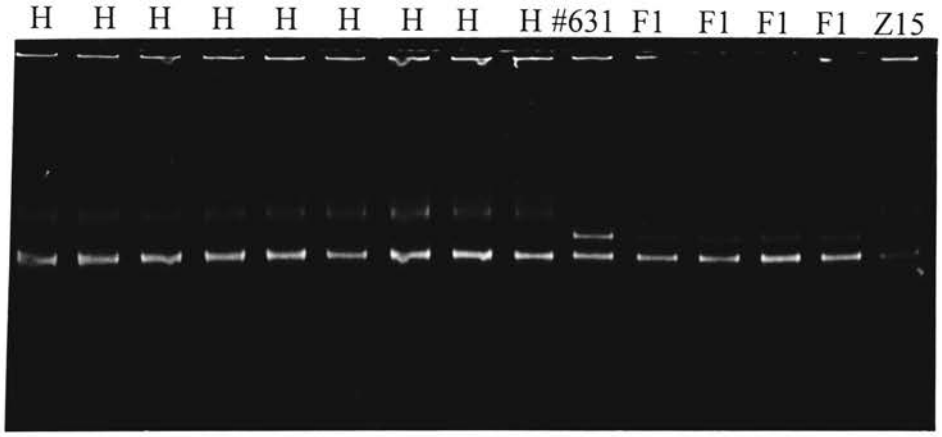
              301       311       321       331       341       351
consensus 301  TTCCAGATCGAAAATAATAAAGATCATTCTAAAAACTAGTAAAAATACCTTTTTACTTCT 360
P.taeda         ............................................................
P.echinata      ............................................................
P.elliotti  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

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

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

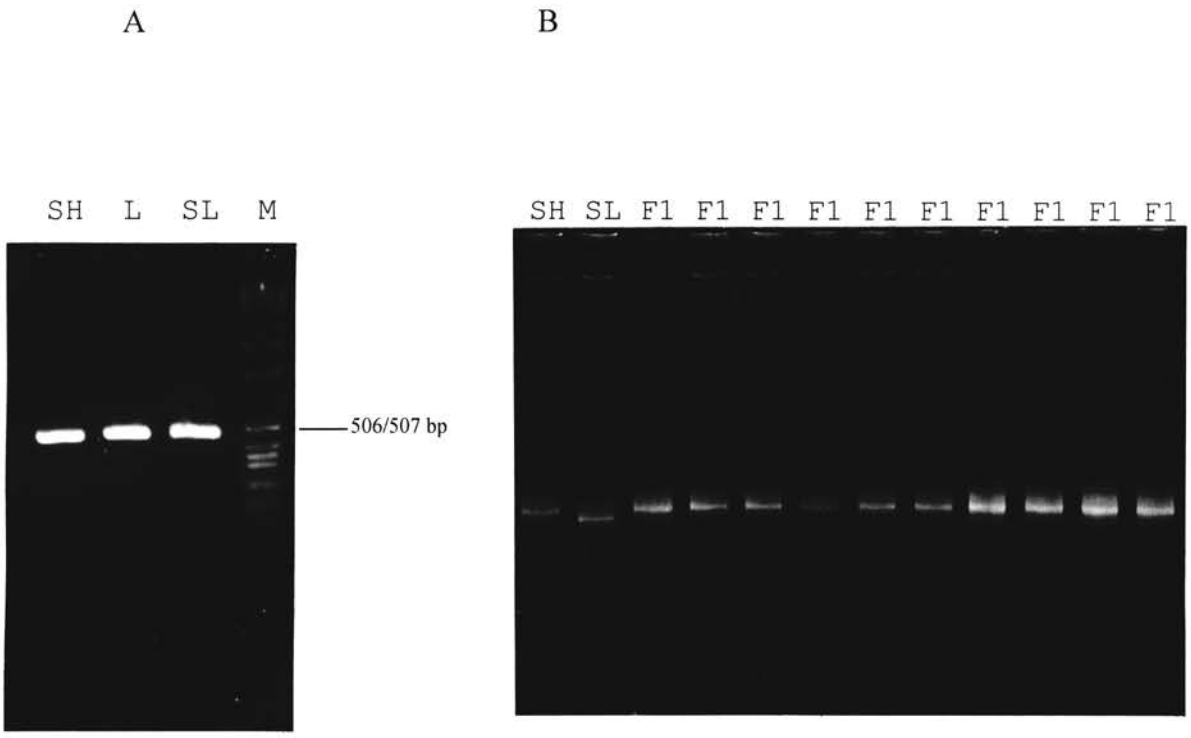
Figure 2.

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).

Figure 3.
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^{TM})

CHAPTER 3

MITOCHONDRIAL DNA INHERITANCE AND VARIATION AMONG THREE *PINUS* SPECIES

Abstract

Mitochondrial DNA inheritance and variation were studied among three *Pinus* species: loblolly pine (*Pinus taeda* L.), shortleaf pine (*Pinus echinata* Mill.) and slash pine (*Pinus elliottii* Engelm.). Maternal inheritance of mitochondrial DNA in these *Pinus* species was confirmed by the artificial cross of slash pine (seed parent) X shortleaf pine (pollen parent). For shortleaf and loblolly pine, PCR-RFLP analyses of nad1 b/c and cox1 revealed no differences; and a PCR-SSCP assay of the nad3-rps12 intergenic region and a mitochondrial microsatellite sequence revealed no variation between the two species. However, one mitochondrial DNA marker varied among individuals of both shortleaf pine and loblolly pine from widely separate populations. No variation for this *mt*DNA marker was observed in eighty individuals of both species within one Arkansas shortleaf –loblolly pine sympatric population. This marker can not be used for genetic introgression studies of shortleaf and loblolly pine. It is apparent that variation of *mt*DNA markers among populations within species should be examined before their application to maternal analysis or natural genetic introgression studies. Our results also indicate that *mt*DNA variation among the pine species studied is from gene rearrangements or microsatellite length differences.

Keywords: mitochondrial DNA (*mt*DNA), maternal inheritance, *Pinus echinata* Mill., *Pinus taeda* L., *Pinus elliottii* Engelm.

Introduction

Genetic introgression between loblolly pine (*Pinus taeda* L.) and shortleaf pine (*Pinus echinata* Mill.) has been studied for a long time (Zobel 1953; Huneycutt and Askew 1989; Edwards and Hamrick 1995; Raja *et al.* 1997; Edwards *et al.* 1997). The artificial hybrids (F1) between shortleaf pine and loblolly pine are morphologically intermediate but most reported that putative hybrids are more similar to shortleaf pine (Edwards *et al.* 1997; Raja *et al.* 1997). Why the natural hybrids are morphologically more similar to shortleaf pine and not intermediate is not clear, but perhaps F1s are rather rare, but do produce many later generation backcross(es) with shortleaf pine. Maternally-inherited *mt*DNA markers between shortleaf and loblolly pine could be useful to prove the existence of the backcrosses when combined with other molecular marker and allozyme data. However, when *mt*DNA markers are used for genetic introgression studies between two different species, it is necessary to examine variation of the *mt*DNA markers both among and within populations of each species because *mt*DNA variation maybe exist at both levels. Wu *et al.* (1998) studied three close-related *Pinus* species and found strong *mt*DNA variation occurs among populations within species.

In this study, one natural shortleaf-loblolly pine sympatric population and two artificial hybrid populations were selected to examine the variation of *mt*DNA markers within and among shortleaf and loblolly pine populations. The two artificial crosses (parents and F1s) were shortleaf pine (seed parent) X loblolly pine and slash pine (*Pinus elliottii* Engelm.) X shortleaf pine (pollen parent). These artificial crosses were used to develop *mt*DNA markers to distinguish the three *Pinus* species and confirm *mt*DNA inheritance in the genus *Pinus*. Because the two shortleaf pine trees in the two artificial

crosses were from different populations, and the loblolly pine tree in one artificial cross was not from the natural population studied, a limited measure of the nature of the stability of *mt*DNA markers among populations for shortleaf or loblolly pine was available.

Traditionally, *mt*DNA markers were developed by RFLP (restriction fragment length polymorphism) analyses with radioactively labelled probes (Neale and Sederoff 1989), but more recently PCR-RFLP analysis of mitochondrial DNA genes or gene fragments have been used to study *mt*DNA polymorphisms (Grivet *et al.* 1999; Wang *et al.* 1996; Wantano *et al*. 1996). In addition, DNA sequencing or PCR-SSCP analysis of some short *mt*DNA fragments (Soranzo *et al.* 1999; Jensen-Seaman and Kidd 2001) have also been used to detect *mt*DNA variation. Thus, we first tried restriction enzymes to digest a PCR-amplified intron b/c region of subunit 1 of NADH dehydrogenase (nad1 b/c) and the subunit 1 of cytochrome oxidase (cox1) of shortleaf pine, loblolly pine and their hybrids to detect *mt*DNA polymorphisms. A mononucleotide mitochondrial microsatellite (Soranzo *et al.* 1999) located within the the intergenic region between nad3 (subunit 3 of NADH dehydrogenase) and rps12 (the S12 subunit protein of the mitochondrial ribosome) of shortleaf pine, slash pine and loblolly pine was sequenced. PCR-SSCP analyses of the nad3-rps12 intergenic region were also performed for the three *Pinus* species. Since *mt*DNA variation could not be found, traditional RFLP analyses with specific *mt*DNA probes were used to identify *mt*DNA polymorphisms because gene rearrangement events are common in the pine mitochondrial genome (Wu *et al*. 1998).

Materials and Methods

Plant materials

Two controlled crosses, shortleaf pine (Z15, seed parent) x loblolly pine (#631, pollen parent), and slash pine (#1204, seed parent) x shortleaf pine (#1351, pollen parent), were kindly supplied by USDA-Forest Service, Southern Institute of Forest Genetics, USA and Dr. Bruce Bongarten, Warnell School of Forest Resources, The University of Georgia. Twenty F1 hybrids from each cross were used to verify the mode of inheritance of *mt*DNA. Shortleaf pine Z15 was from North Carolina; loblolly pine #631 was from the west central piedmont of Georgia County, GA; shortleaf pine #1351 (alias WO33) is a selection from Ouachita, Arkansas; slash pine #1204 (alias W-1-5) is a selection from Wayne County, Mississippi planting but its origin is Georgia.

One natural population was also studied. This population was defined as the pine stands of Montgomery County, Arkansas. Sixteen stands (five individuals per stand) were sampled on a southeast to northwest transect across the county. Stands were located at approximately equal distances across the transect. The southeast stands (#1-8) are mixed with loblolly and shortleaf pine, while the northwest stands (# 9-16) are only shortleaf. Raja *et al.*(1997) showed that about sixteen percent of the individuals within a central population near Mt. Ida are hybrids. These results were based on the heterozygosity of one allozyme marker (IDH) reported by Huneycutt and Askew (1989) to be indicative of a hybrid between shortleaf and loblolly pine. Mt. Ida is approximately the central point of the transect we sampled, and a few miles distant from any known stands of loblolly pine.

To assure there were no sample identification errors, the status of every individual was confirmed by one diagnostic DNA marker from the nuclear ribosomal DNA internal transcribed spacer 1 (ITS-1) (Chen *et al.* 2001b).

DNA extraction

Needles from the parent trees, artificial hybrids (F1) and the eighty samples from the natural population were collected and stored at –80 °C. Total DNA was extracted from needles using a CTAB protocol (Doyle and Doyle 1988).

PCR-RFLP analyses of nad1 b/c and cox1

The nad1 b/c and cox1 regions of the above materials were amplified by PCR in a DNA thermocycler (PTC100, MJ Research Inc) with universal primers (Table 1). Conditions for PCR amplification were: 10 mM Tris-HCl (pH 9.0 at 25 °C), 50 mM KCl and 0.1% Triton X-100, 1.8 mM $MgCl_2$, 0.16 mM dNTP mix, 1.6 μM of each primer, 1 unit DNA *Taq* polymerase, with 20 ng of DNA in a final reaction volume of 25 μl. Cycling conditions for nad1 b/c amplification were as follows: 3 min at 70 °C, two cycles of 2 min at 94 °C, 40 sec at 55 °C, 3 min at 72 °C. Then 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 3 min at 72 °C, finally followed by 8 min at 72 °C and a 4°C soak. The cycling conditions for cox1 amplification were similar to nad1 b/c conditions with the exception that the annealing temperature of cox1 amplification was 50°C and the extension time was 2 min. Nineteen restriction endonucleases that recognize 4-bp and 6-bp sites (*Alu*I, *Dra*I, *Hae*III, *Hinf*I, *Rsa*I, *Pst*I, *Kpn*I, *Msp*I, NciI, *Pvu*II, *Pst*I, *Sac*I, *Sma*I, *Taq*I, *Eco*RI, *Bam*HI, *Apa*I, XbaI, *Xho*I) were used to digest nad1 b/c and cox1. Two percent agarose gel electrophoresis and ethidium bromide staining were used to check the digested PCR products.

PCR-SSCP analyses of nad3-rps12 intergenic region and a mitochondrial microsatellite sequence

The nad3-rps12 intergenic region was amplified by PCR with two universal primers (Table 1). The cycling conditions are the same as those for cox1 with the exception that the extension time is 1 min. SSCP analysis in an undenatured polyacrylamide gel were conducted for the amplified nad3-rps12 intergenic spacer based on the protocol of Chen *et al.* (2001a).

A mitochondrial Gn mononucleotide microsatellite located within the nad3-rps12 intergenic region (Soranzo *et al.* 1999) was amplified by PCR with two universal primers (nad3-1, Table 1). The PCR amplification conditions were from Soranzo *et al.* (1999). The amplified mitochondrial microsatellite segment for each of shortleaf pine, loblolly pine and slash pine was cut from a low-melting agarose gel (1.5%) and gel-purified with Qiaquick columns (Qiagen, Chatsworth, CA). The Oklahoma State University Recombinant DNA/Protein Resource Facility sequenced the purified PCR products. The two universal primers (nad3-1) 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 AF426453 for loblolly pine, AF426454 for shortleaf pine and AF426452 for slash pine).

Probe preparation and RFLP analysis

Three probes (cox1, nad1 b/c and nad3-rps12) were used in the restriction fragment length polymorphism (RFLP) analysis. The probes cox1 and nad1 b/c were specific for single *mt*DNA genes, cox1 and nad1. The nad3-rps12 probe was from the

intergenic region between nad3 and rps12. The nucleotide sequences of the universal primers used for amplification of these probes are given in Table 1.

Probes were amplified by PCR with universal primers, the products were recovered from 1.5% low-melting agarose gel under UV light, and purified using the Wizard™ purification system (Promega). The purified probes were radioactively labeled with ^{32}P by primer extension using a random hexamer labeling kit (Moehringer Mannheim GmbH, Mannheim, Germany).

Ten micrograms of genomic DNA was used for restriction-enzyme digestion. Based on the results of Wu *et al.* (1998), *Bam*HI and *Xba*I were combined to digest genomic DNA for the nad3-rps12 and cox1 probes; and BamHI to digest genomic DNA for the nad1 b/c probe. The protocols and procedures for restriction digestion, agarose gel electrophoresis and Southern blotting were as described by Wu *et al.* (1998).

Results

Polymorphism of nad1 b/c and cox1

The sizes of PCR-amplified nad1 b/c and cox1 in the three *Pinus* species are approximately 2600bp and 710bp, respectively. PCR-RFLP analyses of nad1 b/c and cox1 with nineteen restriction enzymes revealed no variation among the slash, shortleaf and loblolly pine trees sampled.

The mitochondrial microsatellite organization

PCR amplification with the nad3-1 primers of the three *Pinus* species produced fragments of two different sizes. A 110-bp product was observed for slash pine, while a 109-bp product was found in shortleaf pine and loblolly pine. Alignment between the sequences of the loblolly pine parent (#631), the shortleaf pine parent (Z15, #1351) and

the slash pine parent (#1204) (Figure 1) revealed no nucleotide substitutions and only one microsatellite length difference between shortleaf ((G)10) and slash pine ((G)11). Shortleaf pine and loblolly pine share the same microsatellite length and the same nucleotide sequences flanking this microsatellite region.

nad3-rps12 intergenic region variation

Direct electrophoresis using 8% undenaturated polyacrylamide gel and PCR-SSCP analysis (Figure 2) of the nad3-rps12 intergenic spacer region showed only one haplotype corresponding to shortleaf pine, slash pine, loblolly pine and the artificial hybrids (F1) between shortleaf pine and loblolly pine. No polymorphism was observed in this intergenic region among the three *Pinus* species.

***mt*DNA inheritance in shortleaf pine X slash pine**

RFLP analyses with the cox1 and nad3-rps12 probes produced the same hybridization patterns between shortleaf pine (#1351) and slash pine (#1204). When the probe nad1 b/c was hybridized with BamHI-digested genomic DNA, all the artificial hybrids (F1) show the same hybridization pattern as slash pine (#1204), but shortleaf pine (#1351) shows a different pattern (Figure 3). This confirms maternal inheritance of mitochondrial DNA in this *Pinus* cross.

mitochondrial DNA variation in shortleaf pine and loblolly pine

RFLP analyses with cox1 and nad3-rps12 probes produced the same hybridization patterns for shortleaf pine, loblolly pine and their hybrids. But with nad1 b/c probing, Z15 and #1351 (both shortleaf pine) showed different hybridization patterns (Figure 3, 4). Shortleaf pine #1351 shows the same hybridization pattern as the shortleaf pine trees in the natural population sampled (Figure 3,4). Loblolly pine #631 and the other loblolly

pine sampled in the natural population also have different hybridization patterns (Figure 4).

The eighty individuals sampled from the Arkansas population have been characterized as 16 loblolly pine, 53 shortleaf pine and 10 hybrids based on molecular data and morphological data (Chen *et al.* 2001b). Among the ten hybrids, two hybrids are morphologically similar to loblolly pine and are identified as HL. The remaining eight hybrids are morphologically similar to shortleaf pine and identified as HS. When the probe, nad1 b/c, hybridized with BamHI-digested genomic DNA, HL and HS have the same hybridization pattern (Figure 4). All the other individuals sampled from this population also share the same hybridization pattern.

Discussion

Our results show no *mt*DNA variation within the shortleaf-loblolly pine sympatric population studied. RFLP analyses using cox1, nad1 b/c and nad3-rps12 probes shows no difference among all samples from the natural population including shortleaf pine, loblolly pine and their putative hybrids. However, when the nad1 b/c probe was hybridized with BamHI-digested genomic DNA of Z15 and #1351 (both shortleaf pine), different hybridization patterns were found. Loblolly pine parent #631 and loblolly pine from the Arkansas population we selected also show different hybridization patterns. Since Z15 and #1351 are from different shortleaf pine populations, and loblolly pine parent #631 is not from the Arkansas population, our results indicate that *mt*DNA variation exists within different populations of each species. If this *mt*DNA marker variation among populations were not checked, the seed parent of the natural hybrids identified from the Arkansas population would be either shortleaf pine or unknown

because the hybrids (HL or HS) share the same hybridization pattern with shortleaf pine #1351 but differ from shortleaf pine Z15. However, Edwards *et al.* (1997) reported shortleaf pine always sired the putative hybrids between shortleaf pine and loblolly pine. Thus, this mtDNA marker can not be used for genetic introgression studies between shortleaf and loblolly pine. We suggest that variation of *mt*DNA markers within any pine species be examined before the *mt*DNA markers are used for maternal analysis or natural genetic introgression studies among *Pinus* species. If this variation is not examined, one could reach erroneous conclusions.

Our results also indicate that *mt*DNA variation within the pine species studied is from gene rearrangements or microsatellite length difference in the mitochondrial genome. PCR-RFLP analyses of the nad1 b/c intergenic region and the cox1 gene with nineteen restriction enzymes showed no difference between shortleaf pine and loblolly pine. However, when the nad1 b/c was as a probe to hybridize with BamHI-digested genomic DNA, polymorphism was observed among shortleaf pine individuals from different populations. This may be due to the gene rearrangement event. Nucleotide sequences of the mitochondrial microsatellite fragment located within the nad3-rps12 intergenic region showed no nucleotide substitutions but the microsatellite length differs between shortleaf pine and slash pine. In addition, PCR-SSCP analyses of the nad3-rps12 intergenic region showed no variation among the three *Pinus* species. Based on these data, an extremely close phylogenetic relationship between shortleaf pine and loblolly pine is suggested.

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Table 1.

Nucleotide sequences of the primers for the amplification of mtDNA probes

Probe name	5′ primer (5′→3′)	3′ primer (5′→3′)	size[1]
Cox1[2]	TTATTATCACTTCCGGTACT	AGCATCTGGATAATCTGG	712bp
Nad3-rps12[3]	AATTGTCGGCCTACGAATGTG	GCTCG(A=I)GTACGGTC(C=I)GTGCG	~ 370bp
Nad3-1[4]	TTCCCCATGAATGGAAGAAG	ATTGATTCGATGTAGGCATCG	~ 109bp
nad1 Exon B/C[5]	GCATTACGATCTGCAGCTCA	GGAGCTCGATTAGTTTCTGC	~2600bp

1 expected size of PCR product relative to the reference sequence

2 primers published by Glaubitz and Carlson (1992)

3 primers published by Wu *et al.* (1998)

4 primers published by Soranzo *et al.* (1999)

5 primers published by Demesure *et al.* (1995)

Figure 1.

Multiple sequence alignment of a mitochondrial microsatellie located within nad3-rps12 intergenic region from the three *Pinus* species. The polymorphic microsatellite region is shown in bold and the primer annealing sites are shown in italics.

```
                1         11        21        31        41

consensus       TTCCCCATGAATGGAAGAAGGGTGCTTCAGATCGGGAGTAACCACCAATG
P.taeda         .............................................
P.echinata      .............................................
P.elliotti      .............................................

                51        61        71        81        91
consensus       ATAGGGCAACAATCGGGGGGGGGG-AAGGACGGGAAGAGCGATGCCTACA
P.taeda         .............................................
P.echinata      .............................................
P.elliotti      ......................G......................

                101
consensus       TCGAATCAAT
P.taeda         ..........
P.echinata      ..........
P.elliotti      ..........
```

Figure 2.

PCR-SSCP analysis of the *nad3-rps12* intergenic region of slash pine, loblolly pine, shortleaf pine and the hybrid (F1) between shortleaf pine and loblolly pine. The first four lanes show undenaturated PCR product; the last four lanes show denaturated PCR product. SH: shortleaf pine (Z15, seed parent); F1: artificial hybrid between shortleaf pine (Z15) and loblolly pine (#631); L: loblolly pine (#631, pollen parent); SL: slash pine (#1204).

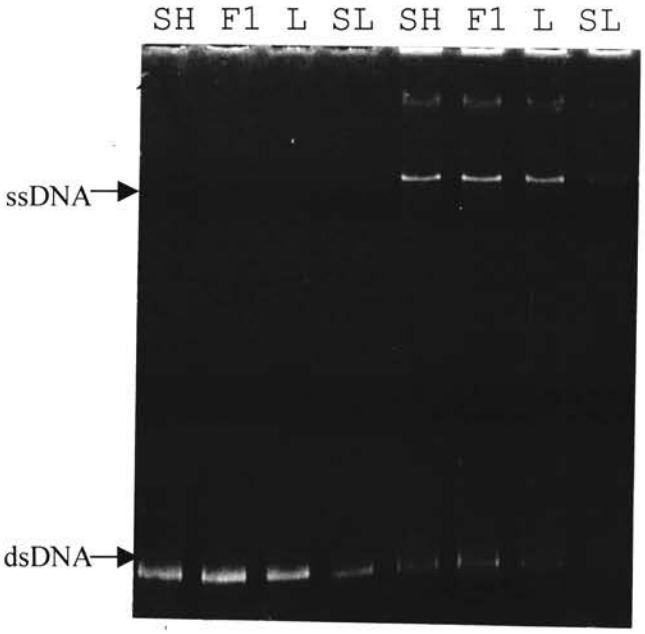


Figure 3.

Hybridization of nad1 b/c to the BamHI-digested genomic DNA of slash pine, shortleaf pine and their artificial hybrids (F1). SH: shortleaf pine (#1351, pollen parent); SL: slash pine (#1204, seed parent); F1: artificial hybrids of slash pine (#1204) x shortleaf pine (#1351)

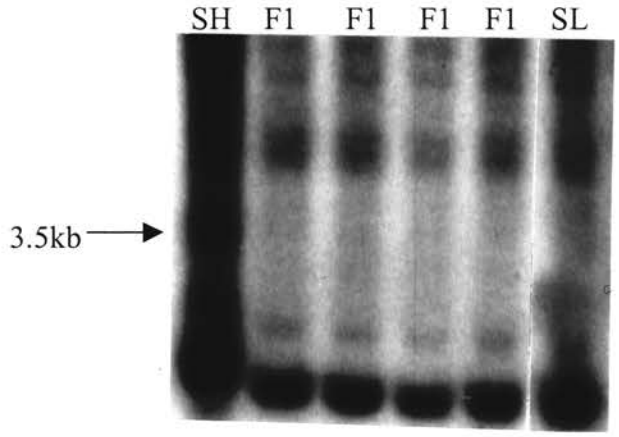
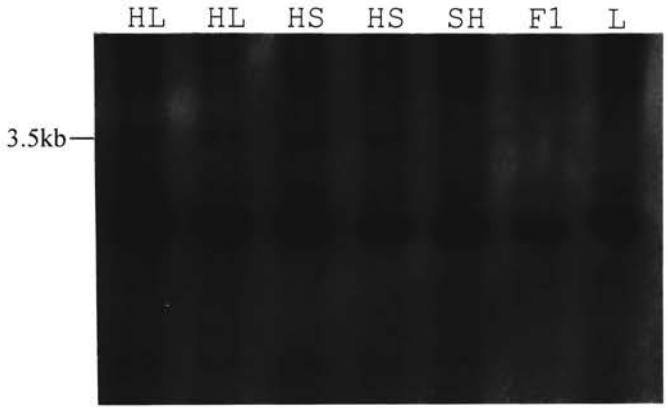


Figure 4.

Hybridization of nad1 b/c to the BamHI-digested genomic DNA of shortleaf pine, loblolly pine and their artificial hybrids (F1). SH: shortleaf pine (#Z15, seed parent); L: loblolly pine (#631, pollen parent); F1: artificial hybrids (F1) of shortleaf pine (Z15) X loblolly pine (#631); HL: the hybrids existing in a natural population and morphologically similar to loblolly pine; HS: the hybrids existing in a natural population and morphologically similar to shortleaf pine.



CHAPTER IV

BIDIRECTIONAL INTROGRESSION BETWEEN *PINUS TAEDA* L. AND *PINUS ECHINATA* MILL.: EVIDENCE FROM MORPHOLOGICAL AND MOLECULAR DATA

Abstract

The frequency and direction of natural hybridization between loblolly pine (*Pinus taeda* L.) and shortleaf pine (*Pinus echinata* Mill) were studied within one sympatric population from central Arkansas. A codominant DNA marker from the nuclear ribosomal internal transcribed spacer region revealed ten hybrids from the eighty trees sampled. Two hybrids were morphologically similar to loblolly pine, and the other hybrids were morphologically similar to shortleaf pine. PCR-RFLP analysis of their *rbc*L gene showed that loblolly pine and the two hybrids morphologically similar to loblolly pine contain an identical chloroplast genome, while the others contain the chloroplast genome similar to that of shortleaf pine. Based on microsatellite analysis, the expected heterozygosity of the putative hybrids is higher than shortleaf pine or loblolly pine. UPGMA (the Unweighted Pair Group Method with Arithmatic Mean) analysis based on genetic distance showed that the hybrids morphologically similar to loblolly pine were clustered with loblolly pine, and the hybrids morphologically similar to shortleaf pine were clustered with shortleaf pine. These results indicate that bidirectional genetic introgression existed between the two species within this population, and the hybrids were likely derived from later generations backcross(es) with either shortleaf pine or loblolly pine.

Keywords: Introgression, *Pinus taeda* L., *Pinus echinata* Mill., nuclear ribosomal DNA, *cp*DNA, microsatellite

Introduction

Loblolly pine (*Pinus taeda* L.) and shortleaf pine (*Pinus echinata* Mill.) are economically important species in the United States, and they have widely overlapping geographic ranges. Hybridization between the two species has interested tree breeders for long time because the hybrids are more resistant to both littleleaf disease and fusiform rust, and also often grow faster, however the extent of occurrence of natural hybrids is still unresolved. Morphological characters, which were used to characterize species and identify hybrids in the past, offer limited help when the genotypes of the parents and their probable hybrids are compounded by environmental factors such as disease or drought stress, resulting in a wide range of phenotype variability. This confusion is aggravated by the fact that subsequent backcrossing of the hybrids to either of the parent species results in morphological characters exhibiting progressively greater similarity to the backcrossed parent species. The limitations of morphological characters resulted in the identification of the allozyme marker IDH (*Isocitrate dehydrogenase*) to identify hybrids (Huneycutt and Askew, 1989). The high frequency of IDH variation seen in natural shortleaf pine populations outside the natural range of loblolly pine (Raja *et al.,* 1997) suggests either profuse hybridization between the two species or that IDH is an unreliable marker. These data required us to look for new markers to confirm the identity of putative hybrids of the two pine species. Highly polymorphic markers such as SSRs (simple sequence repeats) should prove useful in examining the relationship between the putative hybrids and their parent species.

The direction of natural hybridization between the two species is still unclear. Artificial hybridizations in both directions have been successful between the two species

(Edwards *et al.,* 1997). Generally, it is most convenient to use shortleaf pine or shortleaf X loblolly pine trees as the female parents because fresh pollen is available from the earlier flowering loblolly pine trees (Schultz, 1997). Our controlled crosses with shortleaf pine as pollen parent resulted in no seed yield while hybridization with loblolly pine as the pollen parent resulted in many viable seeds. However, Edwards *et al.* (1997) reported that shortleaf pine sired the putative hybrids they identified in the two natural shortleaf-loblolly pine sympatric populations.

For this study, one shortleaf-loblolly pine sympatric population located in Arkansas was sampled. Our previous study using the IDH allozyme marker (Raja *et al.*, 1997) showed that sixteen percent of the trees in a stand from the central part of the population were hybrids. Morphological traits, a codominant nuclear DNA marker, a paternal-inherited chloroplast DNA marker and SSR analyses were used to determine: (1) the frequency of natural hybridization within this population; (2) the direction of natural hybridization between the two species; (3) the genetic relationship between the putative hybrids and the two species.

Materials and Methods

Plant materials

The sample population was defined as the pine stands of Montgomery County, AR. Sixteen stands (five individuals per stand) were sampled on a southeast to northwest transect across the county, at approximately equal distances across the transect. The southeast stands are mixed loblolly and shortleaf pine, while the northwest stands are only shortleaf pine. Raja *et al.* (1997) showed that sixteen percent of the individuals within a central population near Mt. Ida are hybrids. These results were based on the

heterozygosity of one allozyme marker (IDH) reported by Huneycutt and Askew (1989) to be indicative of a hybrid between shortleaf and loblolly pine. Mt. Ida is the approximate central point of the transect we sampled, and a few miles north from any known stands of loblolly pine.

In addition to the population, parents of one controlled cross, shortleaf pine (Z15, seed parent) x loblolly pine (#631, pollen parent), and 20 F1 hybrids from this cross were used to confirm the utility of one diagnostic codominant DNA marker from the nuclear ribosomal internal transcribed spacer region.

Morphological analysis

All samples were measured for four needle or cone traits previously determined to distinguish shortleaf pine and loblolly pine. These traits include the number of needles per fascicle, needle length, fascicle sheath length and cone length. The mean values and the standard deviations of these traits for the eighty population samples were calculated.

DNA extraction

Needles from the parent trees, artificial hybrids (F1) and the eighty samples from the natural population were stored at –80 ^{o}C. Total DNA was extracted from needles using the CTAB protocol (Doyle and Doyle, 1988).

PCR-RFLP analysis of the nuclear ribosomal ITS-1 region

The primer 26S-25R (5'-TATGCTTAAACTCAGCGGGT-3') and the modified ITS-5 primer (5'-GGGAGGAGAAGTCGTAACAAGG-3') (Quijada *et al.*, 1997; Nickrent *et al.,* 1994; White *et al.*, 1990) were used to amplify the nuclear ITS-1 region. Conditions for PCR amplification were: 10 mM Tris-HCl (pH 9.0 at 25 ^{o}C), 50 mM KCl and 0.1% Triton X-100, 1.8 mM $MgCl_2$, 0.16 mM dNTP mix, 1.6 μM of each primer, 1

unit DNA *Taq* polymerase (Promega company), 1% BSA, 5% DMSO and 20 ng of DNA in a final reaction volume of 25 µl. Cycling conditions for ITS-1 amplification were as follows: 3 min at 70 °C, two cycles of 2 min at 94 °C, 40 sec at 55 °C, 3 min at 72 °C. then 35 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 3 min at 72 °C, finally followed by 8 min at 72 °C and a 4 °C soak. Restriction digests of the amplified ITS-1 region DNA were accomplished with 500 ng(12 µl) of unpurified PCR product and the addition of the recommended buffer. Sixteen endonucleases that recognized 4-bp or 6-bp sites (*Alu*I, *Hae*III, *Hinf*I, *Rsa*I, *Pst*I, *Msp*I, *Pst*I, *Sac*I, *Sma*I, *EcoR*I, *Bam*HI, *Apa*I, *Xho*I) were used separately to digest an amplified ITS-1 fragment. Agarose gel electrophoresis (2.0%), ethidium bromide staining were used to check PCR-RFLP bands.

PCR-RFLP analysis of chloroplast DNA *rbcL* gene

The primer A1010 (5'-GTAGTAGGTAAACTTGAAGG-3') and the *rbc*L 3' primer (5'-ATTGGTAGAACGAAAGTCACTGGA-3') (Edwards *et al.*, 1997) were used to amplify the chloroplast *rbcL* region (from bases 961 to 1428). Conditions for PCR amplification were similar to the amplification of the nuclear DNA ITS-1 region but the extension time was 2 minutes. Approximately 12 µl unpurified PCR product was digested with HindIII. The separation and staining of PCR-RFLP bands were the same as the above.

Microsatellite amplification

Eleven highly polymorphic genomic microsatellite markers were selected from http://forestry.tamu.edu/genetics/microsatellite_primers.html. Selected primers were PtTX 2001, PtTX 2006, PtTX 2033, PtTX 2119, PtTX 3016, PtTX 3024, PtTX 3025, PtTX 3035, PtTX 3117, PtTX 3125 and PtTX3104. Each 15 µl PCR reaction was

composed of 35 ng of template DNA, 10mM Tris-HCl (pH9.0 at 25 °C), 50mM KCl and 0.1% Triton X-100, 0.16 mM dNTP mix, 1.6 μM of each forward and reverse primer, 0.9 unit DNA *Taq* polymerase (Promega company), and 1% DMSO. After a denaturing step of 2 min at 94 °C, a touch-down program was used, including a denaturing step of 40 sec at 94°C, an annealing step of 30 sec and an extension step of 50 sec at 72°C. The initial annealing temperature, which is available at http://silva.tamu.edu/genetics/AlleleSizes.rtf for a specific SSR primer, was for two cycles and was subsequently dropped by 1°C every one cycle until a final temperature of 50°C, was reached. The annealing temperature of 50°C was employed for the last 20 cycles of the amplification. The concentrations of $MgCl_2$ for different primers are also available from the above website. PCR products were first checked by 2% agarose gel electrophoresis in order to ensure successful amplification. Then run in a 6% polyacrylamide denaturing gel to separate microsatellites. A silver staining method (from Dr. Bai Guihua's laboratory, Oklahoma State University) was used to visualize microsatellite bands. The allele size was determined by 100 bp DNA ladder (Life Technologies™).

Microsatellite data scoring

Microsatellite loci were selected based on their molecular sizes as given at http://silva.tamu.edu/genetics/AlleleSizes.rtf. Allele frequencies were determined by direct manual count. The frequency of each allele per locus, the observed heterozygosity (He), the expected heterozygosity and deviations from Hardy-Weiberg equilibrium (HWE) were computed by POPGENE3.2 (Yeh and Boyle, 1997).

The eighty individuals in the natural population were grouped into four groups based on the codominant DNA markers and morphological data. All SSR data were then

combined as four groups within one population and the genetic distance was calculated between the four groups. The relationship between groups has been depicted by dendrograms obtained from Nei's (1978) unbiased genetic distance using UPGMA (the Unweighted Pair Group Method with Arithmatic Mean). The level of gene flow was estimated by F_{st} (Slatkin and Barton, 1989).

Results

Morphological data

Mean values of the morphological data for the 80 samples from the natural population are shown in Table 1. Loblolly pine can be easily distinguished from shortleaf pine. Loblolly pine has longer needles, cones and fascicle sheaths. Although most loblolly pine trees have three needles per fascicle, it is not a good discriminator because some shortleaf pine and putative hybrids morphologically similar to shortleaf pine also have mostly three needles per fascicle. Our data agree with other reports that shortleaf and loblolly pine can be grossly distinguished by combining all three discriminating traits (needle length, sheath length and cone length). However, the hybrids identified from the natural population are morphologically either similar to shortleaf pine or loblolly pine, and they could be easily misclassified without molecular marker data.

A codominant DNA marker developed from the nuclear DNA internal transcribed spacer region (ITS)

From the sixteen restriction enzymes used to digest the PCR-amplifed nuclear DNA internal transcribed spacer region, only *Msp*I produced polymorphic patterns among the parental species (Fig1.). The artificial hybrids showed codominant restriction site patterns concordant with patterns of parental species.

This diagnostic nuclear ribosomal DNA marker was used to screen the natural population materials we selected. Of the eighty samples in the natural population, ten hybrids were identified to have both bands from two parents. Among the ten hybrids, two hybrids are morphologically similar to loblolly pine and identified as HL, the others are morphologically similar to shortleaf pine and identified as HS.

Chloroplast DNA inheritance

The HindIII-digested PCR amplified *rbc*L fragment produced polymorphic patterns which can be used to distinguish shortleaf pine and loblolly pine (Fig2.). All of the artificial hybrids (F1) show their pollen parent (loblolly pine) pattern, which confirms that chloroplast DNA is paternally inherited in the cross of shortleaf pine (seed parent) X loblolly pine (pollen parent). This diagnostic marker was also used to screen the population samples. The ten putative hybrids identified from the natural population show two different patterns, two putative hybrids (HL), morphologically similar to loblolly pine, show the loblolly pine pattern, while the other putative hybrids (HS), morphologically similar to shortleaf pine, show the shortleaf pine pattern.

Genetic diversity, gene flow and genetic relationships among the four groups within the natural population

In all samples, observed and expected genotypic compositions conformed to HW expectations. Genetic diversity levels for the four groups were high in this natural population. The total SSR allele number was 104 for the 80 individuals. The 16 loblolly pine trees share 78 alleles, the 53 shortleaf pine trees have 95 alleles, while the two hybrids morphologically similar to loblolly pine have 41 alleles, and the 8 shortleaf-like hybrids have 66 alleles. The allele number is not a good indicator of genetic variation

because the sample sizes of the four groups are different. The observed and expected heterozygosity are shown in Table 2. Both the observed and expected heterozygosities (Ho) for either hybrids (HL) or hybrids (HS) were larger than shortleaf pine or loblolly pine. The heterozygosity (H_0 or H_e) for loblolly pine was larger than shortleaf pine, and this result is consistent with the report of Edwards *et al.* (1997) based on allozyme data. Mean F_{st} value is 0.1079.

Nei's (1978) genetic identity and genetic distance measures are presented in Table 3, genetic identity between loblolly pine and the loblolly-like hybrids (HL) was 0.9370 and 0.9742 between shortleaf pine and the shortleaf-like hybrids (HS). Based on Nei's (1978) genetic distance, the cladistic relationship among the four groups was drawn as presented in Fig 3. This dendrogram indicated that the loblolly-like hybrids (HL) share one clade with loblolly pine, while the shortleaf-like hybrids (HS) share another clade with shortleaf pine.

Discussion

The codominant DNA marker found from PCR-RFLP analysis of the nuclear ribosomal internal transcribed spacer 1 was reported for the first time to distinguish two species. We used this marker to evaluate twelve putative hybrids found in eight natural shortleaf populations. These trees were identified as hybrids (Raja *et al.,* 1997) because they were heterozygous at the IDH locus (Huneycutt and Askew, 1989). In this study, all of these putative hybrids showed both bands from two parental species.

The ten hybrids identified from the eighty population samples using the above nuclear marker showed two types of chloroplast DNA. Two hybrids (HL), morphologically similar to loblolly pine, have loblolly pine chloroplast DNA, suggesting

the two hybrids may be derived from later generation backcross(es) with loblolly pine. The other hybrids (HS) are morphologically similar to shortleaf pine and have shortleaf pine chloroplast DNA, suggesting that these hybrids may be derived from later generation backcrosses with shortleaf pine. We attempted to develop mitochondrial DNA markers to confirm that the putative hybrids are from later generation backcross(es) with shortleaf pine or loblolly pine, but the three mitochondrial DNA (mtDNA) markers we studied either show the same patterns between shortleaf and loblolly pine or show variability within species (Chen *et al.,* 2001).

Based on highly polymorphic microsatellite DNA marker analyses, the observed and expected heterozygosities of two types of hybrids are both higher than either loblolly pine or shortleaf pine. The observed heterozygosity of the loblolly-like hybrids (HL) is much higher (0.8611), which may be due to a sample size of two. The genetic identity between loblolly pine and HL hybrids is relatively close, as is the identity between shortleaf pine and shortleaf-like hybrids (HS). Mean Fst value is not very high (0.1079) among the four groups indicating that gene flow can occur among these groups.

Combining the morphological data and molecular data of the putative hybrids found in the natural population, we conclude that the first type of hybrids (HL) may be from backcross(es) of later generation hybrids with loblolly pine while the second type of hybrids (HS) may be either from the backcross(es) of later generation hybrids with shortleaf pine.

Acknowledgment

This study was supported by the Oklahoma State University Agricultural Experimental Station. DNA primer synthesis was performed by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Needles of parents of the controlled cross and 20 F1 hybrids from this cross were kindly supplied by the USDA-Forest service, Southern Institute of Forest Genetics, USA and Dr. Bruce Bongarten, Warnell School of Forest Resources, The University of Georgia. Thanks to Dr. Guihua Bai, and Dr. Peiguo Guo in Oklahoma State University and Dr. Zhou Yi in Texas A & M University, for help with the SSR experiment, SSR data scoring and analysis.

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Table 1.

Mean values for morphological characters of 80 samples from a natural mixed population of shortleaf and loblolly pine.

Trait	Mean value (standard deviation)			
	L[1]	HL[2]	HS[3]	S[4]
Number of needles/fascicle	3.0 (0.17)	3.0 (0.00)	2.4 (0.3)	2.31(0.08)
Needle length (cm)	17.96 (5.23)	19.54 (0.28)	10.75(0.90)	10.17(6.64)
Cone length (cm)	6.94(4.53)	6.22(0.20)	4.84(0.6)	4.28(0.60)
Fascicle sheath length(mm)	1.92 (0.00)	1.91(0.18)	1.45(0.25)	1.30(0.50)

1 loblolly pine

2 the putative hybrids morphologically similar to loblolly pine

3 the putative hybrids morphologically similar to shortleaf pine

4 shortleaf pine

Table 2.

Observed and expected heterozygosity estimated based on 11 low-copy microsatellite loci assayed for one Arkansas population

Group	Sample size	observed heterozygosity (H0)	expected heterozygosity (He) [1]
Pinus taeda	16	0.5631 (0.2463)	0.5606 (0.2100)
HL[2]	2	0.8611(0.2304)	0.6852 (0.1512)
HS[3]	8	0.7583 (0.2114)	0.6550 (0.1949)
Pinus echinata	53	0.4605 (0.2023)	0.5540 (0.2210)

1 expected heterozygosity (He) was computed using Leven (1949), which is the same to Nei's (1978) unbiased heterozygosity.

2 putative hybrids morphologically similar to loblolly pine.

3 putative hybrids morphologically similar to shortleaf pine

Table 3

Nei's (1978) Unbiased Measures of Genetic Identity (above diagonal) and Genetic distance(below diagonal) among the four groups within the natural population sampled.

pop ID	L	HL	HS	S
L	****	0.9370	0.8474	0.8808
HL	0.0651	****	0.8734	0.8279
HS	0.1655	0.1354	****	0.9742
S	0.1269	0.1888	0.0261	****

L= loblolly pine

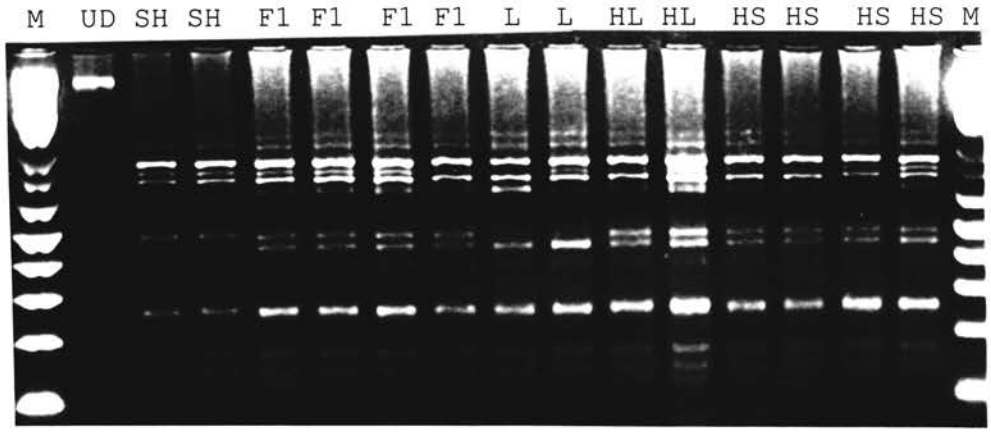
HL=putative hybrids morphologically similar to loblolly pine

HS= putative hybrids morphologically similar to shortleaf pine

S= shortleaf pine

Figure 1.

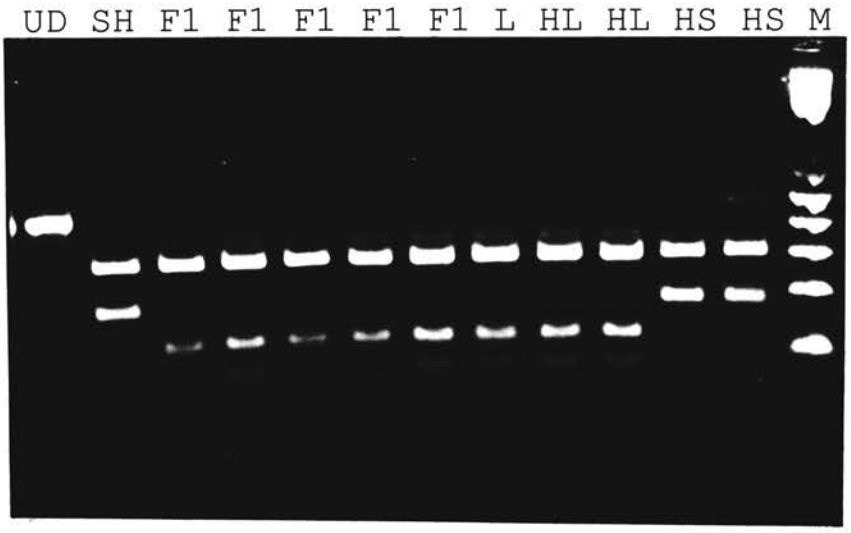
PCR-RFLP analysis of the nuclear ribosomal DNA internal transcribed spacer 1 for shortleaf pine, loblolly pine, their artificial hybrids (F1) and putative hybrids.



M: 1kb plus DNA marker; UD: undigested PCR product; SH: shortleaf pine (Z15); L: loblolly pine (#631); F1: artificial hybrids between Z15 (seed parent) X #631 (pollen parent); HL: putative hybrids morphologically similar to loblolly pine; HS: putative hybrids morphologically similar to shortleaf pine.

Figure 2.

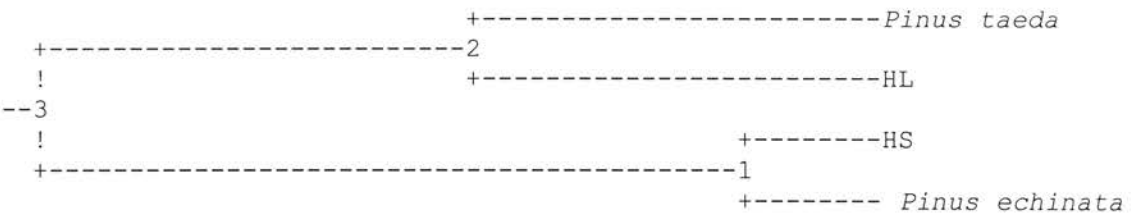
PCR-RFLP of the partial *rbc*L chloroplast gene from shortleaf pine, loblolly pine, their artificial and natural hybrids.



M: 100bp DNA marker; UD: undigested PCR product; SH: shortleaf pine (Z15); L: loblolly pine (#631); F1: artificial hybrids between Z15 (seed parent) X #631 (pollen parent); HL: putative hybrids morphologically similar to loblolly pine; HS: putative hybrids morphologically similar to shortleaf pine.

Figure 3.

The dendrogram based on Nei's(1978) genetic distance using UPGMA for 80 trees of the population sample.



HS: putative hybrids morphologically similar to shortleaf pine; HL: putative hybrids but morphologically similar to loblolly pine; *Pinus taeda* and *Pinus echinata* are the loblolly pine and shortleaf pine collections from the natural population, respectively.

VITA

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Candidate of the Degree of

Doctor of Philosophy

Thesis: INTROGRESSION BETWEEN SHORTLEAF PINE (*PINUS ECHINATA* MILL.) AND LOBLOLLY PINE (*PINUS TAEDA* L.)

Major Field: Plant Science

Biographical:

Personal Data: Born in Tongchen City, Anhui Province, the People's Republic of China on December 15, 1972, the son of Zhouhua Chen and Xiumei Huang.

Education: Graduated from Tongchen High School, Tongchen City, Anhui Province in July 1990; received Bachelor of Science degree in Biological Education from Anhui Normal University, Wuhu City, Anhui Province in July 1994. Completed the requirements for the Master of Science degree with a major in Plant Science in Institute of Botany, Chinese Academy of Sciences in July 1997. Completed the requirements for the Doctor of Philosophy degree with a major in Plant Science in Oklahoma State University in December, 2001.

Personal Experience: Research associate, Laboratory of Systematic & Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing, China, from July 1997 to August 1998; Graduate Research Assistant, Department of Forestry, Oklahoma State University, Stillwater, Oklahoma, USA, from August 1998 to present.

Professional Memberships: Society of American Foresters