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Molecular genetics of growth and development in *Populus*. III. A genetic linkage map of a hybrid poplar composed of RFLP, STS, and RAPD markers

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Abstract We have evaluated three DNA-based marker types for linkage map construction in *Populus*: RFLPs detected by Southern blot hybridization, STSs detected by a combination of PCR and RFLP analysis, and RAPDs. The mapping pedigree consists of three generations, with the F_1 produced by interspecific hybridization between a *P. trichocarpa* female and a *P. deltoides* male. The F_2 generation was made by inbreeding to the maximum degree permitted by the dioecious mating system of *Populus*. The applicability of STSs and RAPDs outside the mapping pedigree has been investigated, showing that these PCR-based marker systems are well-suited to breeding designs involving interspecific hybridization. A *Populus* genome map (343 markers) has been constructed from a combination of all three types. The length of the *Populus* genome is estimated to be 2400-2800 cM.

Key words Cottonwood · Tacamahaca · Aigeiros *Salix* · Genome

Abbreviations RFLP restriction fragment length polymorphism · STS sequence-tagged site · PCR polymerase chain reaction · RAPD random amplified polymorphic DNA

Introduction

Genetic linkage maps of moderate resolution have been developed recently for many plant species important for agriculture, forestry, and/or basic research. The information gained from plant genome mapping has been substantial, especially in the study of genome structure and evolution (Helentjaris et al. 1988; Bonierbale et al. 1988; Whitkus et al. 1992) and in the identification of Mendelian components of quantitative genetic variation (Paterson et al. 1988, 1991; Lander and Botstein 1989; Keim et al. 1990; Stuber et al. 1992). With these same goals in mind, we have constructed a genetic linkage map for the genome of a hybrid *Populus*, a model forest tree.

The genus *Populus* is comprised of about 30 species of poplars, cottonwoods, and aspens. *Populus* has many attributes of a model system in forest tree biology. Chief among these is that breeding and genetics can be brought to bear upon all aspects of *Populus* biology, including anatomy, physiology (Hinckley et al. 1989), pathology (Thielges and Adams 1975; Pinon 1992), reproductive biology (Villar et al. 1993), ecology (Keim et al. 1989), and molecular biology (Parsons et al. 1989; Davis et al. 1991; Coleman et al. 1992; Subramaniam et al. 1993). The ease of vegetative propagation of individual *Populus* genotypes has contributed to the standardization of genetic materials used in these various disciplines, permitting the direct comparison of experiments carried out at different times in different places and serving many of the same roles as recombinant inbred lines in crop and model herbaceous plants (Burr et al. 1988; Reiter et al. 1992).

Both traditional and molecular genetic manipulations are simpler in *Populus* than in most forest trees. Sexual propagation may be carried out in the greenhouse with abundant seed set in 6–8 weeks. The chromosome number of all species is identical (2n = 38), and the nuclear genome is relatively small (2C = 1.2 pg; Dhillon 1987; Bradshaw and Stettler 1993). Transgenic *Populus* can be produced with a variety of methods (Fillatti et al. 1987).

Perhaps most important for the present study, interspecific hybrids can be produced from many species

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pairs (Stettler et al. 1980), and these hybrids are generally fertile. The high level of genetic polymorphism between species results in substantial segregating variation in the F_2 generation, facilitating genome mapping. Since interspecific hybrids are the most commercially important forms of *Populus* in many parts of the world (Zsuffa 1975), such a map is likely to be useful for understanding the genetic basis of the heterosis observed in the F_1 generation (Zsuffa 1975; Heilman and Stettler 1985).

We have evaluated three DNA-based marker types for their utility in *Populus* genome mapping in an F_2 progeny of interspecific hybrids: RFLPs detected by Southern blot hybridization, sequence-tagged sites (STSs; Olson et al. 1989) detected by a combination of PCR and RFLP analysis, and RAPDs (Williams et al. 1990). The applicability of STSs and RAPDs outside the mapping pedigree has also been investigated. A *Populus* genome map has been constructed from a combination of all three marker types.

Materials and methods

The mapping pedigree

Our three-generation *Populus* mapping pedigree (Bradshaw and Stettler 1993, 1994) was founded by interspecific hybridization between a female *P. trichocarpa* (T; clone 93-968) and a male *P. deltoides* [D; clone 59-129-17 (also known as ILL-129)] to produce F_1 hybrid (T × D) family 53. The F_2 (TD × TD; family 331) resulted from sibmating the female 53-246 and the male 53-242. Approximately 350 F_2 trees in this family are maintained at Washington State University Farm 5 in Puyallup, Washington.

RFLP probes and Southern blot hybridization

DNA was extracted from *Populus* leaves and Southern blot hybridizations were carried out essentially as reported previously (Bradshaw and Stettler 1993). The RFLP probes used for linkage mapping are described in detail in Table 1. Plasmid clones of *Populus* genomic DNA were given the prefix "pPOP".

STS determination, PCR amplification, and RFLP analysis

The terminal sequence of cloned RFLP probes was determined (Bradshaw and Stettler 1994). On the basis of STS data, primers suitable for amplification of the cloned locus were chosen using OLIGO (Rychlik and Rhoads 1989). Primer selection criteria were approximately equal T_m for both primers of about 55 °C, minimal internal duplex formation, and minimal complementarity between primers. PCR was carried out in a final volume of 10 µl in 10 m*M* Tris-HCl pH 8.3/50 m*M* KCl/2.5 m*M* $MgCl_2$/0.01% gelatin with 20–200 µ*M* of each of the 4 dNTPs, 0.5 U Amplitaq (Perkin-Elmer Cetus), 100 ng of each primer, and 10-50 ng of *Populus* genomic DNA or 10 pg of plasmid DNA. Amplifications were performed in COY or MJ Research thermocyclers. Many of the *Populus* and *Salix* leaf samples were collected from the University of Washington Arboretum or campus. *Nicotiana tabacum* cv 'Xanthi' was a gift of Milt Gordon, *Arabidopsis thaliana* was a gift of Liz van Volkenburgh, and *Dendrobium nobile* was a gift of Doug Ewing.

RAPD markers

Primers for RAPD amplification were obtained from Operon Technologies, and used according to Williams et al. (1990), except that reaction volumes were scaled down to 10 µl with 1–5 ng of *Populus* genomic DNA as template.

Linkage analysis and map construction

MAPMAKER 3.0 (Lander et al. 1987; Lincoln et al. 1992) was used to analyze linkage data. All known triploid offspring (Bradshaw and

Table 1 RFLP probes used for mapping in *Populus*. The locus name (anon. = anonymous), source tree from which the probes were cloned (referenced by the US/WSU code composed of the family number and clone number), taxon, and the restriction enzyme used for cloning genomic DNA fragments are given

Probe number	Locus	Source clone	Taxon[a]	Cloning enzyme	Genomic or cDNA	Fragment length	Number tested	Number single- or low-copy nuclear probes	Percentage single- or low-copy nuclear probes	Reference
1–700	anon.	50–188	T × D	*Pst*I	Genomic	Random	93	56	60%	This paper
751–818	anon.	93–968	T	*Pst*I	Genomic	2–3 kb	60	40	67%	This paper
831–898	anon.	93–968	T	*Xba*I	Genomic	2–3 kb	35	21	60%	This paper
899–1016	anon.	93–968	T	*Pst*I	Genomic	2–3 kb	53	25	47%	This paper
1017–1343[b]	anon.	93–968	T	*Pst*I	Genomic	2–3 kb	258	186	72%	This paper
	*win*8	11–11	T × D		cDNA		1	1		Parsons et al. 1989
	*win*3	11–11	T × D		cDNA		1	1		Hollick and Gordon 1993
	*win*4	11–11	T × D		cDNA		1	1		Parsons et al. 1989
	*win*6	11–11	T × D		Genomic		1	1		Davis et al. 1991
	4CL	11–11	T × D		cDNA		1	1		Douglas et al. 1992
	PAL	11–11	T × D		cDNA		1	1		Subramaniam et al. 1993
	ADH	–	D		cDNA		1	1		D. E. Harry personal communication
	BSP	–	D		cDNA		1	1		Coleman et al. 1992
	*chi*D	11–11	T × D		Genomic		1	1		J. M. Davis personal communication
Total							508	337		

[a] T, *Populus trichocarpa*; D, *P. deltoides*; T × D, F_1 hybrid
[b] These probes were pre-screened with radiolabelled total *Populus* DNA as described in text

Stettler 1993) were excluded from the linkage analysis. Genotypic data were coded as though they were derived from inbred lines represented by the parental species *P. trichocarpa* and *P. deltoides*. Only three genotypes were recognized: *TT* homozygous, *TD* heterozygous, and *DD* homozygous. Although in many cases more than two alleles were segregating in the F_2 due to heterozygosity in the parental trees (and the resulting allelic differences in the male and female F_1s mated to form the F_2), this additional information was not used for estimation of recombination fractions. Provisional linkage groups were identified at a LOD threshold of 4.0, and 1 or 2 anchor loci chosen from each of the linkage groups for subsequent mapping. The remaining loci were assigned to linkage groups using an initial LOD of 4.0 and a relaxed threshold LOD of 3.0. Within linkage groups, locus order was determined with a starting LOD of 2.0 and a minimum LOD of 1.3. Loci ordered with these criteria formed the framework of each linkage group. Additional loci usually mapped to each linkage group, but could not be ordered unambiguously at a LOD threshold of 1.3. Current genotypic data, marker, and map information may be obtained through the Poplar Molecular Network via gopher (poplar1.cfr.washington.edu) or by contacting the PMN database manager (editor@poplar1.cfr.washington.edu).

Genome length estimation

The recombinational length of the *Populus* genome was estimated using the method described by Hulbert et al. (1988).

Results

RFLP probes for Southern blots

RFLP probe numbers, sources, proportion of single- and low-copy nuclear probes, and other details are given in Table 1. The genetic locus recognized by a probe was given the designation "P" followed by the probe number. In addition to the single- and low-copy number probes, one probe that gives an intense signal on Southern blots but segregates as a nuclear marker (P771) was mapped. As is the rule in plants, the vast majority of *Populus* RFLPs resulted from insertion/deletion polymorphisms as opposed to single-base substitutions (McCouch et al. 1988). All RFLPs were inherited in a codominant manner.

At many RFLP loci there was severe segregation distortion, generally resulting in an excess of heterozygotes. The extent of segregation distortion in the mapping pedigree, and an explanation for distortion at one locus, has been documented (Bradshaw and Stettler 1993b).

Of the 232 RFLP probes used for mapping in the F_2, 25 (11%) showed clear evidence of hybridization to more than one polymorphic locus; 23 revealed two loci and 2 recognized three loci (Fig. 1). Duplicated loci were named after the primary locus, with an extension to signify the degree of duplication (e.g. P1274, P1274.2, P1274.3). In cases where there was a distinct difference in the intensity of the signal on Southern blots, the darker locus was designated as the primary locus (e.g. P757), and the less intense hybridization pattern was attributed to the duplicated locus (e.g. P757.2).

Heterozygosity in the parental *P. trichocarpa* and *P. deltoides* trees was estimated by calculating the proportion of probes ($n = 100$) showing at least one distinct band in a parental genome that was missing in at least one of the F_1 hybrids. The values obtained were 30% for *P. trichocarpa* 93-968 and 15% for *P. deltoides* ILL-129.

STS-based markers

STS data were obtained for 89 of the cloned *Populus* genomic DNA fragments found to be single- or low-copy and polymorphic by Southern blot hybridization.

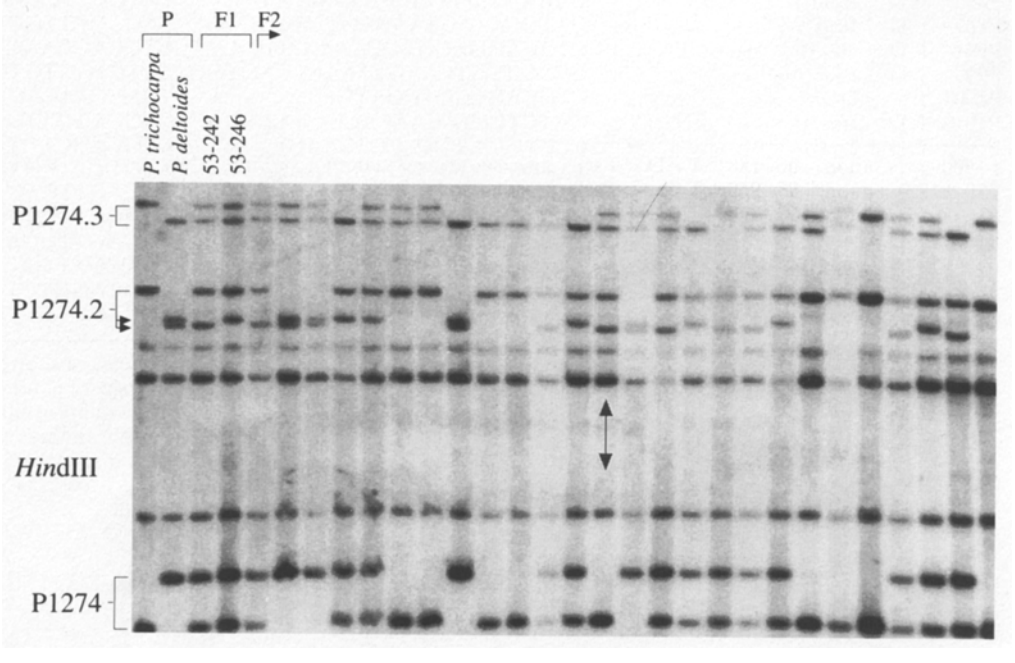


Fig. 1 Segregation of RFLPs in the *Populus* mapping pedigree detected by Southern blot hybridization. Three loci are recognized by the probe 1274: P1274, P1274.2, and P1274.3. The RFLP pattern from the parental *P. trichocarpa* and *P. deltoides*, the F_1 clones 53-242 and 53-246, and a panel of 26 F_2 offspring are shown following digestion with *Hin*dIII. Both parents are homozygous for alternate alleles at P1274 and P1274.3, but the *P. deltoides* parent is heterozygous at P1274.2 (*two arrows* in *left margin*) and the F_1s inherit alternate *P. deltoides* alleles. P1274 and P1274.2 are closely linked, but a single recombinant between them is indicated by the *double-headed vertical arrow* in *lane 17*; this F_2 is homozygous for the T allele at P1274 and heterozygous at P1274.2

Primer pairs were synthesized for 44 genomic STSs. Each primer pair was tested with several template types to determine its utility for genome mapping in *Populus*. PCR conditions were held constant across all template types. First, the primer pair was tested for its ability to direct amplification of a product from the plasmid clone from which the STS data were obtained. Because of the low complexity of the plasmid DNA and the acceptable quality of the DNA sequence data, all 44 primer pairs worked well at this stage. Next, each primer pair was checked using the genomic DNA from which the clone was derived (usually *P. trichocarpa* 93-968); 29 (66%) of the primer pairs were able to direct strong amplification of a PCR product of the expected size (based on the fragment amplified from the plasmid clone; Table 2).

Primers were then examined for their ability to direct amplification of homologous sequences from the *P. deltoides* male parent of the mapping pedigree in order that codominant PCR-based markers useful for mapping in the F_2 could be identified. The results of these tests for amplification, along with key aspects of the PCR conditions used for amplification from each primer pair, are given in Table 2. Despite the fact that *P. trichocarpa* and *P. deltoides* are in two different taxonomic sections of the genus *Populus* (Tacamahaca and Aigeiros, respectively; Eckenwalder 1977), 26/29 (90%) of the primer pairs that were successful in *P. trichocarpa* were able to amplify a fragment from *P. deltoides* (Table 2). The other 3 primer pairs amplified only from the *P. trichocarpa* parent, and these STS markers were inherited in a dominant manner. Only 8% (2/26) of the codominant STS-based polymorphisms were detectable as length variation (amplified sequence polymorphisms; ASPs) in the amplification products observed in the parents of the mapping pedigree, in contrast with what was found for RFLPs detected by Southern blot hybridization. One of these ASPs, that for the *win3* gene (Bradshaw et al. 1989; Hollick and Gordon 1993), was predicted in advance of primer synthesis based on DNA sequence information from the cloned

Table 2 STS markers developed in *Populus*

Locus	Amp.[a]	Polymorphism[b]	Segregation[c]	Linkage group[d]	Forward primer	Reverse pimer	PCR conditions
P164	T,D	–	na	na	CTGCAGGATGGGTATCATC	CTGCAGTGATATCAATGTC	1[g]
P753	T	DOM	na	na	GTTGATGGTTTTGGAAGG	CTGCAGGTGAAAAATCGT	1
P754	T,D	*Mbo*I	+	P	TTGGCAAGACAATAGGCC	CTGCAGGCGGAACTGTAC	1
P755	T,D	*Hae*III	+	C	AATTTAGCTGATAGGTTTTTTC	AGGGCTGATTTTTCTTCG	2[h,l]
P757	T,D	*Hae*III	+	P	TGGCAGCAAGAAAGAATG	TGCAGGATCAATTCCCTC	1
P762	T,D	*Sca*I	nd	na	TATGCTTGGAATGACATGGC	CTGCAGCCTGCCTGTGTC	3[i]
P763	T,D	–	na	na	TGCAGTACCAAACATAGAAAGGG	TGCAGAATAGATGGCTAATGGC	1
P767	T,D	*Hae*III	+	T	CTGCAGGCTAATGTACCCCTG	CTGCAGAAGAGGAACTTCACGA	1
P770	T,D	*Dde*I	–	na	CCAGTTTTTCGTTGTGGCA	TGGGTCCATAAGCATGCAAG	1
P771	T,D	*Hin*dIII	–	na	GCGCTCCGGGCATCG	TGCAGGTCGGCAATCG	4[j,l]
P781	T,D	*Hae*III	+	H	TGCAGGACTAGCATTGGAACTC	CTGGGAGGCAACTATCCAAAAG	5[k]
P783	T,D	*Msp*I	–	na	CCGGTCAAGCTTATGATTTCTG	ATGGGCATACGAGCTTTTGG	3
P800	T,D	*Msp*I	+	none	CCAGAATTCCATTGAAGATTGC	GCACTGCTACATCAGCTTGAGG	1
P832	T,D	*Msp*I	nd	na	AGGAGAATCGTGTTGGCAGC	CTAGAATGAAGATTGCTGGCAG	1
P849	T	DOM	na	na	TATGTGATGAAAGCCCGTCC	CCGACCAATAAACTTTTTCAAACC	1
P856	T,D	*Hin*dIII	+	I	GAAAAAAGTTTGCCCAGCAT	CATGCTTCATTTGATGCAATTG	1
P869	T,D	*Dra*I	+	B	GTTGCATTGTAACAGTCAGAGTGC	TAGAGCTTGCATTCATCATTGC	1
P991	T,D	nd	na	na	CTGCAGGACGTGCAAAAAGA	CTGCAGCAAATTAACAAAAGCC	2
P993	T,D	*Rsa*I	+	G	GCAGTTGTCAACATAAATGAGTGG	AATCTGGTGTCCATGTAACCATG	5
P1004	T	DOM	na	na	CTGCAGCCGTCGATGCTA	TCAGCAACAGGAAGGACGAA	1
P1010	T,D	*Hae*III	+	X	GATCTCCTCCAACACCAGAAAA	CTGCAGGTTCTGTTGTCCCG	1
P1013	T,D	*Ssp*I	+	W	CCCTGCAACAAATGGAACT	CTGCAGCTTCTTTTTGAGAAAA	1
P1018	T,D	ASP	+	E	TATTGTTGGTGGAGCTAACATG	AAGGGCGATGTAAATGAAAAAT	1
P1027	T,D	*Bcl*I	+	R	GTAGCTCAGCAAAAGAATTCGC	TGCAGTTCACTTCAGGGTCAA	1
P1046	T,D	*Bcl*I	+	F	CGAATCCTCTTGGAATGAGATC	GCAGCTCCCTTTAACTTTATCG	2
P1054	T,D	*Alu*I	+	B	CTACTGCCGCTTCCGAATTAAG	TGCAGTTGGAAACACTTACTCG	1
P1079	T,D	*Hin*dIII	–	E[e]	CTGCAGTGGCTTTCACCTTC	TGCAGATTGTACAGGGCTGG	1
P1086	T,D	*Dde*I	+	J	AGCTTGGGCTTATGCCATTC	CTGCAGGTCTCTTGAACCTGG	1
win3	T,D	ASP	na[e]	AB	CCCGAAGTGTCCAGAGC	CCCACTCAAATAGTCTAC	4

[a] Amp indicates whether an amplification product was generated from the *Populus trichocarpa* (T) and *P. deltoides* (D) parents of the mapping pedigree

[b] The restriction enzyme used to generate STS/RFLPs in the mapping pedigree is indicated. DOM, dominant marker, amplified only from the *P. trichocarpa* parent of the mapping pedigree; ASP, amplified sequence polymorphism (see text); –, not polymorphic; nd, not done

[c] Segregation data were compared with those from Southern blot hybridizations. +, segregated according to expectation; –, did not segregate as expected, na, not applicable

[d] Linkage group assignment corresponds to the map in Fig. 4 and to smaller linkage groups not shown

[e] *win3* was not mapped by Southern blot hybridization

[f] A locus not corresponding to either P1079 or P1079.2 was identified by this pair of primers, and was designated P1079.3

[g] 30 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 2 min

[h] 5 cycles of 94 °/15 s, 55°/15 s, 72 °/2 min; 30 cycles of 94 °/15 s, 50°/15 s, 72 °/2 min

[i] 5 cycles of 94°/15 s, 60°/15 s, 72°/2 min; 30 cycles of 94°/15 s, 55°/15 s, 72°/2 min

[j] 30 cycles of 94 °/15 s, 50 °/15 s, 72 °/2 min

[k] 30 cycles of 94 °/15 s, 60 °/15 s, 72 °/2 min

[l] [dNTPs] = 20 μM

homologous loci from *P. trichocarpa* and *P. deltoides*. Because of the general lack of ASPs, most STS-based markers required restriction enzyme digestion of the amplification product to reveal polymorphisms; these were termed STS/RFLPs. As many as 30 restriction enzymes were used in the search for polymorphisms between the parents of the mapping pedigree, but eventually a polymorphism was found in 21/23 (91%) of the markers examined (Table 2). A typical pattern of segregation for an STS/RFLP marker is shown in Fig. 2. Twenty codominant STS/RFLP and STS/ASP markers were mapped in the F_2 to verify concordance with the original Southern blot mapping data; 16 (80%) mapped as expected, but 4 (20%) gave segregation data that differed from expectation (Table 2). Thus, the overall success rate in conversion of STS data to codominant markers in the interspecific mapping pedigree can be estimated as the fraction of primers able to amplify from both *P. trichocarpa* and *P. deltoides* (26/44; 59%) multiplied by the level of polymorphism (ASPs and RFLPs; 23/25; 92%), multiplied by the proportion of markers segregating as predicted from the Southern blot data (16/20; 75%), for a product of 41%.

Finally, 20 primer pairs were used with a panel of DNA templates made from angiosperms of varying relatedness to *P. trichocarpa*. Typical species from the three most commercially important sections of the genus *Populus* were included: *P. trichocarpa* from the Tacamahaca section, *P. deltoides* from the Aigeiros section, and *P. tremuloides* from the Leuce section (Eckenwalder, 1977). A willow, *Salix matsudana* var 'Tortuosa', was used to represent the other large genus (with *Populus*) in the family Salicaceae. Two herbaceous dicotyledonous species, *Nicotiana tabacum* and *Arabidopsis thaliana*, and a monocot (orchid), *Dendrobium nobile*, were also tested (Table 3). To determine whether the PCR amplification products from the different angiosperm species were homologues of the *P. trichocarpa* amplicon or artifacts arising from mispriming, Southern blot hybridizations were performed using the original cloned sequences from *P. trichocarpa* as probes. Autoradiograms made after washing the blots at relatively low stringency (calculated T_m −20 °C) showed that all of the amplified fragments observed in the Tacamahaca, Aigeiros, and Leuce sections of *Populus* and all but 1 (P757) observed in the *Salix* species appear to be authentic homologues; however, the occasional visible bands amplified from the herbaceous angiosperms are likely to be the result of mispriming, with the exception of P164 (Fig. 3; Table 3).

RAPD markers

One hundred and eighty commercial available primers (Operon Technologies; primer sets A-I) were screened for their ability to detect polymorphisms between the parents of the mapping pedigree. Because the parents are themselves highly heterozygous, to use the F_2 segregation data in MAPMAKER 3.0 it was necessary to verify that both F_1s had inherited the expected bands to assure an F_2 pattern of segregation (3:1 in the absence of segregation distortion). Other possible patterns of RAPD inheritance include pseudotestcross (1:1) segregation of the marker (in the case where only one of the F_1s inherits the band due to parental heterozygosity) or the absence of the marker in the F_2 progeny (in the case where neither of the F_1s inherits the band due to parental heterozygosity). Sixty-three primers were used to determine the F_2 genotypes at 111 loci. None of the markers appeared to be inherited in a codominant manner.

The parental *P. trichocarpa* 93-968 and *P. deltoides* ILL-129, along with the two F_1s, 53-242 and 53-246, were screened at 230 presumptive loci (RAPD bands) for heterozygosity. The female *P. trichocarpa* is obviously heterozygous at 27% of the loci examined; the corresponding value for the male *P. deltoides* parent is 22.5%. The estimates of heterozygosity in the parental *P. trichocarpa* and *P. deltoides* are biased downward since only two F_1s were checked for correspondence of bands

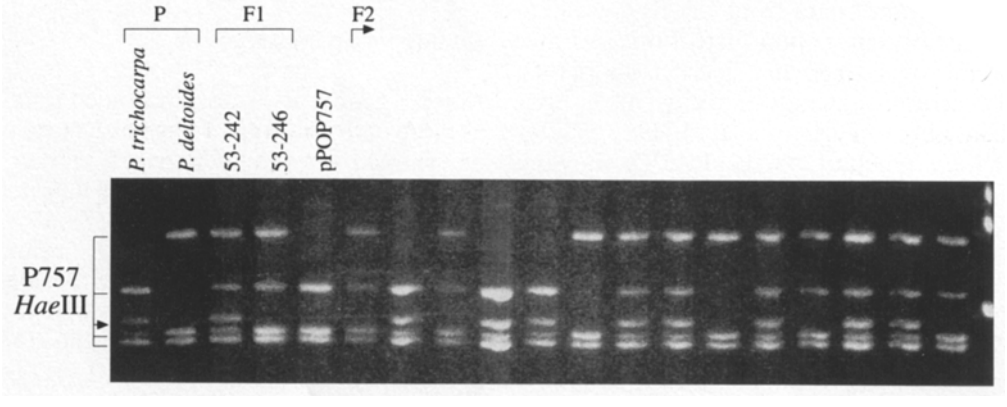


Fig. 2 Segregation of an STS/ RFLP in the *Populus* mapping pedigree. DNA from the *P. trichocarpa* and *P. deltoides* parents, the two F_1s, the plasmid clone used for Southern blot hybridizations (pPOP757), and a panel of 14 F_2 offspring was amplified with STS primers 757F and 757R (Table 2) and cleaved with *Hae*III. The female *P. trichocarpa* is heterozygous (*arrow*), as shown by the fact that only one allele has been cloned into pPOP757 and that the two F_1s have inherited alternate *P. trichocarpa* alleles.

Table 3 Application of STS/RFLPs to angiosperms of varying relatedness to *Populus trichocarpa*. DNA from *P. trichocarpa* (sect. Tacamahaca), *P. deltoides* (sect. Aigeiros), *P. tremuloides* (sect. Leuce), *Salix matsudana* var 'Tortuosa', *Nicotiana tabacum* cv 'Xanthi', *Arabidopsis thaliana*, and *Dendrobium nobile* was amplified using a subset of the primers listed in Table 2. PCR conditions were as described in Table 2. The first symbol (+ or −) indicates whether an amplification product was visible on an ethidium-stained agarose gel following PCR, and the second symbol shows whether a Southern blot hybridization signal was detected using the radiolabelled RFLP mapping probe from which the STS primers were derived (see text) (+/+ amplification products were detected homologous to the mapping probe, +/− amplification products were found that failed to hybridize to the cognate probe, −/− neither amplification products nor hybridization signals were found)

Locus	*Populus trichocarpa*	*Populus deltoides*	*Populus tremuloides*	*Salix matsudana*	*Nicotiana tabacum*	*Arabidopsis thaliana*	*Dendrobium nobile*
P164	+/+	+/+	+/+	+/+	+/+	+/+	+/+
P753	+/+	−/−	+/+	+/−	+/−	+/−	+/−
P754	+/+	+/+	+/+	+/+	−/−	−/−	−/−
P757	+/+	+/+	+/+	+/−	−/−	−/−	−/−
P762	+/+	+/+	+/+	+/+	+/−	+/−	−/−
P767	+/+	+/+	+/+	+/+	−/−	−/−	−/−
P770	+/+	+/+	+/+	−/−	+/−	+/−	+/−
P781	+/+	+/+	+/+	−/−	−/−	−/−	−/−
P783	+/+	+/+	+/+	+/+	+/−	+/−	+/−
P800	+/+	+/+	+/+	+/+	+/−	+/−	+/−
P832	+/+	+/+	+/+	+/−	+/−	+/−	+/−
P1010	+/+	+/+	+/+	+/+	−/−	−/−	+/−
P1012	+/+	+/+	−/−	−/−	−/−	−/−	−/−
P1013	+/+	+/+	+/+	+/+	−/−	−/−	−/−
P1027	+/+	+/+	+/+	−/−	−/−	−/−	−/−
P1046	+/+	+/+	+/+	−/−	−/−	−/−	−/−
P1054	+/+	+/+	+/+	−/−	−/−	−/−	−/−
P1079	+/+	+/+	+/+	+/−	+/−	+/−	+/−

with each of the parents. Each F_1 has a probability of 1/2 of inheriting the "with band" RAPD allele at each locus where one parent is heterozygous, so that both F_1s will receive this allele 1/4 of the time even when the parent is truly heterozygous. Hence, the heterozygosity estimates of 27% for *P. trichocarpa* 93-968 and 22.5% for *P. deltoides* ILL-129 should be divided by 3/4, thus increasing the estimated heterozygosities to 36% and 30%, respectively. We could have used more F_1 progeny in the assessment of parental heterozygosity to reduce the downward bias, but rather chose to use the available marker screening data involving only the parental clones and the two F_1s used in advanced-generation breeding. The reason for the large discrepancy in the *P. deltoides* ILL-129 heterozygosity estimate made from RFLP and RAPD data is unknown, but the competitive nature of the RAPD reaction and the occasional observation of non-parental bands in the F_1 cause us to give more credence to the RFLP-based estimate.

The RAPD inheritance data from the F_2 were examined for evidence of segregation distortion. In other mapping work in forest trees and herbaceous plants, RAPDs with aberrant segregation ratios have been excluded from analysis (Tulsieram et al. 1992; Reiter et al. 1992). We were reluctant to reject RAPDs showing segregation distortion, since this phenomenon is common in our mapping pedigree (Bradshaw and Stettler 1994). Of the 111 RAPD markers mapped in the F_2, 16 (14%) were skewed significantly (χ^2 test; $df = 1; P < 0.01$) from the expected 3:1 ratio. This value is about twice as high as the 14/229 (6%) reported for segregation distortion in this F_2 family using RFLP markers (Bradshaw and Stettler 1994). However, only 3 of the 16 (19%) skewed RAPD markers failed to map to linkage groups containing RFLP markers, a proportion not very different from the overall fraction of unlinked RAPD markers (19/111; 17%), indicating that RAPDs showing segregation distortion are as likely to be mapped as those with Mendelian segregation ratios.

To determine the fraction of mapped RAPD markers that might be applicable across most or all F_2 progenies derived from hybridization between any pair of *P. trichocarpa* and *P. deltoides*, 8 additional *P. trichocarpa* and 10 additional *P. deltoides* (to our knowledge unrelated to the parents of the mapping pedigree, and largely unrelated to each other) were screened with a subset of 50 of the primers used in the mapping pedigree. Of 64 mapped markers revealed by the 50 RAPD primers, 26 (40%) appeared fixed for the "with-band" allele within one parental species and for the null allele in the other.

Linkage map construction

Marker genotypes were determined for as few as 26 and as many as 90 F_2 trees. In general, more genotypes were determined for the PCR-based marker systems (STS and RAPD) than for the RFLPs detected by Southern blotting. Segregation of 215 RFLPs, 17 STSs (including an additional locus recognized by primers 1079F and 1079R in Table 2; this locus was designated P1079.3), and 111 RAPDs has been followed for linkage map construction in the F_2. When the 343 markers are grouped by MAPMAKER at a LOD threshold of 3.0

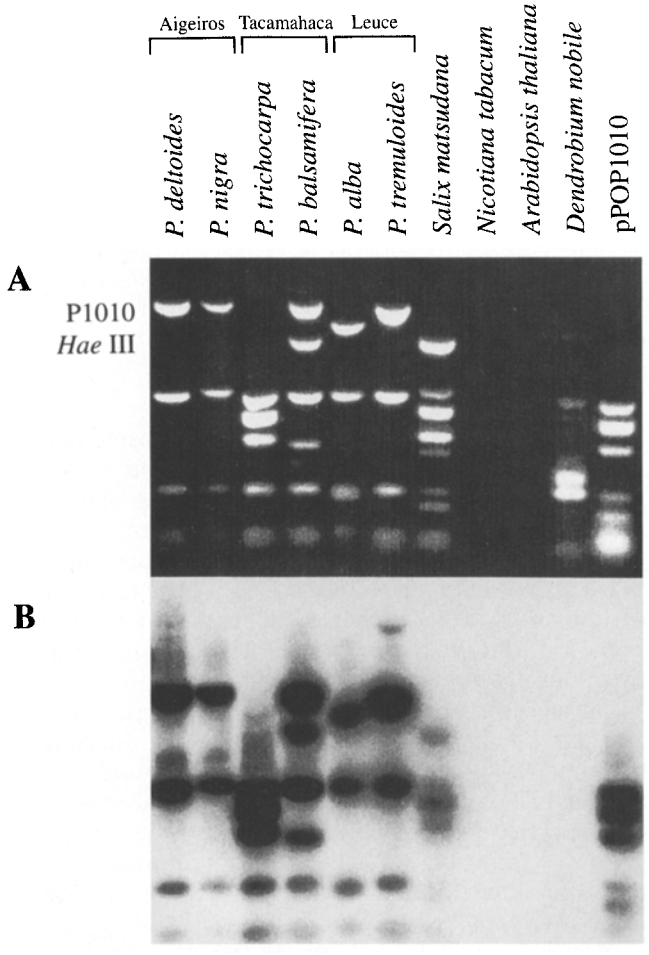


Fig. 3A, B STS/RFLP analysis and Southern blot hybridization of angiosperms related to *P. trichocarpa*. STS primer 1010F and 1010R (Table 2) were used to direct amplification from templates of the angiosperm species shown, and the PCR products were digested with *Hae*III. Members of three sections of *Populus*, a willow (*Salix*), and the original plasmid clone pPOP1010 yielded amplification products (**Panel A**) capable of hybridizing to the pPOP1010 probe (**Panel B**). While amplification products were visible in the lane containing *Dendrobium* DNA (Panel A), these products failed to hybridize to the relevant probe (Panel B). Thus, the *Dendrobium* received a rating of +/− for locus P1010 in Table 3. Note that the patterns of *P. trichocarpa* and pPOP1010 are identical, as expected since *P. trichocarpa* 93-968 is the source of the insert in pPOP1010 (Table 1)

and a maximum Haldane map distance of 35 cM, there are 35 linkage groups (vs. 19 chromosomes in the *Populus* genome) and 31 unlinked markers (Fig. 4). Twelve of the unlinked markers are RFLPs, none are STSs, and 19 are RAPDs. The total map distance contained within the 19 largest linkage groups is 1261 cM. The relationship between mapped linkage groups and *Populus* chromosomes is unclear, as there is no classical genetic or cytogenetic map of the *Populus* genome available.

Genome length estimation

Maximum likelihood estimates of Haldane map distances were calculated for all unique pairs of 100 (N) RFLP markers having approximately equal numbers of informative meioses (52 meioses resulting in 26 F_2 trees). The maximum observed value of two-point map distance at LOD thresholds of 2.0 (30 cM) was substituted for X in the equation

$$G = N(N-1)X/K$$

where G is the genome length and K is the number of markers at or above the LOD threshold (Hulbert et al. 1988). From our linkage data for values of K at LOD scores of 2.0 and 3.0, we estimate that the genome length of *Populus* is 2400–2800 cM. The lower estimate is based upon a LOD threshold of 3.0 and the higher is based upon a LOD threshold of 2.0. For purposes of discussion, we will consider the genome to be approximately 2600 cM. The proportion of the genome contained within the largest 19 mapped linkage groups is thus 48.5% (1261/2600).

Discussion

RFLP probes

The first probes used for mapping were random *Pst*I fragments of an F_1 hybrid (50–188). While adequate for mapping, these random clones were seldom longer than 300 bp and caused several problems related to their small size. Such short fragments gave best results as probes only when isolated from the plasmid vector, generally by PCR amplification. Because of the relatively small probe size, signals on the Southern blots were not as intense as desired. Further disadvantages of the short clones became apparent when we began to use STSs as markers (see below). When 2–3 kb *Pst*I or *Xba*I fragments were cloned for use as RFLP probes the entire plasmid was radiolabelled and used for hybridization, saving the additional step of insert isolation and resulting in improved signals on Southern blots.

Since about two-thirds of the RFLP probes were single- or low-copy, we did not feel that it was necessary to routinely pre-screen candidate plasmid clones for the presence of organelle DNA or nuclear repeated DNA sequences. However, when we used total *Populus* DNA as a hybridization probe to pre-screen plasmid clones (probe numbers 1017–1343) for high-copy number sequences, a worthwhile increase in the proportion of useful probes was realized (Table 1). We have no firm estimate of the proportion of single- or low-copy probes polymorphic between the parents of the mapping pedigree, since no systematic effort was made to screen every probe. A minimum estimate of polymorphism is obtained by noting that of 186 single- or low-copy probes found using probe numbers 1017–1343 (Table 1), 121 (65%) were used for mapping.

There was no difference between *Pst*I and *Xba*I in the proportion of single-copy loci or polymorphic loci

found, despite the fact that the former enzyme is sensitive to cytosine methylation and the latter is not (Table 1). This may be a consequence of the compact genome of *Populus*, reflective of a relatively low abundance of repeated DNA sequences; however, very little is known about repeated sequences or DNA methylation in *Populus*.

A significant fraction (11%) of RFLP probes revealed multiple loci in *Populus*, a situation observed in other "diploid" plants (Helentjaris et al. 1988; Keim et al. 1990). This fraction is probably an underestimate of the true level of genome duplication in *Populus*, since not all duplicated loci will be polymorphic (Keim et al. 1990) or will retain sufficient sequence identity to hybridize at high stringency. Duplication of some regions of the *Populus* genome could lead to complications in mapping, such as apparent segregation distortion due to digenic inheritance ratios if the duplicated loci are on different chromosomes but share a common RFLP; however, we have found no evidence for this in our mapping effort. RFLPs detected by Southern blot hybridization were especially informative for revealing duplications since both fragment size and signal intensity on the autoradiograms gave clues as to which bands were allelic and which were at different loci.

RFLP markers proved exceptionally useful for identifying triploid F_2 trees (Bradshaw and Stettler 1993). Triploids violate the basic premise of genome mapping, that of a regular meiosis. Triploids are recognized by their inheritance of two alleles at which the female parent (in all cases that we have observed in *Populus*) is heterozygous. Since some *Populus* females are capable of producing a high proportion of triploid or aneuploid offspring (Bradshaw and Stettler 1993), it may be necessary as well as desirable to eliminate triploids from the analysis of genetic linkage. STS (or other codominant) markers can serve the same function, but dominant RAPDs would seem to be almost useless for distinguishing triploids from diploids.

STS-based markers

The development of STS markers was initiated in order to provide a framework linkage map composed of codominant, multiallelic markers that could be assayed quickly, easily, and with fairly crude preparations of DNA from small tissue samples (Tragoonrung et al. 1992). Traditional RFLP mapping by Southern blot hybridization was used to identify markers spaced evenly around the *Populus* genome, and probes of known map position were converted to STS/RFLPs. Fewer than half (41%) of the first attempts to convert markers from Southern blot detection to a PCR-based system proved informative for mapping in the interspecific *Populus* F_2. The failure of several STS/RFLPs to segregate as predicted from the original Southern blot mapping data appears to be due in part to the amplification of duplicated, unlinked loci. When these anomalously-segregating STS amplification products are digested with restriction enzymes, the sum of the molecular weights of the digestion products exceeds that of the amplified fragment itself, implying that the single band observed on gels of the undigested amplification product is a mixture of related sequences. The heterozygosity of individual trees further complicates the analysis of such loci and makes interpretation of the resulting fragment patterns difficult.

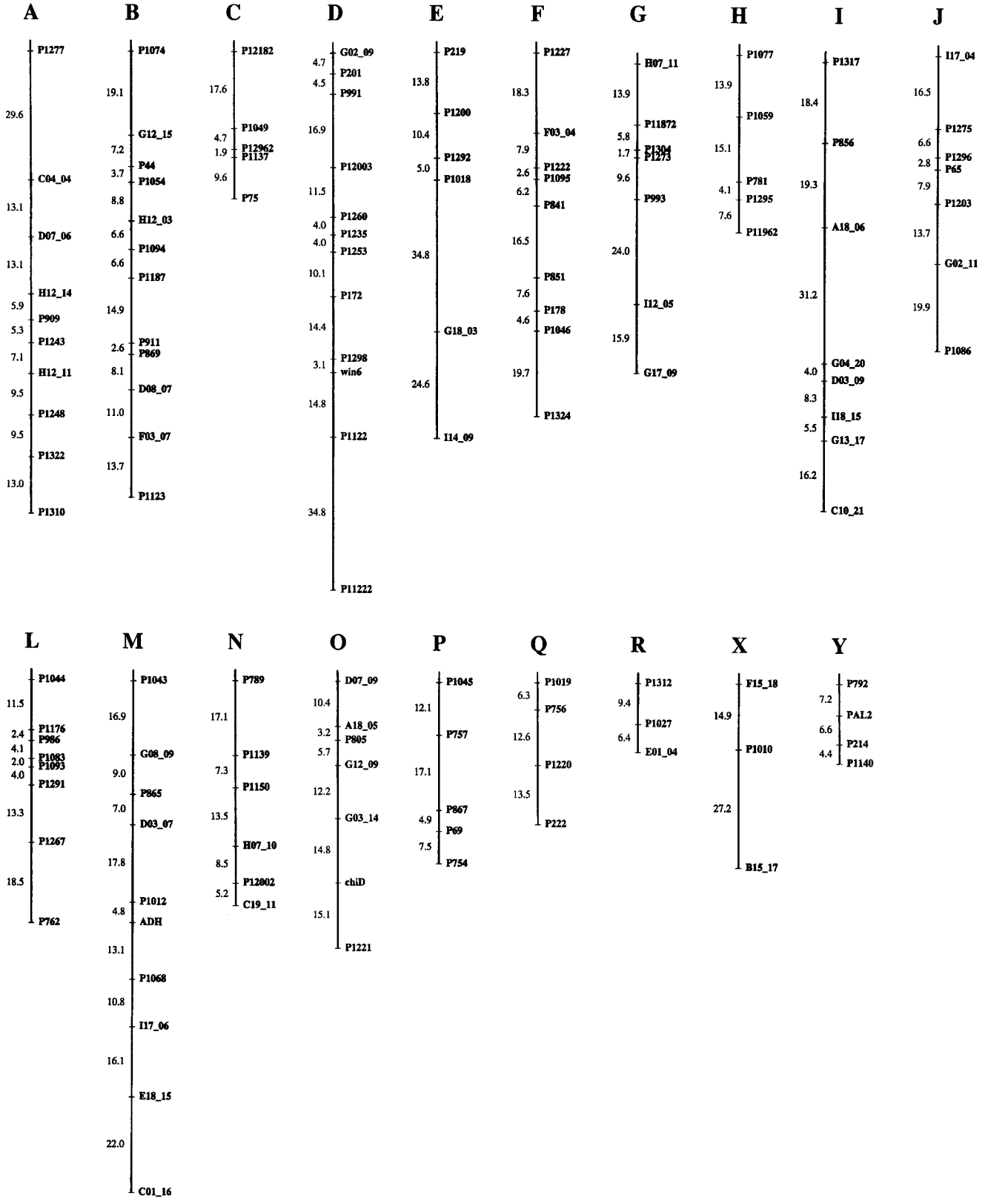
Simulations have shown that a relatively wide marker spacing can be used for mapping quantitative trait loci (QTLs; Darvasi et al. 1993), so it may be worthwhile to place one or two STS polymorphisms on each of the *Populus* chromosomes to permit rapid genome scans for QTLs. In our preliminary experiments, we have located at least one STS on 11 of the 19 largest mapped linkage groups (Table 2).

While our STS/RFLP sample size is relatively small, it seems that a large fraction of the STS/RFLPs developed from fragments of *P. trichocarpa* genomic DNA will be useful for mapping in other sections of *Populus* and many will be satisfactory for *Salix*. Such markers may also be helpful in studies of the population genetics, ecology, and evolution of the Salicaceae, complementing work already done with organelle DNA (Smith and Sytsma 1990), ribosomal DNA (Faivre-Rampant et al. 1990), and random RFLPs (Keim et al. 1989).

RAPD markers

The relative simplicity, speed, and low cost of RAPDs have led to their widespread and increasing use in experimental genetics (Tingey and Deltufo 1993), especially for those organisms with poorly developed classical genetics. As markers for linkage mapping of whole genomes, RAPDs suffer from two principal drawbacks; dominant inheritance and the fact that only two alleles ("with-band" and null) are generally present in the population (Williams et al. 1993). A further complication is that a RAPD band of the same apparent size in two individuals may be at an entirely different locus in each, an issue that can be resolved unambiguously only by resorting to those techniques (cloning, sequencing, Southern blot hybridization) that one uses RAPDs to

Fig. 4 Linkage map derived from segregation of 343 RFLP, STS, and RAPD markers in the *Populus* mapping pedigree. Only the 19 largest linkage groups are shown. Loci on the *right* of each vertical bar representing a linkage group are defined by the RFLP markers shown in Table 1, the STS markers shown in Table 2, and the RAPD markers named according to their Operon letters and approximate fragment size (e.g. D07_06 on linkage group A is a 600bp product of Operon primer D7). The Haldane map distance between adjacent markers is shown on *the left side* of each linkage group. MAPMAKER has omitted the decimal point from duplicated loci, so that the locus P1200.2 appears as P12002 on the map (linkage group N)


A
P1277
29.6
C04_04
13.1
D07_06
13.1
H12_14
5.9
P909
5.3
P1243
7.1
H12_11
9.5
P1248
9.5
P1322
13.0
P1310
B
P1074
19.1
G12_15
7.2
P44
3.7
P1054
8.8
H12_03
6.6
P1094
6.6
P1187
14.9
P911
2.6
P869
8.1
D08_07
11.0
F03_07
13.7
P1123
C
P12182
17.6
P1049
4.7
P12962
1.9
P1137
9.6
P75
D
G02_09
4.7
P201
4.5
P991
16.9
P12003
11.5
P1260
4.0
P1235
4.0
P1253
10.1
P172
14.4
P1298
3.1
win6
14.8
P1122
34.8
P11222
E
P219
13.8
P1200
10.4
P1292
5.0
P1018
34.8
G18_03
24.6
I14_09
F
P1227
18.3
F03_04
7.9
P1222
2.6
P1095
6.2
P841
16.5
P851
7.6
P178
4.6
P1046
19.7
P1324
G
H07_11
13.9
P11872
5.8
P1304
1.7
P1273
9.6
P993
24.0
I12_05
15.9
G17_09
H
P1077
13.9
P1059
15.1
P781
4.1
P1295
7.6
P11962
I
P1317
18.4
P856
19.3
A18_06
31.2
G04_20
4.0
D03_09
8.3
I18_15
5.5
G13_17
16.2
C10_21
J
I17_04
16.5
P1275
6.6
P1296
2.8
P65
7.9
P1203
13.7
G02_11
19.9
P1086
L
P1044
11.5
P1176
2.4
P986
4.1
P1083
2.0
P1093
4.0
P1291
13.3
P1267
18.5
P762
M
P1043
16.9
G08_09
9.0
P865
7.0
D03_07
17.8
P1012
4.8
ADH
13.1
P1068
10.8
I17_06
16.1
E18_15
22.0
C01_16
N
P789
17.1
P1139
7.3
P1150
13.5
H07_10
8.5
P12002
5.2
C19_11
O
D07_09
10.4
A18_05
3.2
P805
5.7
G12_09
12.2
G03_14
14.8
chiD
15.1
P1221
P
P1045
12.1
P757
17.1
P867
4.9
P69
7.5
P754
Q
P1019
6.3
P756
12.6
P1220
13.5
P222
R
P1312
9.4
P1027
6.4
E01_04
X
F15_18
14.9
P1010
27.2
B15_17
Y
P792
7.2
PAL2
6.6
P214
4.4
P1140

avoid. In typical forest tree pedigrees, with heterozygous parents and little or no inbreeding, these factors make it difficult to construct RAPD maps from segregating diploid offspring and to align RAPD maps made from different segregating families. Several clever strategies to overcome some of these obstacles have been implemented, including the use of haploid tissues from single trees (Carlson et al. 1991; Tulsieram et al. 1992) and pseudotestcross configuration of RAPDs in diploid progenies (Grattapaglia et al. 1992). These methods still suffer from a poor ability to map markers linked in repulsion and from a lack of concordance among maps derived from different individuals and/or families.

In the case of *Populus*, however, we found RAPDs to be especially useful for two reasons beyond those given above. First, MAPMAKER is able to infer the haplotypes of individual trees from a mixture of dominant and codominant markers, thus reducing the negative impact of dominance for mapping in a population of F_2s when substantial RFLP data are included in the analysis. RAPDs linked in repulsion were placed on a framework of codominant markers so that a single map was produced from the segregating diploid family (Fig. 4), as has been done on a limited scale in other plants (Torres et al. 1993). Second, since a large fraction (40%) of the RAPDs mapped in the F_2 appear substantially fixed for alternate alleles in the two parental species, it appears that these same RAPD bands can serve as reference loci in most or all F_2 populations resulting from hybridization of *P. trichocarpa* and *P. deltoides*. By judicious screening of more primers, it should be possible to construct a framework map entirely of RAPD markers, with the expectation that such a map would be easily transferred among interspecific F_2s. Paradoxically, those RAPD markers most informative in all F_2 populations, i.e, invariant within the parental species, will be useless for mapping in the F_1 generation for the same reason. This could prove to be a limitation in studies of interspecific hybrids where the correlations among parental, F_1, and F_2 phenotypes are being investigated.

Linkage map construction

Because the marker genotype data were encoded as though the F_2 progeny were the result of selfing an F_1 derived from two inbred lines, the *Populus* genome map in Fig. 4 represents linkage analysis only of "interspecific" recombinations between the chromosomes of the *P. trichocarpa* and *P. deltoides* parents and ignores recombinations that might have been useful for making "single-tree" maps of each of the F_1s (Tulsieram et al. 1992). Although a much smaller fraction of all markers is informative for such "single-tree" mapping, it may be useful in the future to compare map distances based on meioses in female and male *Populus* hybrids. Further, the level of heterozygosity in the parental *P. trichocarpa* and *P. deltoides* is sufficienly high that "intraspecific" recombinations could be assessed in a large F_1. It may be that the "interspecific" map is compressed, expanded, or non-collinear with the "intraspecific" maps and that such aberrations might be useful indicators of divergence in genome structure.

Previous work has demonstrated the feasibility of identifying linkage groups in *Populus* using allozymes (Müller-Starck 1992; Liu and Furnier 1993) and RFLPs (Liu and Furnier 1993). The *Populus* linkage map shown in Fig. 4, while certainly incomplete, represents a significant advance in our knowledge of *Populus* genome structure. The map is of sufficient scope and density to permit the mapping of QTLs governing growth, form, and phenological traits in the F_2 (Bradshaw and Stettler, unpublished). Both the cloned probes and vegetative propagules of each tree in the mapping pedigree serve as genetic resources for forest tree biologists. The mapped probes serve as a framework for other *Populus* (and perhaps *Salix*) genomes. The individual trees in the mapping pedigree, with their cumulative marker genotypic data, can be distributed much as recombinant inbred lines in crop plants to replicate experiments in time and space.

Relative merits of each marker type

The most desirable genetic markers are codominant, multiallelic, abundant in the genome of the chosen organism, and applicable across reasonably wide phylogenetic distances. Ideally such markers are also easily, rapidly, and inexpensively assayed. None of the three marker types we have used meets all these criteria. Perhaps the STS-based RFLPs are the best of the three marker types tested, but the expense of primer synthesis, the long recombinational length of the *Populus* map, and the somewhat inefficient conversion process are drawbacks of this approach. The same argument applies to microsatellite-based markers, which exist in *Populus* (Bradshaw, unpublished) but have not been further developed for the same reasons. It does appear that locus-specific PCR primers from one species of *Populus* would be generally useful in other species, sections, and genera of the Salicaceae. Standard RFLP analysis by Southern blot hybridization remains a viable method since fairly large quantities of DNA can be extracted from trees and the small genome size of *Populus* makes blot hybridizations technically simpler than similar experiments in conifers with genome sizes 10–20 times as large. RAPDs are doubtless the simplest and least expensive DNA-based markers but are relatively uninformative compared with codominant, multiallelic markers. A mixed marker map composed of a large number of RAPDs and a smaller number of codominant markers, such as RFLPs and/or allozymes, could take advantage of the best attributes of both marker types.

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