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ORIGINAL PAPER

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Genetic mapping in (*Populus tomentosa* × *Populus bolleana*) and *P. tomentosa* Carr. using AFLP markers

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Abstract The AFLP genetic linkage maps for two poplar cultivars were constructed with the pseudo-test-cross mapping strategy. The hybrids were derived from an interspecific backcross between the female hybrid clone "TB01" (*Populus tomentosa* × *Populus bolleana*) and the male clone "LM50" (*P. tomentosa*). A total of 782 polymorphic fragments were obtained with a PCR-based strategy using 49 enzyme-nested (*Eco*RI/*Mse*I) primer combinations. Six hundred and thirty two of these fragments segregated in a 1:1 ratio ($P<0.01$), indicating that these DNA polymorphisms are heterozygous in one parent and null in the other. The linkage analysis was performed using Mapmaker version 3.0 with LOD 5.0 and a maximum recombination fraction (θ) of 0.3. Map distances were estimated using the Kosambi mapping function. In the framework map for "LM50" (*P. tomentosa*), 218 markers were aligned in 19 major linkage groups. The linked loci spanned approximately 2,683 cM of the poplar genome, with an average distance of 12.3 cM between adjacent markers. For "TB01" (*P. tomentosa* × *P. bolleana*), the analysis revealed 144 loci, which were mapped to 19 major linkage groups and covered about 1,956 cM, with an average distance of 13.6 cM between adjacent markers. These maps covered about 87% and 77% of the estimated genome size of parents "LM50" and "TB01", respectively. The maps developed in this study lay an important foundation for future genomics research in poplar, providing a means for localizing genes controlling economically important traits in *P. tomentosa*.

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Introduction

Genetic linkage maps, based on molecular markers, now provide a powerful tool for detecting loci controlling a number of traits and for studying genome organization and evolution in many forest trees species (Bradshaw and Stettler 1995; Devey et al. 1995; Grattapaglia et al. 1995; Marques et al. 1999; Sewell et al. 2002). These results, in turn, are the basis for map-based gene cloning and for marker-assisted selection of important traits related to faster growth, better stress adaptation or better disease resistance. The advent of robust molecular-marker systems greatly facilitates the construction of genetic linkage maps in various tree species.

The four principal types of molecular markers used in mapping include restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), simple sequence repeats (SSRs) and amplified fragment length polymorphisms (AFLPs). AFLP markers are generated through a PCR-based approach that does not require either prior knowledge of sequence information or probe preparation, both of which are required for generating RFLP and SSR markers. AFLPs are more reliable compared to RAPDs and, being dominant markers, supply as much information as co-dominant markers for analysis of backcross or pseudo-test-cross populations (Staub and Serquen 1996). Currently, the AFLP marker system has largely been used in tree genetic mapping (Marques et al. 1998; Remington et al. 1999; Cervera et al. 2001).

The genus *Populus* is comprised of more than 30 species classified into five or six sections. Poplar is one of

the most economically and intensively studied forest tree species due to its importance as a timber source and a model forest tree. The Chinese white poplar (*Populus tomentosa* Carr.), belonging to the section *Populus*, is a fast-growing timber species native to China, with an excellent wood quality and outstanding resistance to many diseases and insects. Consequently, the Chinese have cultivated it more than any other poplar species, mainly in northern China, on an area of approximately 1 million km^2. However, it is difficult to generate a large segregating population of intraspecific hybrids for genetic mapping in *P. tomentosa* because its overall ability to reproduce sexually is poor, and some individuals are sterile. To deal with this problem, an inter-specific backcross breeding approach strategy (e.g. the hybrid *P. tomentosa* × *Populus bolleana* is utilized as the maternal parent and backcrossed with *P. tomentosa*) was therefore proposed (Zhu and Zhang 1997). This strategy was then used to easily create a large segregating population by crossing (*P. tomentosa* × *P. bolleana*) with *P. tomentosa*.

Various genetic maps have been developed for genomic research in poplar to date. The first poplar genetic map was developed with RFLP and allozyme markers (Liu and Furnier 1993). Much more detailed and complete linkage maps have also been established by combining hundreds of RAPD, RFLP, STS (sequence-tagged site), SSR and AFLP markers (Bradshaw et al. 1994; Wu et al. 2000; Cervera et al. 2001; Yin et al. 2001). However, new genetic maps based on distinct populations are still needed for special objectives such as QTL detection and map based cloning.

In this paper, we report an application of AFLP markers in a two-way pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994), to map the genome of an elite clone of "LM50" (*P. tomentosa*) and its hybrid clone "TB01" (*P. tomentosa* × *P. bolleana*) from progeny data. This work could be exploited as a potential reference map for QTL analysis of *P. tomentosa* for important traits.

Materials and methods

The mapping pedigree

The BC1 lines of *Populus* (696 offspring) used in this study were derived from the interspecific backcross TB01×LM50. TB01 was derived from the *P. tomentosa* clone 3082 × *P. bolleana,* while LM50 was from *P. tomentosa* clone 3075. *P. tomentosa* clones 3082 and 3075 belong to the same family. One hundred and twenty progenies were randomly selected and used for genetic mapping.

AFLP procedures

Total genomic DNA was extracted from frozen young leaves of 120 inter-specific backcross hybrids and their parents, by the methods described by Murray and Thompson (1980). AFLP analysis was performed essentially as described by Vos et al. (1995). In the present study, this method was modified by Zhang et al. (2002). Products of the selective amplification reaction were detected as discrete bands on a polyacrylamide gel, using electrophoresis and the silver-staining method as described by Tixier et al. (1997).

Heterozygosity analysis

The average heterozygosity was defined as the ratio of bands segregating in the offspring to the total number of bands observed (Cervera et al. 2001). The average heterozygosity was estimated by analyzing the 120 individuals and their parents, using 30 different AFLP primer combinations.

Data analysis and map construction

Segregation of the markers was scored only for clear, unambiguous bands having electrophoretic migration patterns that obviously represented polymorphisms between the parents. For each marker, Chi-square tests (df=1, P<0.01) were conducted to check deviations from the expected 1:1 Mendelian ratio. All markers deviating at the 1% significance level were excluded from the linkage analysis. Heterozygous AFLP markers present in one parent, but not in the other, were used to construct separate genetic linkage maps for the male clone "LM50" parents and the female clone "TB01" parents using the two-way pseudo-testcross strategy (Grattapaglia and Sedroff 1994). The data set was duplicated, to allow the detection of linkage for markers in the repulsion phase. Both parents' genetic linkage maps were constructed using MAPMAKER version 3.0 (Lincoln et al. 1992). Markers were first grouped using a minimum LOD score of 5.0 and a maximum recombination frequency (θ) of 0.30. For each linkage group, markers were ordered by using a minimum LOD score of 3.0 and a maximum θ of 0.40 using the First-Order command. The ordered marker sequences were confirmed using the Ripple command. Markers ordered with low confidence were placed again, using the Try command. New markers were placed at appropriate positions of the maps with the Place command. Linkage maps were generated with the Map command. Possible errors or double-crossovers were checked with the Genotype command before map construction. Map distances in centiMorgans were computed using Kosambi's mapping function.

Genome length estimation

The recombination length of the *Populus* genome was estimated from partial linkage data using the equation

$$G_e = N(N-1)X/K, \quad (1)$$

with a confidence interval given by equation

$$G_e \Big/ \left(1 \pm 1.96 \Big/ \sqrt{K}\right), \quad (2)$$

where Ge is the estimated genome length, N is the number of framework markers, X is the maximum map distance between two adjacent framework markers in centiMorgans at a certain minimum LOD score of 3.0, and K the number of marker pairs at the same minimum LOD score (Hulbert et al. 1988).

Results

Polymorphisms

The system of *Eco*RI/*Mse*I primers, consisting of pre-amplification with E00/M00 and selective-amplification with E+3/M+3, was utilized for detection of DNA markers in poplar, which has a relatively small genome size (approximately 550 Mbp). With modified reaction conditions, a total of 200 AFLP primer combinations, with the selective bases at the 3′-end of each primer, were tested to determine the quality of the AFLP fingerprinting and the levels of polymorphism. Of the 200 primer pairs

Table 1 Total number of AFLP markers generated with 49 *Eco*RI+3/*Mse*I+3 primer combinations

Primer combinations		Total visible bands	Total markers	Size of markers	Skewed markers	Framework map markers	
*Eco*RI+3	*Mse*I+3					LM50	TB01
E33(AAG)	M32(AAC)	50	15	68–396	2	6	3
E33(AAG)	M35(ACA)	40	10	98–705	2	3	2
E33(AAG)	M36(ACC)	40	7	68–473	1	3	1
E33(AAG)	M37(ACG)	45	10	65–469	0	1	3
E33(AAG)	M38(ACT)	78	23	59–518	3	9	7
E33(AAG)	M39(AGA)	46	10	69–354	3	1	0
E33(AAG)	M40(AGC)	48	13	51–364	0	3	3
E33(AAG)	M41(AGG)	53	21	48–702	9	3	5
E33(AAG)	M42(AGT)	70	19	50–517	2	6	3
E33(AAG)	M44(ATC)	68	17	48–660	4	4	2
E33(AAG)	M47(CAA)	74	15	46–432	0	5	4
E33(AAG)	M48(CAC)	68	12	97–684	2	1	3
E33(AAG)	M50(CAT)	42	6	99–382	1	3	0
E33(AAG)	M58(CGT)	44	12	97–344	1	5	0
E33(AAG)	M61(CTG)	72	19	69–472	5	6	3
E33(AAG)	M62(CTT)	66	11	98–502	6	0	2
E33(AAG)	M65(GAG)	72	17	40–567	8	3	1
E33(AAG)	M66(GAT)	58	12	85–330	1	0	4
E33(AAG)	M79(TAA)	70	16	96–755	4	3	7
E33(AAG)	M82(TAT)	60	11	84–767	2	4	2
E33(AAG)	M86(TCT)	50	8	98–555	1	4	2
E34(AAT)	M44(ATC)	110	26	79–604	4	8	6
E34(AAT)	M47(CAA)	68	12	98–542	3	2	2
E34(AAT)	M48(CAC)	60	7	67–492	2	1	3
E35(ACA)	M50(CAT)	48	4	99–411	1	0	1
E35(ACA)	M52(CCC)	68	17	57–487	4	6	2
E44(ATC)	M40(AGC)	72	16	73–324	3	6	3
E44(ATC)	M44(ATC)	66	15	99–693	1	9	1
E44(ATC)	M46(ATT)	80	26	98–658	8	7	2
E44(ATC)	M50(CAT)	74	18	91–650	6	5	3
E44(ATC)	M60(CTC)	76	18	59–644	2	3	8
E60(CTC)	M32(AAC)	50	11	67–453	1	1	7
E60(CTC)	M33(AAG)	53	20	46–473	2	13	2
E60(CTC)	M34(AAT)	56	15	58–477	2	3	1
E61(CTG)	M31(AAA)	65	19	70–649	4	6	2
E61(CTG)	M41(AGG)	68	19	52–473	4	8	1
E63(GAA)	M32(AAC)	70	17	99–396	3	1	5
E63(GAA)	M33(AAG)	64	16	93–373	4	5	3
E63(GAA)	M36(ACC)	66	18	66–517	0	11	5
E63(GAA)	M39(AGA)	76	27	49–436	10	4	4
E63(GAA)	M40(AGC)	72	20	61–651	3	8	2
E63(GAA)	M43(ATA)	62	16	98–673	1	7	0
E63(GAA)	M46(ATT)	68	20	58–432	2	4	5
E63(GAA)	M52(CCC)	60	21	71–961	7	7	3
E63(GAA)	M61(CTG)	64	26	60–336	8	3	3
E63(GAA)	M67(GCA)	67	17	64–650	1	3	1
E65(GAG)	M31(AAA)	73	23	40–642	3	10	2
E65(GAG)	M34(AAT)	70	21	74–827	2	3	8
E65(GAG)	M39(AGA)	60	13	98–454	2	1	2
Total		3,100	782	40–961	150	218	144

tested, 49 were effective in revealing visually clear polymorphic loci between "LM50" and "TB01". Altogether, 3,100 bands were detected using the selected 49 primer combinations, of which 782 (25.2%) bands were polymorphic, with sizes ranging from approximately 40 to 961 bp (note: bands longer than 800 bp were rarely detected, at about 0.4%). The number of bands varied among the different pairs of selective primers (Table 1). The combination of EAAG/MACA and EAAG/MACC gave the smallest number of bands (40); while more than 42 bands were generally detected with other primer combinations. The average number of bands per primer pair was 63 and the number of polymorphic bands was 16, with a range of 4–27 (Table 1).

Segregation distortion

In our study, segregation distortion is defined as the deviation from the expected Mendelian 1:1 segregation ratio (Cervera et al. 2001). Forty nine pairs of AFLP primers generated 782 markers, with 510 (65%) heterozygous for the male "LM50" and 272 (35%) heterozygous for the female "TB01" (Table 1). One hundred and fifty

(19%) of the 782 AFLP markers displayed a skewed null hypothesis of the Mendelian 1:1 segregation ratio, based on 120 progeny in the mapping population ($P<0.01$). Of these 150 markers, 114 (22%) and 36 (13%) were derived from "LM50" and "TB01", respectively. These deviating markers were excluded from subsequent linkage analysis in order to avoid false linkage. As a result, 632 test-cross markers were employed for the construction of clone "LM50" and clone "TB01" linkage maps.

Heterozygosity levels

The estimated average heterozygosity of clones "LM50" and clone "TB01" were 18.71% and 8.47%, respectively, based on the 120 progeny and 30 AFLP primer combinations.

AFLP linkage maps

The linkage analysis was based on 632 testcross AFLP markers with 396 in the male "LM50" and 236 in the female "TB01". For "LM50", 25 linkage groups, 12 triplets, 23 doublets and 74 unlinked markers were obtained at a LOD score of 5.0 and $\theta=0.30$ using the MAPMAKER 3.0 linkage program. Nineteen of the 25 linkage groups were classified as "major" groups (>50 cM), which constituted the framework map for "LM50". The male map spanned a total length of 2,682.8 cM, and included 218 markers, with an average interval of 12.3 cM between adjacent marker loci. Linkage groups ranged in length from 57.1 cM (TLG19) to 326.5 cM (TLG1), while the number of markers mapped in each linkage group varied from 6 (TLG16, TLG17, TLG18 and TLG19) to 24 (TLG2).

The same criteria were used to establish the linkage map of the maternal tree, "TB01". The 236 markers were assigned to 19 major groups (>40 cM), including 1 triplet, 20 doublets, with 49 unlinked markers. These 19 groups in the female map covered 1,956.3 cM, and 144 markers were located on the map, with an average distance between marker loci of 13.6 cM. The length of the groups ranged between 42.2 cM (TBLG19) and 274.8 cM (TBLG1), and the number of markers located in each linkage group varied from 4 (TBLG12, TLG18 and TLG19) to 17 (TBLG1).

Estimated genome length

Using Eqs. (1) and (2), the estimated genome lengths of "LM50" and "TB01" were Ge=3,097 cM, with a 95% confidence interval of 2,854–3,385 cM, and Ge=2,552 cM with a 95% confidence interval of 2,284–2,891 cM, respectively. The observed genome length based on framework maps was 2,683 cM for "LM50", and 1,956 cM for "TB01", respectively. These results showed that the linked markers used to construct our genetic maps would provide 87% coverage of the estimated genome length for "LM50" and 77% for "TB01".

Discussion

Characteristics of AFLPs

Unlike *Populus deltoides*, *Populus trichocarpa* or *Populus nigra* that have been intensively studied on the molecular level, very few studies have been carried out for *P. tomentosa* on the DNA level. In this study, the AFLP marker system was successfully used in genetic mapping for *P. tomentosa*. AFLP is a PCR-based method that offers an efficient and reliable means of generating the DNA markers needed for linkage map construction (Vos et al. 1995). A highly informative pattern of 4 to 27 polymorphic bands per primer pair was obtained, providing a convenient and reliable tool for the construction of genetic maps based on an interspecific backcross population. As compared to RFLP and RAPD analyses, the labor required in detecting polymorphisms with AFLPs is considerably reduced. On average, each primer pair could produce 13 test-cross markers, approximately five times more than that obtained using the RAPD approach in a different interspecific cross-population in section *Populus* (Yin et al. 2001). AFLPs are dominant markers but the reduced information content of dominant markers is compensated for by the use of a backcross or testcross design; many marker loci will have only two genotypes segregating among the backcross offspring: heterozygotes (Aa) and recessive homozygotes (aa). We note that the efficiency of the AFLP marker system might sometimes be affected by the constitution of the selective bases at the 3′-end of each primer. In our study, we found that more informative AFLP markers were generated when the primer combinations contained AG/GA or AT/TA.

Segregation distortion

Segregation distortion of molecular markers has commonly been observed in mapping populations of crops (Bert et al. 1999), forest trees (Bradshaw and Stettler 1994) and fruit trees (Lu et al. 1998). In our study, 22% of AFLP markers in *P. tomentosa* and 13% in *P. tomentosa* × *P. bolleana*, respectively, were found to deviate from the expected 1:1 Mendelian segregation ratio. Similar or lower-distorted segregation ratios were observed in previous efforts to construct genetic linkage maps using inter- or intra-specific crossing populations and AFLP markers: 16.4% in *Lolium perenne* (Bert et al. 1999), 15% in *Eucalyptus globulus* and *Eucalyptus tereticornis* (Marques et al. 1998), and 15% in *Prunus persica* (Lu et al. 1998). Forest trees are outcrossing organisms typically characterized by a high genetic load. It was reported that a recessive lethal allele in the F2 population of *P. trichocarpa* × *P. deltoides* affecting embryonic development was tightly linked to the deviating markers

(Bradshaw and Stettler 1994). Cervera et al. (2001) reported that markers co-segregating with the *Melampsora larici-populina* resistance gene showed a significant deviation in *P. deltoids* due to missing genotypes resulting from the death of susceptible trees. Reasons for skewed segregation ratios of molecular markers are still not well understood, but are generally believed to be related to genetic factors such as: chromosome loss and structural rearrangements (Williams et al. 1995; Kuang et al. 1999), genetic isolating mechanisms (Zamir and Tadmor 1986), the presence of an allele for pollen lethality (Bradshaw and Stettler 1994) or gametic selection (Zamir et al. 1982), as well as other non-biological factors such as sampling in finite mapping populations or scoring errors (Plomion et al. 1995). The high level of segregation distortion obtained in our study indicates that *P. tomentosa* × *P. bolleana* and *P. tomentosa* have close affinity.

Because the grandparent *P. tomentosa* 3082 and the backcrossed parent *P. tomentosa* 3075 originated in the same family, inbreeding depression was a possible reason for segregation distortion in our study. Further studies with additional crosses, larger populations or more molecular markers, would be helpful in investigating the potential reason responsible for segregation distortion of the molecular markers in Chinese white poplar. Regardless of the cause for skewed segregation ratios, such skewing inevitably leads to increased difficulty in both linkage determination and recombination frequency estimation, thereby eventually affecting the map construction (Whitkus 1998). Therefore, all markers deviating at the 1% significance level were excluded from the linkage analysis in order to minimize false linkage during map construction in our study.

Map construction

Grattapaglia and Sederoff (1994) put forward the mapping strategy of pseudo-testcross through testcross configuration in forest trees. Genetic maps have been established for two closely related poplar species, "LM50" and its hybrid "TB01", using a pseudo-testcross strategy and AFLP markers. The genetic maps of "LM50" and "TB01" were comprised of 19 major linkage groups, some minor linkage groups and some unlinked markers, respectively. Ideally, a complete genetic linkage map should contain 19 linkage groups, consistent with the 19 haploid chromosomes in *Populus*. The presence of small groups and unlinked markers in both maps, indicates that some regions are missing in the maps constructed in this study. This may in part be due to the absence of neighboring markers, innate limits of using a single marker technique, or the relatively small size of the mapping population. Therefore, additional efforts will be required to map RFLPs, SSRs, ESTs, allozymes and other valuable markers in a larger mapping population, to coalesce some of these linkage groups together.

Analysis of distributions of informative markers in our maps indicated that certain primer combinations produced more informative polymorphic markers, than others, for mapping. This was the case for E33M38, E63M36, E60M33 and E34M44, although many of the markers generated by E33M38 mapped to the same linkage group TLG13. Apparently, both marker screening experiments and considerable effort in searching for suitable primer combinations are needed for detection of AFLPs on the entire genome. Markers on most linkage groups are randomly distributed in these maps, with obvious gaps ($\geq$30 cM) in linkage groups of TLG1, TBLG1, TBLG2, TBLG3, TBLG6, TBLG7 or TBLG11, which could be eliminated by increasing the number of markers.

To our knowledge, this is the first report on genetic mapping in *P. tomentosa*. As compared to most previous genetic maps constructed for poplar, the population size sampled in this study was larger. The genetic map of *P. tomentosa*, i.e. "LM50", was the better-covered and saturated map. Many co-dominant markers such as SSR and EST have been developed for *P. trichocarpa* and *Populus tremuloides*, unlike that for *P. tomentosa*. Therefore, it is currently difficult to construct a genetic map of *P. tomentosa* with these co-dominant markers due to species' specificity. However, in the future these AFLP "scaffold" maps will be saturated with SSR and EST markers that are specific to *P. tomentosa*, resulting in a much more comprehensive genetic map. Such a map will provide a basis for adding other genetic markers, and ultimately the cloning of major genes and QTLs controlling economically important traits in *P. tomentosa*.

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