COMPARISON OF RAPD LINKAGE MAPS CONSTRUCTED FOR A SINGLE LONGLEAF PINE FROM BOTH HAPLOID AND DIPLOID MAPPING POPULATIONS

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ABSTRACT

Considerable concern has been voiced regarding the reproducibility/transferability of RAPD markers across different genetic backgrounds in genetic mapping experiments. Therefore, separate gametic subsets (mapping populations) were used to construct individual random amplified polymorphic DNA (RAPD) linkage maps for a single longleaf pine (*Pinus palustris* Mill.). A haploid mapping population consisting of megagametophytic DNAs from 88 wind-pollinated seeds of longleaf pine (clone 3-356), and a diploid population including 86 F_1 progeny from a controlled cross [longleaf pine 3-356 (♀) × slash pine H-28 (♂)] were employed. Seventy-one RAPD primers selected for this study identified a total of 137 mapped loci in longleaf pine 3-356 based on amplification of megagametophytic DNAs. Of the 137 loci useful for comparative purposes, 62 loci (45.3%) could not be scored when DNAs of F_1 progeny from the controlled cross were amplified. Of the 75 loci that were scorable, 26 loci (34.7%) were fixed in slash pine H-28, and 49 loci (65.3%) were segregating (42 1:1, and seven 3:1). Comparisons were made using the 49 loci common to both the haploid- and diploid-based maps. The 49 loci allowed us to determine homologous counterparts between maps. Orders were conserved for those groups containing three or more loci. Genetic distance estimates were found to vary considerably, but not in any systematic manner.

Key words: random amplified polymorphic DNAs (RAPDs), *Pinus palustris* Mill., genetic linkage maps, megagametophytic DNA

INTRODUCTION

Linkage studies in the genus *Pinus* have traditionally been conducted with isozyme markers (CONKLE 1981). However, the paucity of available isozyme markers and their low level of polymorphism have precluded the construction of detailed (saturated) genetic maps. Two-dimensional (2-D) electrophoresis and staining of total proteins has significantly increased the number of protein polymorphisms that can be identified (BAHRMAN & DAMERVAL 1989). Using 2-D techniques, GERBER *et al.* (1993) placed 65 protein loci identified in megagametophytic tissues of maritime pine (*Pinus pinaster* Ait.) into 10 groups (3+ loci) and 7 pairs, covering 530 centiMorgans (cM). Karyological studies have revealed that pine species contain 12 similar-sized pairs of homologous chromosomes (SAYLOR 1972; KORMUTAK 1975), and current genome size estimates based on partial genetic linkage data vary from ~1350 cM for maritime pine (PLOMION *et al.* 1995b) to ~3300 cM for longleaf pine (NELSON *et al.* 1994). Although 2-D electrophoretic techniques have significantly increased the number of available protein loci (approximately three-fold over isozyme systems), protein markers alone will not allow for effective coverage of the entire pine genome.

In contrast to protein markers, DNA markers are potentially unlimited in number as they allow direct access to coding and non-coding regions of the genome (SOLLER & BECKMANN 1983). Several methods have been developed to detect DNA polymorphisms. One common approach applies restriction fragment length polymorphisms (RFLPs), which use conserved or variable regions of the genome as DNA probes. While genetic maps based on RFLPs have been constructed for a number of agronomic crop species, progress in long-lived perennial tree species lags behind. The use of RFLPs to map conifer genomes has recently been proposed (NANCE & NELSON 1989; NEALE & WILLIAMS 1991), and is currently underway for loblolly pine

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(*Pinus taeda* L.) where nearly 100 RFLP markers have been developed and are starting to be used for linkage analyses (AHUJA *et al.* 1994; DEVEY *et al.* 1994; GROOVER *et al.* 1994).

Several factors have delayed the widespread use of RFLPs in genetic linkage studies in the genus *Pinus*. The large physical size of pine genomes [2C = 33 to 57 pg, OHRI & KHOSHOO (1986)], can complicate the autoradiographic detection of single-copy genes (NEALE & WILLIAMS 1991; SHATTUCK-EIDENS *et al.* 1992). Due to the long generation times associated with many tree species, there is also a lack of well-defined pedigrees (beyond two generations) with which to work. Possibly as a result of such complications, only a limited number of tree species have been mapped using RFLP markers. Two RFLP -based genetic maps for citrus (DURHAM *et al.* 1992; JARRELL *et al.* 1992), one for apple (WEEDEN & HEMMAT 1993), two for poplar (LIU & FURNIER 1993; BRADSHAW *et al.* 1994), and one for an interspecies cross between peach and almond (FOOLAD *et al.* 1995) have recently been published. In comparison to pines, these species have relatively small genomes [for example; citrus 2C = 1.24 pg (GUERRA 1984) and aspen 2C = 1.6 pg (DHILLON *et al.* 1984)].

A new technique for generating DNA markers, commonly referred to as the random amplified polymorphic DNA (RAPD) technique, the arbitrary primed polymerase chain reaction (AP-PCR), or the DNA amplification fingerprinting (DAF) technique, has recently been developed (WILLIAMS *et al.* 1990; WELSCH *et al.* 1990; CAETANO-ANOLLES et al. 1991). Due to their rapidity and simplicity of detection, RAPDs are making quick advances in genetic mapping possible. The method applies the polymerase chain reaction (PCR) with a short oligonucleotide primer randomly amplifying fragments of template DNA (WILLIAMS *et al.* 1990). The procedure requires only very small amounts of template DNA, and can rapidly amplify sequences that are inherited in a Mendelian fashion. The RAPD procedure holds great promise for quickly placing markers on linkage groups, even for species with large genomes such as conifers (GRATTAPAGLIA *et al.* 1992; TULSIERAM *et al.* 1992).

An advantage of the RAPD technique with conifers is the availability of haploid DNA in the megagametophytic tissue of conifer seeds. The megagametophyte is derived from repeated mitotic divisions of a single meiotic product (USDA 1974), and sufficient DNA for several hundred to several thousand RAPD reactions can be extracted from a single megagametophyte (TULSIERAM *et al.* 1992; NELSON *et al.* 1993). The use of haploid tissues is beneficial because the RAPD technique does not readily differentiate between heterozygotes (+/–; + = band present, – = band absent) and dominant homozygotes (+/+) in diploid individuals. Low- to medium-density RAPD maps have recently been constructed for several conifer species such as white spruce (TULSIERAM *et al.* 1992), Norway spruce (BINELLI & BUCCI 1994), loblolly pine (GRATTAPAGLIA *et al.* 1992), slash pine (NELSON *et al.* 1993; KUBISIAK *et al.* 1995), longleaf pine (NELSON *et al.* 1994; KUBISIAK *et al.* 1995), Turkish red pine (KAYA & NEALE 1995), and maritime pine (PLOMION *et al.* 1995a), using the haploid megagametophyte system.

In contrast to the haploid system, a strategy employing diploid progeny from a specific cross provides data on both parents simultaneously (GRATTAPAGLIA & SEDEROFF 1994; HEMMAT *et al.* 1994). The efficiency of such an approach, however, is limited by the number of loci found to be in testcross configuration between parents (+/– ×–/– or –/– ×+/–). Testcross locus configurations appear to be quite common in matings between highly-heterozygous outcrossed tree species (CARLSON *et al.* 1991; ROY *et al.* 1992), and have been used to construct maps for the parents of crosses between the banana cultivars "SF265" × "Banksii" (FAURE *et al.* 1993), the apple cultivars "Rome Beauty" × "White Angel" (HEMMAT *et al.* 1994), by selfing F_1 progeny of the peach cultivars "NC174RL" × "Pillar" (CHAPARRO *et al.* 1994), *Eucalyptus grandis* × *Eucalyptus urophylla* (GRATTAPAGLIA & SEDEROFF 1994), and longleaf pine × slash pine (KUBISIAK *et al.* 1995).

A comparison of RAPD maps constructed for a single maritime pine tree using megagametophytes from self- and open-pollinated seeds, suggests that framework maps constructed using RAPD markers are repeatable in terms of recombination estimates and marker order (PLOMION *et al.* 1995b). Although RAPD markers appear to be repeatable in terms of their amplification and inheritance using haploid megagametophytic DNAs obtained from the same individual, we know nothing about the transferability/reproducibility of RAPD markers in different genetic backgrounds. In order to examine the effects that genetic background might have on the amplification and inheritance of RAPD markers, we examined the linkage relationship among RAPD loci identified in longleaf pine 3-356 based on their inheritance in both a haploid megagametophyte mapping population (NELSON *et al.* 1994) as well as in a longleaf pine 3-356 (♀) × slash pine H–28 (♂) diploid F_1 mapping population (KUBISIAK *et al.* 1995).

MATERIALS AND METHODS

Plant Material

Megagametophytes of longleaf pine clone 3-356 were

dissected from wind-pollinated seeds, and total DNA prepared as outlined in NELSON *et al.* (1994). Needle tissue from F_1 progenies of a cross between longleaf pine 3-356 (♀) and slash pine H-28 (♂) were collected, and total DNA prepared as outlined in KUBISIAK *et al.* (1995). In total, the haploid mapping population included 88 megagametophytes (80 used in NELSON *et al.* 1994), plus 8 additional megagametophytes), and the diploid population included the 86 F_1 progeny originally used in KUBISIAK *et al.* (1995).

Primer selection and DNA amplification

A total of 71 primers that had previously identified heterozygous loci in longleaf pine 3-356 (using the haploid megagametophyte system (NELSON *et al.* 1994) were selected for this study. Primer DNAs were obtained from either Operon Technologies Inc. (Alameda, CA) or J.E. Carlson (Univ. of British Columbia, Vancouver, B.C., Canada). To ensure that the same RAPD fragments were being scored in both populations, DNAs from 14 F_1 progenies (fourth template set in KUBISIAK *et al.* 1995) were amplified along with DNAs obtained from an additional eight megagametophytes and subsequently separated in the same agarose gel. Amplification of template DNAs followed the protocol outlined in NELSON *et al.* (1994), with the only modification consisting of a doubling of the diploid template DNAs to 6.25 ng per reaction.

Segregation analysis

Linkage analysis was performed on the full haploid data set (original data set plus the additional eight megagametophytes) using the program MAPMAKER /EXP version 3.0 (LINCOLN *et al.* 1992). A two-point linkage criteria or log of the odds (LOD) ratio of 5.0 was used to establish linkage groups, and a three-point or multi-point LOD threshold (LOD >1.5) was used to determine marker order.

RESULTS

Revised haploid-based map

Amplification and separation of both haploid and diploid DNAs in the same agarose gel facilitated the scoring of nine additional RAPD markers not previously included in the haploid data set (NELSON *et al.* 1994). This increased the total number of heterozygous loci available for linkage analyses to 183. The additional megagametophytic data increased the size of the mapping population by 10% (from 80 to 88 megagametophytes). As a result of the additional data, linkage was now suggested between groups A and O, H and P, as well as I and K. An additional linkage group (previously a linked pair, now designated group T) was also formed (Figure 1). Three linked pairs identified in NELSON *et al.* (1994) mapped to terminal locations on three different linkage groups (Figure 2). Current linkage analyses suggest a genetic map consisting of 14 groups and two pairs (162 loci) spanning a total of 2023.3 cM. Compared to the previously published map (NELSON *et al.* 1994), twenty-nine additional markers were mapped, increasing the coverage by 388 cM. Of the 29 additional loci mapped, six were new marker loci and 23 were previously unordered in NELSON *et al.* (1994) as they were found to have two or more likely (LOD < 2.0) positions. As long as inconsistencies in linkage were not detected (*e.g.* a locus could just as likely be placed in two or more distantly separated locations) loci were placed in their most likely position. Although some incorrect marker orders might be declared (especially in the case of tight linkage), this comprehensive approach allowed us to maximize the number of marker loci available for comparative purposes.

Comparison of haploid and diploid-based maps

The 71 primers selected for this study identified a total of 148 heterozygous loci in longleaf pine 3-356 based on amplification of megagametophytic DNAs. Of these 148 loci, 133 loci (89.9%) were grouped into 14 groups (three or more loci) and two pairs, four loci (2.7%) were grouped but could not be confidently ordered (even using relaxed LOD scores <2.0), and 11 loci (7.4%) remained unlinked. Of the 137 loci that could be grouped based on two-point analyses, 62 loci (45.3%) could not be scored when DNAs from F_1 progeny were amplified. Thirty-two of these 62 loci (51.6%) did not consistently amplify from F_1 DNAs, and the other 30 loci (48.4%) could not be assessed due to the presence of slash pine bands of similar molecular weight which precluded accurate scoring. Of the 75 loci that were scorable, 26 loci (34.7%) were fixed (assuming slash pine H–28 to be homozygous band -present at the same locus), and 49 loci (65.3%) were segregating (42 loci segregating 1:1, and seven loci segregating 3:1). Comparisons were made using the 49 loci common to both the haploid and diploid maps.

The 49 loci allowed us to determine homologous counterparts between maps. Seven homologous linkage groups (A, B, C, D, E, N, and O) were characterized by three or more loci (Figure 3). Five groups and one linked pair (F, G, H, I, J, Lp3) were found to have two markers in common (Figure 3). Single loci in the linked pairs Lp2 and Lp5 suggested homology with groups Q

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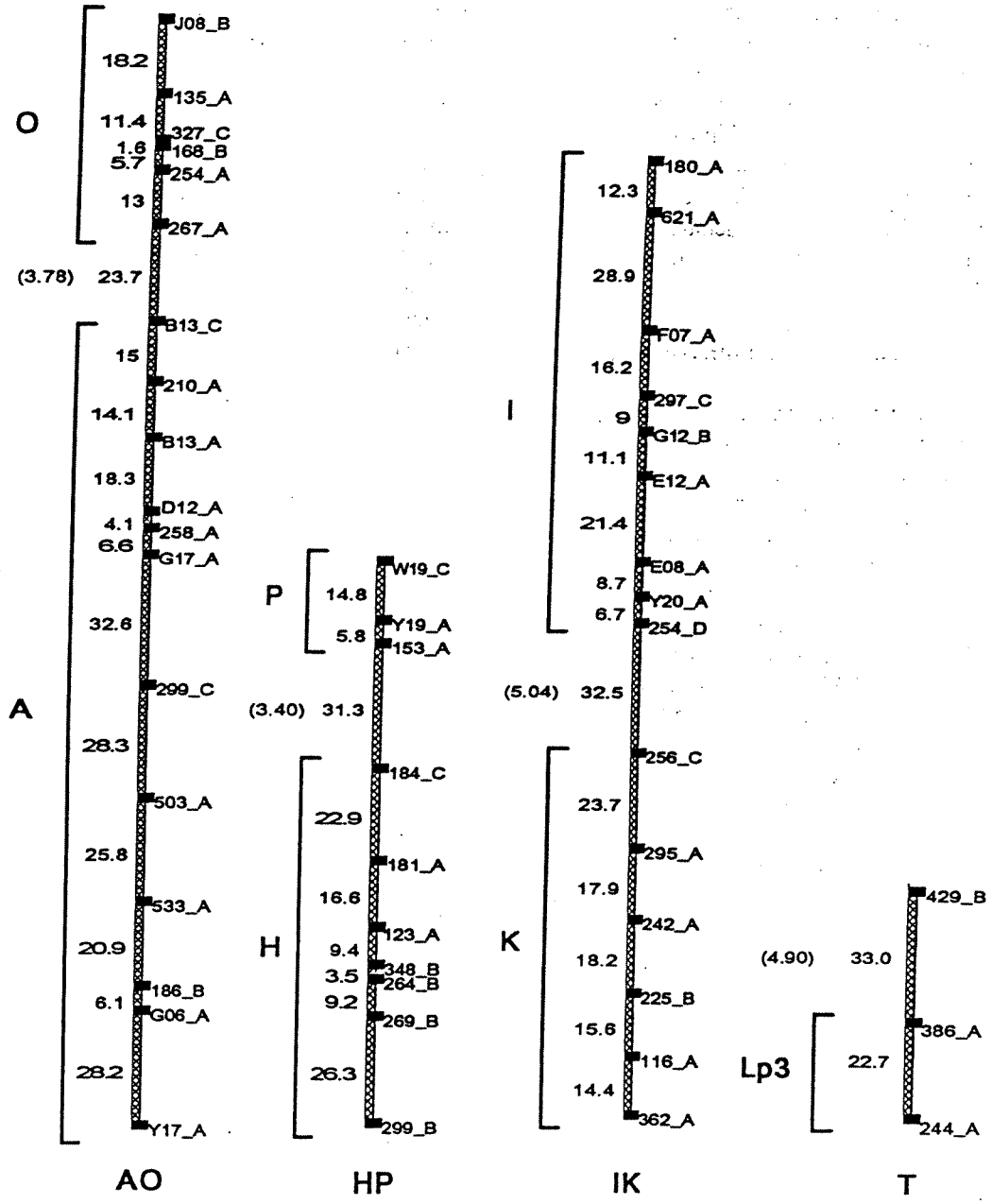

Figure 1 Convergence of linkage groups A and O, H and P, I with K, and an additional linkage group (previously a linked pair, now designated group T) identified in NELSON *et al.* (1994), as a result of additional megagametophyte data. Primer names and Haldane centiMorgan (cM) distance are provided. Log of the odds (LOD) ratios between groups are displayed in parentheses.

and S (Figure 3). Seven of the comparable loci were found to segregate in a 3:1 Mendelian ratio in the longleaf pine (♀) × slash pine (♂) F_1 mapping population, therefore, genetic distances could not be calculated using MAPMAKER/EXP. However, their association with markers on homologous groups was confirmed by chi-square (χ^2) analyses (KUBISIAK *et al.* 1995). Three loci identified in the F_1 mapping population were grouped using two-point analyses, but could not be confidently ordered. In total, 31 loci were placed on each map and used for comparative purposes. These 31 loci allowed us to align 10 homologous linkage groups (designated superscript H and D for haploid- and diploid-based linkage groups; Figure 3).

Although the order of loci in common between the two maps was found to be conserved, there appeared to be some fairly large discrepancies in terms of genetic distance estimates between syntenic loci (Figure 3). A

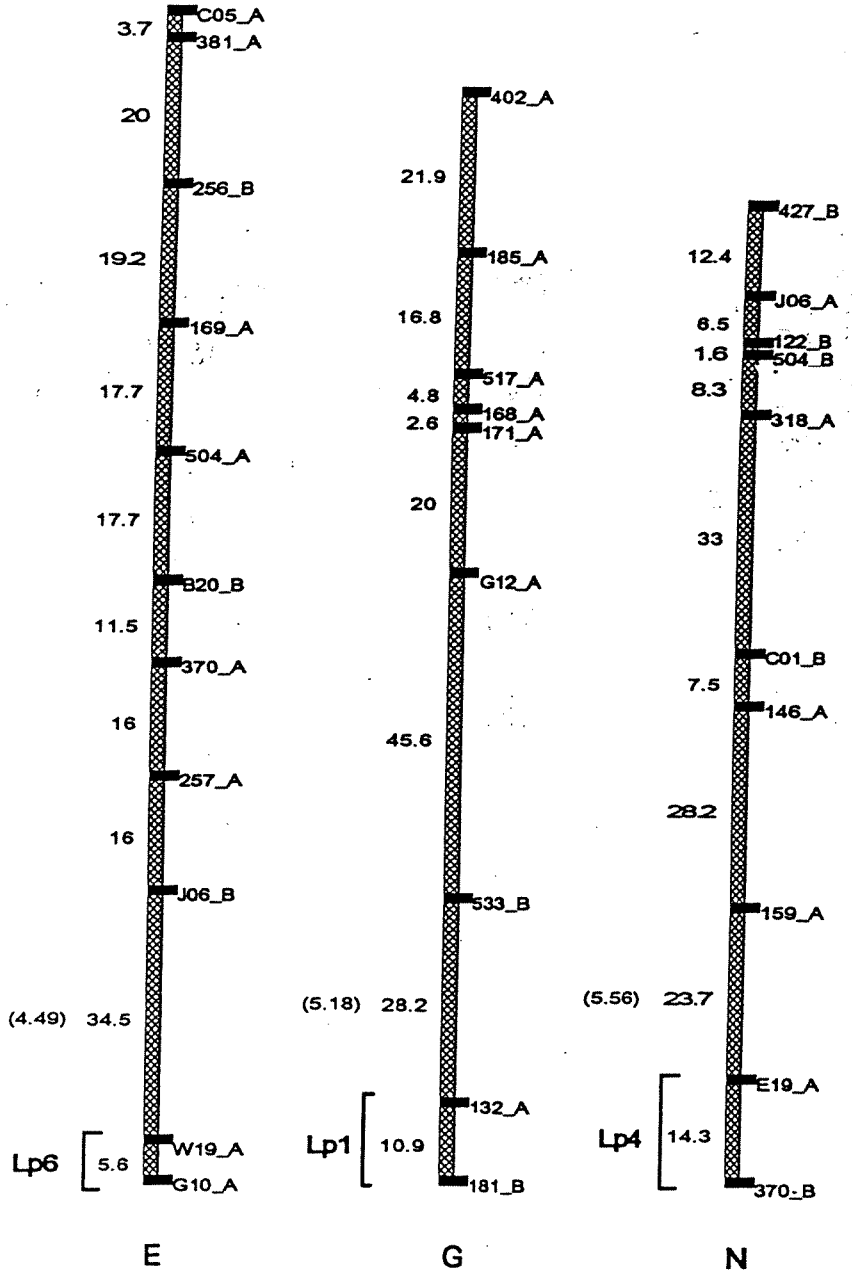

Figure 2 Convergence of linkage groups and linked pairs E and Lp6, G and Lp1, and N with Lp4 identified in NELSON *et al.* (1994), as a result of additional megagametophyte data. Primer names and cM distances are provided. LOD scores are displayed in parentheses.

paired comparison test (paired t-test) was utilized to determine whether the difference in genetic distance estimates between syntenic loci was significantly different from zero. In other words, this test was used to determine if one gametic subset was consistently over-estimating or under-estimating genetic distance estimates. The paired comparison test resulted in an insignificant t-value ($t_{0.025,19} = -0.35803$). Therefore, no evidence exists to indicate that the two gametic subsets produced consistently (significantly) different genetic distance estimates.

DISCUSSION

Overall, a fairly large number of loci that mapped in the haploid megagametophyte population [62 of 137 loci (45.3%)] could not be reproducibly amplified from DNAs obtained from diploid F_1 progenies. Such a low

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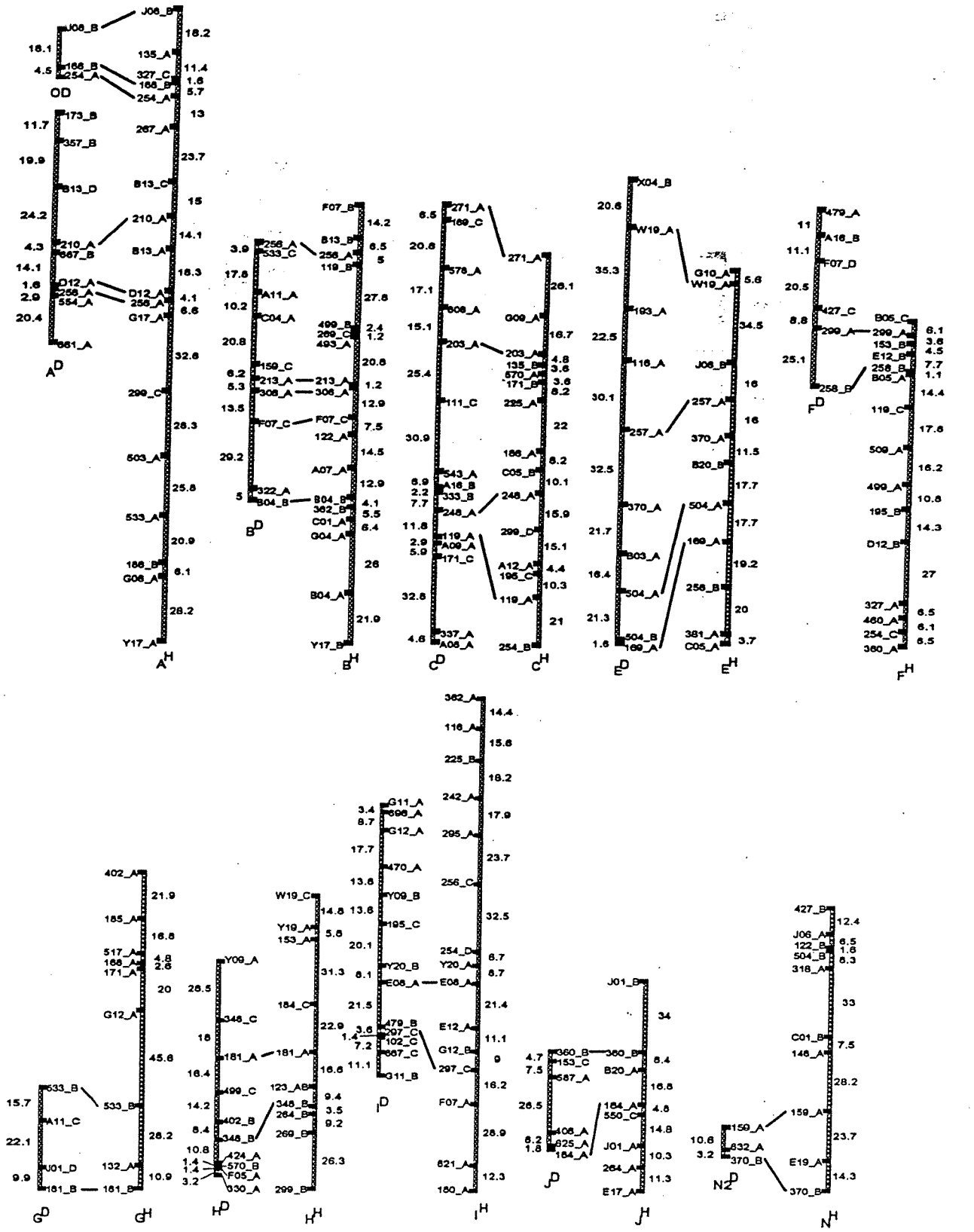

Figure 3 Longleaf pine 3-356 haploid-diploid comparison map. Linkage groups designated with a superscript D (e.g. A^D) indicate diploid-derived groups, those indicated with a superscript H indicate haploid-derived groups. Primer names and cM distances are provided. Markers in common between maps are indicated with heavy lines. Loci segregating 3:1 in the diploid mapping population are not displayed.

number of comparable loci can be partially explained by several observations. First, RAPD fragments of high molecular weight (>2000 bp) or low molecular weight (<400 bp) were often nonreproducible in the diploid DNAs. PLOMION *et al.* (1995a) also noted nonreproducibility of RAPD fragments in these size classes, even within subsets of segregants from the same individual. Secondly, some faint RAPD fragments were not scorable in the diploid DNAs. This result is most likely due to additional fragments simultaneously being amplified in DNAs derived from diploid versus haploid tissues. The presence of additional priming sites enhances primer competition during the initial cycles of amplification (NEWBURY & FORD-LLOYD 1993; CAETANO-ANOLLES *et al.* 1994). Thirdly, additional bands amplified from slash pine (the other genomic complement) prevented accurate scoring of some loci of interest, as they occurred at similar molecular weights. In general, these results suggest that the transferability/reproducibility of RAPD markers in different genetic backgrounds may be limited to only very intensely amplified polymorphisms.

Although there was no evidence to suggest that the two gametic subsets derived from longleaf pine produced consistently different genetic distance estimates, some of the discrepancies in distance estimates were rather large (Figure 3). In this study, the two linkage maps for longleaf pine were not constructed from the megagametophytes and embryos (plants) contained within the same seeds. Such an approach would provide the same genetic distance estimates (given no missing or misclassified data), as the megagametophytic tissue and embryo contained in a single seed receive the same genetic complement from the maternal parent (USDA 1974). As a result, variation associated with random gametic sampling might partially explain such differences in genetic distance estimates (SALL & BENGTSSON 1989; SALL & NILSSON 1994).

Factors that distort segregation can also influence genetic distance estimates. It is obvious that misclassifications may disturb the results of linkage studies. Misclassifications systematically lead to a positive bias in map distances (SHIELDS *et al.* 1991; LINCOLN & LANDER 1992). In mapping experiments using DNA markers, the risk of misclassification is especially pronounced because large data matrices are produced, subsequently interpreted, scored, and entered into a computer in several steps. Furthermore, for RAPD data where reproducible results require extreme experimental care, misclassifications must seriously be considered. WEEDEN *et al.* (1992) reported error rates as high as 10% in segregating pea and apple populations, but with extra care, such as using only high quality template DNA and scoring only clear polymorphisms they were able to keep their error rate below 4%. However, an error rate of 4% can result in substantial differences in genetic distance estimates, especially with limited sample sizes (KUBISIAK *et al.* 1993; SALL & NILSSON 1994).

Segregation distortion at some loci may result due to a reduced viability of some of the resulting phenotypes. Significant deviations from normal Mendelian ratios are detected with the χ^2 test. It has been demonstrated that when only one locus shows distorted segregation the expectation of the recombination estimate is not biased; only the variance of the estimate is influenced (BAILEY 1961; OTT 1991; RITTER *et al.* 1990). However, if more than one locus is experiencing distorted segregation as a result of reduced viability then recombination estimates are influenced. Two linkage groups identified in the diploid map (C and D1) contained regions to which multiple distorted markers mapped (KUBISIAK *et al.* 1995). This might explain the large discrepancy in distance estimates between comparable loci in group C.

Although marker order was conserved between the two maps, in general, the low number of reproducible /transferable markers suggests that RAPDs may not be as efficient as other types of markers [for example RFLPs (AHUJA *et al.* 1994)] for genetic mapping studies. Further refinements in the technique will no doubt be required to increase the reproducibility /transferability of RAPDs across different genetic backgrounds. Conversion of RAPD markers to sequence characterized amplified regions (SCARs) may provide such a means (PARAN & MICHELMORE 1993). The specificity of SCAR primers would greatly simplify the kinetics of the PCR reaction (*i.e.* eliminate the problems associated with primer competition), and result in much simplified product profiles in the agarose gel (amplification of only a single fragment).

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