Genotyping of Longleaf Pine Ramets After Hurricane Hugo by Using DNA and Isozyme Markers

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Isozyme and restriction fragment length polymorphism (RFLP) markers were used to determine the genetic identities of 12 longleaf pine (Pinus palustrus Mill.) *ramets whose identities came into question after Hurricane Hugo. Isozyme assays were performed for 12 enzyme systems representing 15 loci. Variation at 6 loci revealed unique identities for 6 ramets. Four RFLP probes showed that 9 of the 12 ramets were genetically unique. This study confirms the power of isozyme analysis for determining genetic identity of seed orchard clones and, in addition, demonstrates the increased power of genetic discrimination offered by RFLP analysis.*
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In September 1989, Hurricane Hugo swept through the Atlantic seaboard, causing extensive damage to forests, seed orchards, and clone banks. The USDA Francis Marion Seed Orchard near Charleston, South Carolina, presented the National Forest Genetic Electrophoresis Laboratory (NFGEL) in Camino, California, with the challenge of determining the identities of 12 longleaf pine *(Pinus palustris* Mill.) ramets from a clone bank that could be used to replenish the longleaf pine clonal orchard that was damaged by the hurricane. It was unclear whether the 12 ramets were ramets from one clone, individual ramets from each of 12 clones, or something in between. It was known, however, that the clone bank ramets were not included in the damaged clonal orchard. NFGEL asked the molecular genetics laboratory at the Institute of Forest Genetics (IFG) in Placerville, California, to genotype the 12 unlabelled ramets using the restriction fragment length polymorphism (RFLP) technique to test the efficiency of DNA technology for solving practical genetic identity questions. For comparative purposes, isozyme assays were conducted by NFGEL on needle tissues from the same 12 ramets.

Isozyme genetic markers have been used extensively over the past decade for numerous applications in forest tree improvement, including paternity analysis, varietal identification, seed lot certification, and verification of controlled crosses (Adams 1981, 1983; Adams et al. 1988, Cheliak et al. 1987; Miller et al. 1989). Isozyme assays are relatively inexpensive and technically easy to perform on large samples and are thus the preferred marker in cases where they provide adequate discrimination.

DNA technology has developed rapidly in human forensic science in recent years. This technology is now being used for applications in agricultural science and forestry. The recent development of DNA marker provides a new tool to tree breeders that promises to increase the power of genetic discrimination (Nybom and Schaal 1990, Rogstad et al. 1988, Kreike et al. 1991, Neale and Williams 1991, Friedman and Neale 1993, Wagner 1992). Assays of DNA markers are more costly and time consuming to apply than isozyme assays but have several important advantages: (1) there are potentially a large number of DNA markers, (2) DNA assays can be performed on most tissue types, and (3) DNA markers are less affected by environmental variation. Restriction fragment length polymorphisms are simple Mendelian genetic markers that result from various types of mutations and rearrange ments of the DNA. These alterations are detected when the DNA is cleaved with restriction enzymes and the fragments of varying lengths are separated by electrophoresis in an agarose gel. The fragmented DNA is transferred to a nylon membrane and incubated in a solution containing a radioactively labeled DNA probe. Because of "hybridization" between the probe and filter-bound DNA, the location of the probe-specific fragments can be visualized using x-ray film (figure 1) (Neale and Williams 1991).

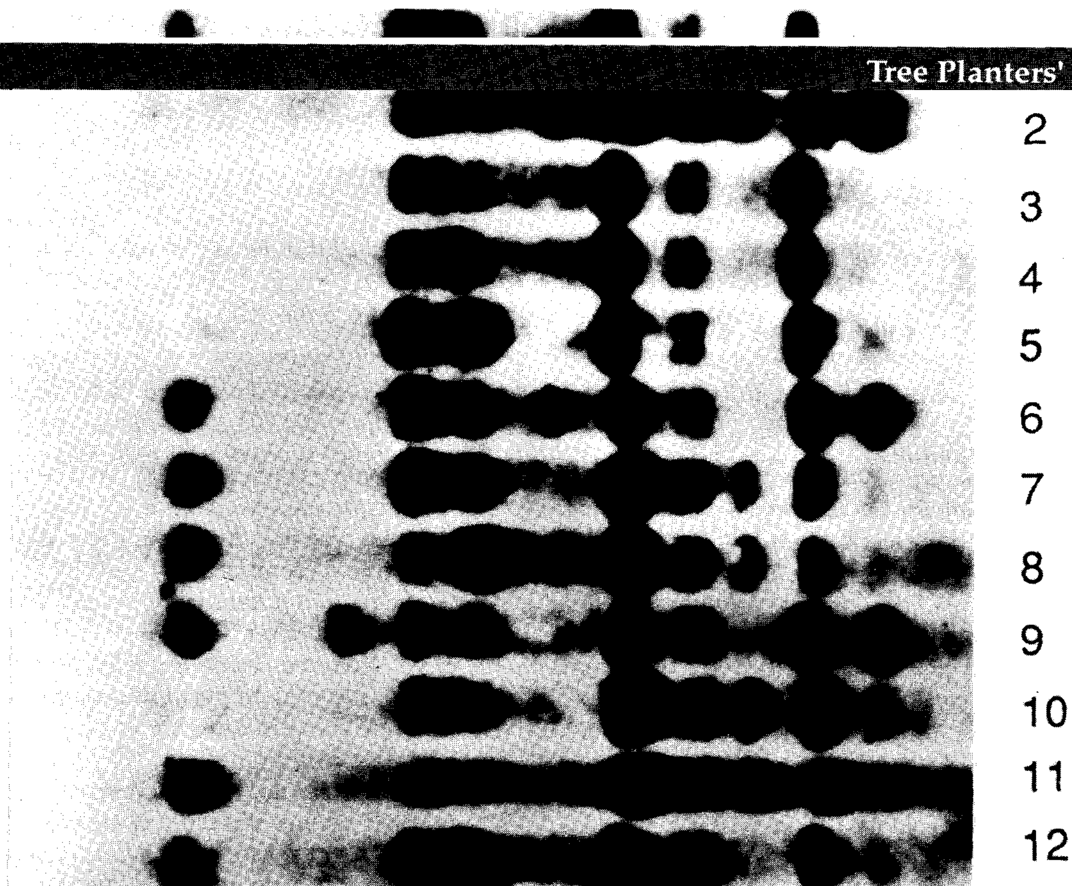

Figure 1—*DNAs of 12 longleaf pine clone bank ramets of unknown genetic identities were cleaved with* HindIII *and hybridized with the loblolly pine RFLP probe pPtIFG669.*

Materials and Methods

Needle tissue from 12 longleaf pine clone-bank ramets was sent by the USDA Forest Service's Francis Marion Seed Orchard in Charleston, South Carolina. The genetic relationship among the ramets was unknown.

RFLPs. *DNA isolation and blot preparation.* Needle tissue was ground to a coarse powder in liquid nitrogen and stored at - 70 /C. Total genomic DNA was extracted from 10 g dry-weight tissue using methods described by Devey et al. (1991). Two 10-µg samples of DNA from each ramet were digested with 50 units of either restriction enzyme *Hind*III or *Bam*HI and fractionated on 0.8% agarose gels submerged in 1 x (TAE) buffer. DNA was transferred to Zetaprobe GT nylon membrane (BioRad) using methods described by Reed and Mann (1985).

DNA probes and southern hybridizations. Four probes (pPtIFG: 669, 1628, 660, 1963) were selected from a loblolly pine cDNA library because they reveal polymorphism in loblolly pine and were known to hybridize with other *Pinus* species. Southern hybridizations were conducted using methods described by Devey et al. (1991).

Isozymes. Isozyme analyses were performed at NFGEL for 12 enzyme systems (DIA, GOT, GLY, SKD, ME, PGI, LAP, UGPP, PGM, IDH, MDH, GPGD) coded by 15 loci. Assays were performed on protein extracts of fresh needle tissues following standard methods (Adams et al. 1990, Conkle et al. 1982). Six loci (Skd, Pgi-2, Ugpp, Pgm-1, Mdh, 6-Pgd) were polymorphic among the 12 ramets, and allozyme genotypes at these loci were determined for each ramet.

Results

RFLP's. The RFLP genotypes of the 12 ramets are shown in table 1. Based on their multilocus genotypes, ramets 1, 2, 6, 7, 8, 9, 10, 11, and 12 had unique genetic identities. Probe pPtIFG669 revealed unique DNA band patterns among ramets 2, 6, 9, 10, and 11

Table 1-RFLP band pattern types in 12 longleaf pine clone-bank ramets treated with 6 probe enzymes

Ramet	669 *Hind*III	1628 *Bam*HI	1628 *Hind*III	660 *Bam*HI	660 *Hind*III	1963 *Bam*HI	1963 *Hind*III	Multi-locus genotype
1	1	ND	1	1	1	1	1	A
2	2	2	2	2	2	2	2	B
3	3	3	3	3	3	3	3	C
4	3	3	3	3	3	3	3	C
5	3	3	3	3	3	3	3	C
6	4	4	4	4	4	4	4	D
7	5	5	5	5	5	5	5	E
8	5	5	5	5	5	10	10	F
9	6	6	6	6	6	6	6	G
10	7	7	7	7	7	7	7	H
11	8	8	8	8	8	8	8	I
12	1	1	1	1	9	9	9	J

ND= no data. Numbers are assigned to unique RFLP band pattern genotypes for each probe-enzyme combination. Letters are assigned to each multi-locus RFLP band pattern genotype.

when hybridized to DNA's restricted with Hind*III* (figure 1). The remaining 7 ramets were clustered into 3 distinct groups: ramets 1 and 12; ramets 3, 4, and 5; and ramets 7 and 8. Southern hybridization with probe pPtIFG1628 gave the same results as probe pPtIFG669. Probe pPtIFG660 revealed variation between ramets 1 and 12 when cleaved with Hind*III*. Lastly, probe pPtIFG1963 gave unique RFLP band patterns for ramets 7 and 8. Repeat hybridizations were conducted for confirmation of results. Ramets 3, 4, and 5 consistently revealed identical RFLP patterns.

Isozymes. Isozyme genotypes of the 12 ramets at the six polymorphic loci are presented in table 2. On the basis of their multilocus genotypes, ramets 1, 6, 7, 9, 10, and 12 had unique genetic identities. The remaining 6 ramets were clustered into two distinct groups: ramets 2, 3, 4, and 5; and ramets 8 and 11.

Discussion

This study reaffirms the power of isozymes for genetic analysis in tree populations but also demonstrates the added discrimination that can be achieved through the use of molecular markers, such as RFLP's. Isozyme analysis was able to resolve the identities of 6 of the 12 ramets; the other 6 ramets were classified into 2 groups of 2 and 4 ramets each. RFLP analysis, however, resolved identities for 9 of the 12 ramets; ramets 3, 4, and 5 had identical RFLP patterns for all probes. On the basis of the results of isozyme and RFLP analysis we conclude that ramets 1, 2, 6, 7, 8, 9, 10, 11, and 12 are genetically unique. Ramets 3, 4, and 5 appear to be from the same clone, but further analysis may reveal unique genetic identities.

Table 2-Isozyme genotypes of 12 longleaf pine clone-bank ramets

	Isozyme locus						
Ramet	*Skd*	Pgi-2	Ugpp	Pgm-1	Mdh	6-Pgd	Multi-locus genotype
1	22	11	11	12	11	11	A
2	11	11	12	11	11	12	B
3	11	11	12	11	11	12	C
4	11	11	12	11	11	12	C
5	11	11	12	11	11	12	C
6	12	12	12	11	11	11	D
7	11	11	11	11	12	12	E
8	11	11	11	11	11	12	F
9	11	12	12	11	11	11	G
10	14	11	11	11	11	12	H
11	11	11	11	11	11	12	I
12	11	11	11	12	11	11	J

Numbers refer to diploid allozyme genotypes at each locus.

Information obtained from this genetic study enabled the USDA Forest Service's Francis Marion Seed Orchard to make practical management decisions regarding genetic resources. Clonal material from the genetically differentiated longleaf pine ramets was included in the restoration of the clonal orchard that had been damaged by Hurricane Hugo. In addition, scions from the differentiated longleaf pine ramets were grafted into a new clone bank, and open-pollinated seed has been collected for future use.

Summary

This study demonstrates the high level of genetic discrimination that can be achieved using just a small number of RFLP probes and restriction enzymes. The genetics laboratory at IFG is attempting to identify additional DNA probes that reveal substantial levels of genetic variability in the tree populations. If such diagnostic probes can be identified, then a cost-effective method for genotyping in tree populations could be devised. The Francis Marion Seed Orchard has demonstrated a useful application of information obtained from analysis with RFLP markers for the purpose of genetic resource management.

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