OBSERVATIONS ON MITOCHONDRIAL DNA INHERITANCE AND VARIATION AMONG THREE *PINUS* SPECIES

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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* Englem.). Maternal inheritance of mitochondrial DNA in these *Pinus* species was confirmed by the artificial cross of slash pine (seed parent) × shortleaf pine (pollen parent). PCR-RFLP analyses of nad1 b/c and cox1, a PCR-SSCP assay of the nad3-rps12 intergenic region and a mitochondrial microsatellite sequence analysis within this spacer region revealed no polymorphism between shortleaf and loblolly pine. Only one microsatellite length difference was revealed between shortleaf and slash pine. However, one mitochondrial DNA marker varied among individuals of both shortleaf pine and loblolly pine from widely separated 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 indicate that *mt*DNA variation among the pine species studied may be from gene rearrangements or microsatellite length differences.

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

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 & ASKEW 1989; EDWARDS & 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 natural putative hybrids are reported to be 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 most putative hybrids are later generation backcross(es) with shortleaf pine. Maternally-inherited *mt*DNA markers between shortleaf and loblolly pine, when combined with other molecular marker and allozyme data, could be useful to prove the existence of the backcrosses. Our objective was to develop *mt*DNA markers to help identify natural hybrids between loblolly and shortleaf pine. 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 may exist at both levels. WU *et al.* (1998) studied three closely-related *Pinus* species and found strong *mt*DNA variation occurs among populations within species.

In this study, the natural shortleaf-loblolly pine population of Montgomery County, Arkansas was sampled to examine the variation of *mt*DNA markers within and among shortleaf and loblolly pine. Two artificial crosses were also utilized, and these were (parents and F_1s) shortleaf pine (seed parent) × loblolly pine and slash pine (*Pinus elliottii* Engelm.) × shortleaf pine (pollen parent). These artificial crosses were used to develop *mt*DNA markers to distinguish the three *Pinus* species and confirm maternal *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)

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analyses with radioactively labeled probes (NEALE & SEDEROFF 1989), but more recently PCR-RFLP analysis of mitochondrial DNA genes or gene fragments has 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) has 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 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 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 analysis of the nad3-rps12 intergenic region was also performed for the three *Pinus* species. Since *mt*DNA variation could not be found using the above methods, traditional RFLP analysis with specific *mt*DNA probes was 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) was a selection from Ouachita, Arkansas; slash pine #1204 (alias W-1-5) was a selection from a Wayne County, Mississippi planting but its origin was Georgia.

A natural population, defined as the pine stands of Montgomery County, Arkansas was also studied. Sixteen stands (five individuals per stand) were sampled on a southeast to northwest transect across the county (Figure 1). Stands were located at approximately equal distances across the transect. The southeast stands (#1–8) are mixed loblolly and shortleaf pine, while the

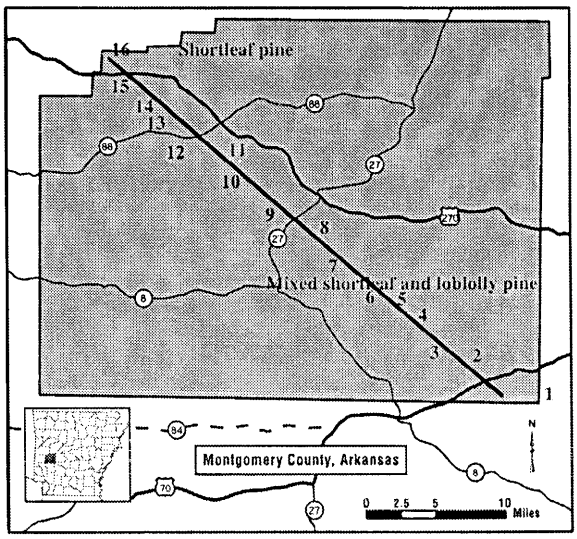

Figure 1. Sample transect across Montgomery County, Arkansas, showing approximate stand location by stand number.

northwest stands (# 9–16) are only shortleaf pine. Only dominant or co-dominant trees, separated by at least 60 meters, were sampled within each stand. RAJA *et al.* (1997) showed that about sixteen percent of the dominant and co-dominant trees within a central population near Mt. Ida are hybrids. These results were based on the heterozygosity of one allozyme marker (IDH: Isocitrate dehydrogenase) reported by HUNEYCUTT & 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 has been confirmed by one diagnostic DNA marker from the nuclear ribosomal DNA internal transcribed spacer 1 (ITS1) and the IDH allozyme marker (CHEN 2001).

DNA extraction

Needles from the parent trees, the artificial hybrids (F_1) 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 & 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. 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	712 bp
Nad3-rps12[3]	AATTGTCGGCCTACGAATGTG	GCTCG (A=I)GTACGGTC (C=I)GTGCG	~ 370 bp
Nad3-1[4]	TTCCCCATGAATGGAAGAAG	ATTGATTCGATGTAGGCATCG	~ 109 bp
nad1 Exon b/c[5]	GCATTACGATCTGCAGCTCA	GGAGCTCGATTAGTTTCTGC	~ 2600 bp

[1] expected size of PCR product relative to the reference sequence,
[2] primers published by GLAUBITZ & 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).

(Table 1). Conditions for PCR amplification were: 10mM Tris-HCl (pH9.0 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, 1unit DNA *Taq* polymerase, with 20 ng of DNA in a final reaction volume of 25 µl. Cycling conditions for nad1 b/c amplification were: 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, followed by 8 min at 72 °C. 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, *Nci*I, *Pvu*II, *Pst*I, *Sac*I, *Sma*I, *Taq*I, *Eco*RI, *Bam*HI, *Apa*I, *Xba*I, *Xho*I) were used to digest nad1 b/c and cox1. Two percent agarose gel electrophoresis and ethidum 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 the Nad3-1 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 was conducted for the amplified nad3-rps12 intergenic spacer based on the protocol of CHEN (2001).

A mitochondrial G_n 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 purified PCR products were sequenced by the Oklahoma State University Recombinant DNA/Protein Resource Facility. 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 genes. 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 were 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 *Bam*HI to digest genomic DNA for the nad1 b/c probe. The protocols and procedures for restriction digestion, agarose gel electrophoresis and

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Southern blotting were as described by Wu *et al.* (1998).

RESULTS

*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 *Bam*HI-digested genomic DNA, all the artificial hybrids (F_1) show the same hybridization pattern as slash pine (#1204), but shortleaf pine (#1351) shows a different pattern (Figure 2). This confirms maternal inheritance of mitochondrial DNA in this *Pinus* cross.

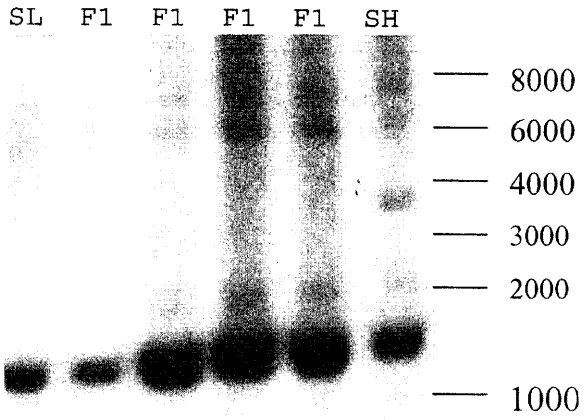

Figure 2. Hybridization of nad1 b/c to the *Bam*HI-digested genomic DNA of slash pine, shortleaf pine and their artificial hybrids (F1). SH: shortleaf pine (#1351, pollen parent); SL: slash pine (#1204, seed parent); F1: artificial hybrids of slash pine (#1204) x shortleaf pine (#1351). DNA marker size (bp) is indicated in the figure.

Polymorphism of nad1 b/c and cox1

The sizes of PCR-amplified nad1 b/c and cox1 in the three *Pinus* species are approximately 2600 bp and 710 bp, 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 of the nad3-rps12 intergenic region 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 parents (Z15, #1351) and the slash pine parent (#1204) (Figure 3) 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.

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                  1         11         21         31         41

consensus         TTCCCCATGAATGGAAGAAGGGTGCTTCAGATCGGGAGTAACCACCAATG
P. taeda          .........................................................
P. echinata       .........................................................
P. elliottii      .........................................................

                  51        61         71         81         91

consensus         ATAGGGCAACAATCGGGGGGGGGG-AAGGACGGGAAGAGCGATGCCTACA
P. taeda          .........................................................
P. echinata       .........................................................
P. elliottii      ......................G..................................

                  101

consensus         TCGAATCAAT
P. taeda          .........
P. echinata       .........
P. elliottii      .........
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Figure 3. Multiple sequence alignment of a mitochondrial microsatellite located within the *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.

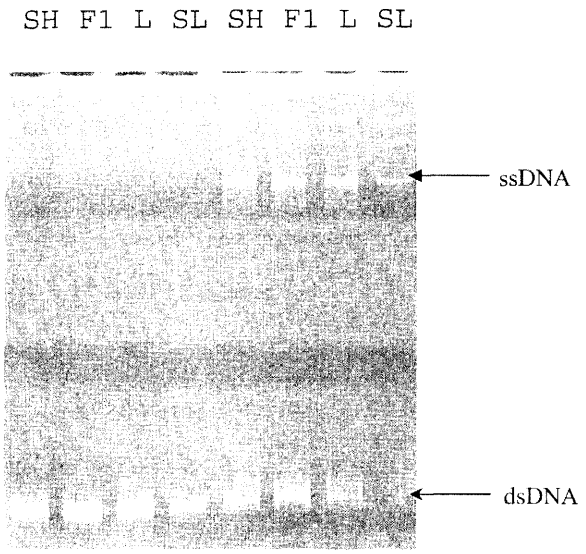

Figure 4. PCR-SSCP analysis of the *nad3-rps12* intergenic region of slash pine, loblolly pine, shortleaf pine and the hybrid (F1) between shortleaf pine and loblolly pine. The first four lanes show undenaturated PCR product; the last four lanes show denaturated PCR product. SH: shortleaf pine (Z15, seed parent); F1: artificial hybrid between shortleaf pine (Z15) and loblolly pine (#631); L: loblolly pine (#631, pollen parent); SL: slash pine (#1204).

nad3-rps12 intergenic region variation

Direct electrophoresis using an 8 % undenaturated polyacrylamide gel and PCR-SSCP analysis (Figure 4) of the nad3-rps12 intergenic spacer region showed only one haplotype corresponding to shortleaf pine, slash pine, loblolly pine and the artificial hybrids (F_1) between shortleaf pine and loblolly pine. The artificial hybrids (F_1) between shortleaf pine and slash pine shared the same PCR-SSCP pattern as their parents (data not shown). No polymorphism was observed in this intergenic region among the three *Pinus* species.

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. However, with the nad1 b/c probe, Z15 and #1351 (both shortleaf pine) showed different hybridization patterns (Figure 2, 5). Shortleaf pine #1351 shows the same hybridization pattern as the shortleaf pine trees in the natural population sampled (Figure 2, 5). Loblolly pine #631 (Figure 5) has a different hybridization pattern from the loblolly pine sampled in the natural population (data not shown).

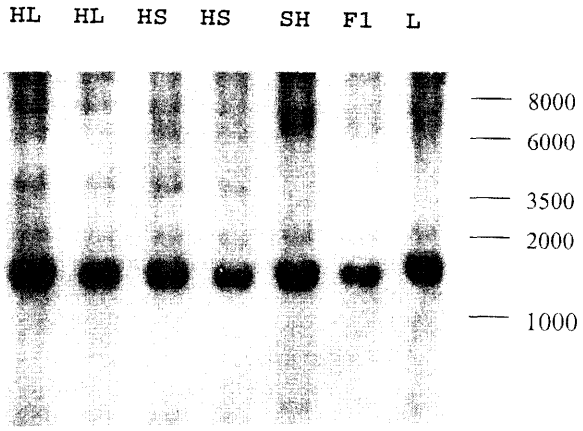

Figure 5. Hybridization of nad1 b/c to the *Bam*HI-digested genomic DNA of shortleaf pine, loblolly pine and their artificial hybrids (F1). SH: shortleaf pine (#Z15, seed parent); L: loblolly pine (#631, pollen parent); F1: artificial hybrids (F1) of shortleaf pine (Z15) X loblolly pine (#631); HL: the 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. DNA marker size (bp) is indicated in the figure.

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 2001). 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 was hybridized with *Bam*HI-digested genomic DNA, HL and HS have the same hybridization pattern (Figure 5). All the other individuals sampled from the population shared this same hybridization pattern.

DISCUSSION

Our results show no *mt*DNA variation within the Montgomery County, Arkansas 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 *Bam*HI-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.

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Since Z15 and #1351 are from different shortleaf pine populations, and loblolly pine parent #631 (North Carolina) is not from the Arkansas population, our results indicate that *mt*DNA variation exists within different populations of each species. This *mt*DNA 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 carefully 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. Since our sample size among populations was quite small, within-species variation may be considerable.

Our results also suggest that *mt*DNA variation within the pine species studied may be 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 used as a probe to hybridize with *Bam*HI-digested genomic DNA, a polymorphism was observed among shortleaf pine individuals from different populations. This may be due to gene rearrangement events. Nucleotide sequences of the mitochondrial microsatellite fragment located within the nad3-rps12 intergenic region showed no nucleotide substitutions but there was a 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. Such a close relationship is supported by PRICE *et al.* (1998) and CHEN *et al.* (2002).

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REFERENCES CITED

CHEN, J.W. 2001: Introgression between shortleaf pine (*Pinus echinata* Mill.) and loblolly pine (*Pinus taeda* L.). Ph.D thesis, Oklahoma State University, Stillwater, OK, Pp. 1-66.

CHEN, J.W., TAUER, C.G., & HUANG, Y.H. 2002: Nucleotide sequences of the internal transcribed spacers and 5.8S region of nuclear ribosomal DNA in *Pinus taeda* and *Pinus echinata*. *DNA Sequen. - J. Sequen. Mapp.* **13**(2):129-131.

DEMESURE, B., SODZI, N., & PETIT, R.J. 1995: A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Mol. Ecol.* **4**: 129-131.

DOYLE, J.J. & DOYLE, J.L. 1988: Isolation of plant DNA from fresh tissue. *Focus* **12**:13-15.

EDWARDS, M.A., HAMRICK, J.L., & PRICE, R.A. 1997: Frequency and direction of hybridization in sympatric populations of *Pinus taeda* and *P. echinata (Pinaceae)*. *Am. J. Bot.* **84**(7): 879-886.

EDWARDS, M.A. & HAMRICK, J.L. 1995: Genetic variation in shortleaf pine, *Pinus echinata* Mill. (*Pinaceae*). *For. Genet.* **2**: 21-28.

GLAUBITZ, J.C. & CARLSON, J.E. 1992: RNA editing in the mitochondria of a conifer. *Current Genet.* **22**: 163-165.

GRIVET, D., JEANDROZ, S., & FAVRE, J.M. 1999: Nad1 b/c intron polymorphism reveals maternal inheritance of the mitochondrial genome in *Picea abies*. *Theor. Appl. Genet.* **99**: 346-349.

HUNEYCUTT, M. & ASKEW, G.R. 1989: Electrophoretic identification of loblolly pine-shortleaf pine hybrids. *Silvae Genet.* **38**: 3-4.

NEALE, D.B. & SEDEROFF, R.R. 1989: Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theor. Appl. Genet.* **77**: 212-216.

PRICE, R.A., LISTON, A., & STRAUSS, S.H. 1998: Phylogeny and systematics of *Pinus*. *In*: Richardson, D.M. (eds), Ecology and Biogeography of *Pinus*. Cambridge University Press, Pp. 49-68.

RAJA, R.G., TAUER, C.G., WITTWER, R., & HUANG, Y. 1997: An isoenzymatic study of the genetic structure in natural populations of shortleaf pine, *Pinus echinata* Mill. *Can. J. For. Res.* **27**(5): 740-749.

SORANZO, N., PROVAN, J., & POWELL, W. 1999: An example of microsatellite length variation in the mitochondrial genome of conifers. *Genome* **42**(1): 158-61.

WANG, X.R., SZMIDT, A.E., & LU, M.Z. 1996: Genetic evidence for the presence of cytoplasmic DNA in pollen and megagametophytes and maternal inheritance of mitochondrial DNA in *Pinus*. *For. Gen.* **3**(1): 37-44.

WATANO, Y., IMAZU, M., & SHIMIZU, T. 1996: Spatial distribution of *cp*DNA and *mt*DNA haplotypes in a hybrid zone between *Pinus pumila* and *P. parviflora* var. *pentaphylla* (Pinaceae). *J. Plant Res.* **109**: 403-408.

WU, J., KRUTOVSKII, K.V., & STRAUSS, S.H. 1998: Abundant mitochondrial genome diversity, population differentiation and convergent evolution in pines. *Genetics* **150**: 1605-1614.

ZOBEL, B.J. 1953: Are there natural loblolly-shortleaf pine hybrids? *J. For.* **51**: 494-495.