Introgression between *Pinus taeda* L. and *Pinus echinata* Mill.

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INTRODUCTION

Loblolly pine (*Pinus taeda* L.) and shortleaf pine (*Pinus echinata* Mill.) have widely overlapping geographic ranges. Hybridization between the two species has interested tree breeders for a long time. Morphologically, the two pine species are different. The needles of loblolly pine are 6 to 9 inches long, usually with three yellow-green needles per fascicle; but shortleaf pine needles are 3 to 5 inches long, with two or three dark yellow-green slender and flexible needles per fascicle. Loblolly pine also has larger cones than shortleaf, as well as other differences, however, these characters offer limited help when the genotypes of the parents and their probable hybrids are compounded by environmental factors 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 (Rajiv *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.

A more extensive study (relative to the study of Rajiv *et al.*, 1997), sampling a larger portion of shortleaf-loblolly pine sympatric population, was conducted to further explore the nature and extent of these hybrids in the native populations. We combined morphological traits, the allozyme marker (IDH), a codominant DNA nuclear marker, a paternally-inherited chloroplast DNA marker and SSR markers to explore natural hybridization between the two species.

MATERIALS AND METHODS

The sample population was defined as the pine stands of Montgomery County, Arkansas. Five trees in each of sixteen stands were sampled on a southeast to northwest transect across the county. The southeast stands are mixed loblolly and shortleaf pine, while the northwest stands are only shortleaf pine. Rajiv *et al*. (1997) showed that about sixteen percent of the individuals within a population near Mt. Ida are hybrids. Mt. Ida is the approximate central point of the transect we sampled, and a few miles north of any known stands of loblolly pine.

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

All samples were measured for 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 trees were calculated. Since these eighty trees were ultimately assigned into four groups of unequal size, a pseudo-t test was used to test for differences among them.

Total DNA was extracted from needles using the CTAB protocol. PCR was used to amplify the nuclear ITS-1 region. Agarose gel electrophoresis (2.0%) and ethidum bromide staining were used to reveal PCR-RFLP bands. PCR-RFLP analysis was also used to amplify the chloroplast *rbcL* region.

The IDH allozyme marker reported by Huneycutt and Askew (1989) to identify shortleaf loblolly pine hybrids was assayed for all of the individuals in the population identified as hybrids.

Eleven highly polymorphic genomic microsatellite markers were selected from http://forestry.tamu.edu/genetics/microsatellite_primers.html for use in this study. Microsatellite loci were selected based on their molecular size. Allele frequencies were determined by direct manual count.

Based on the morphological data and the PCR-RFLP analysis of ribosomal DNA ITS-1 marker, the eighty individuals in the study population were placed in four groups: pure shortleaf pine, pure loblolly pine, hybrids morphologically similar to loblolly pine and hybrids morphologically similar to shortleaf pine. 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 was depicted by a dendrogram obtained from Nei's (1978) unbiased genetic distance using UPGMA (the unweighted pair group method with arithmatic mean). Genetic differentiation was estimated by Fst (Slatkin and Barton, 1989).

RESULTS

Mean values of the morphological data for the 80 trees as placed into four groups are shown in Table 1. The pseudo-t tests comparing each possible paring of the groups showed that the morphological data clearly distinguishes loblolly pine from shortleaf pine. Loblolly pine has longer needles, cones and fascicle sheaths, and essentially 3 needles per fascicle while shortleaf has an average of 2.3. These data also distinguish the pure species from the hybrids that are morphologically similar to the other parent, but do not allow identification of those hybrids morphologically similar to themselves. Since all the hybrids identified from the natural population are morphologically either similar to

shortleaf pine or loblolly pine, they could be easily misclassified as pure species without utilizing molecular marker data.

Trait	Mean value (standard deviation)			
	Group[a] (sample size)			
	L (16)	HL (2)	HS (8)	S (54)
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)

Table 1. Mean values for morphological characters of 80 samples from a natural mixed population of shortleaf and loblolly pine.

[a] Abbreviations: L; loblolly pine, HL; the putative hybrids morphologically similar to loblolly pine, HS; the putative hybrids morphologically similar to shortleaf pine, S; shortleaf pine.

The nuclear DNA internal transcribed spacer region produced a polymorphic pattern between the parental species. The artificial hybrids showed codominant restriction site patterns concordant with the patterns of the parental species. The diagnostic nuclear ribosomal DNA marker was used to screen the 80samples in the natural population, and ten hybrids were identified. Among the ten hybrids, two were morphologically similar to loblolly pine and the others were morphologically similar to shortleaf pine. The HindIII-digested PCR amplified *rbc*L chloroplast DNA fragment produced polymorphic patterns which showed two different patterns, two putative hybrids, morphologically similar to loblolly pine, showed the loblolly pine pattern, while the other putative hybrids, morphologically similar to shortleaf pine, showed the shortleaf pine pattern.

Since our data indicated that the hybrids identified with nuclear markers are morphologically similar to one parent or the other, and not intermediate as expected for an F1, these hybrids are most probably later generation backcrosses or intercrosses. As such, one would expect segregation at the IDH locus, resulting in some of these later generation hybrids being homozygous for one parent or the other at the IDH locus. Consequently, the IDH locus appears to be reliable in identifying hybrids of these two pine species, but that reliability does not extend to later generation hybrids. The same would be true for the nuclear marker we developed, but these markers used in combination should allow the identification of most of the naturally occurring hybrids between loblolly pine and shortleaf pine.

Genetic identity between loblolly pine and the loblolly-like hybrids was 0.9370 and 0.9742 between shortleaf pine and the shortleaf-like hybrids. Based on Nei's (1978) genetic distance, the phenetic relationship among the four groups was drawn. This

dendrogram indicates that the loblolly-like hybrids share one clade with loblolly pine, while the shortleaf-like hybrids share another clade with shortleaf pine.

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