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A genetic linkage map of willow (*Salix viminalis*) based on AFLP and microsatellite markers

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Abstract The genus *Salix* (willow) contains a number of species of great value as biomass crops. Efforts to breed varieties with improved biomass yields and resistances to pests and diseases are limited by the lack of knowledge on the genetic basis of the traits. We have used AFLP and microsatellite markers to construct a genetic map of willow from a full-sib cross of the diploid species *Salix viminalis* (2n = 38). In accordance with a double pseudo-testcross approach, separate parental maps were constructed and merged to produce a consensus map comprising 291 AFLP and 39 willow microsatellite markers. Nineteen poplar microsatellites were also tested in willow. Five of these amplified loci, of which two were mapped. Linkage groups of the consensus map that could be identified in the parental maps are presented here and spanned 1,256.5 cM with an average interval between markers of 4.4 cM.

Keywords Willow · *Salix* · Biomass · AFLP · SSR · Genetic map

Introduction

Increased production from renewable energy sources is currently a high priority in Europe in response to the growing need to replace fossil fuels in ways that are non-polluting. Fast-growing biomass crops contribute to the solution, and the European Union is now committed to reach a target for additional biomass energy of 8.5%, or 90 million tonnes of oil equivalent (mtoe) per year, by 2010 (European Commission White Paper, Anon 1997). This equates to planting biomass crops on 7.1% of all EU agricultural and forestry land (10 million hectares). Breeding of high yielding, pest- and disease- resistant biomass crops urgently needs to be accelerated to help meet these targets.

The genus *Salix* (willow) contains a number of species of great value as biomass crops (Gullberg 1993; Åhman and Larsson 1994; Lindegaard and Barker 1997). Over the past few years, significant advances have been made in the breeding of biomass willows (Zsuffa 1990; Gullberg 1993; Åhman and Larsson 1994; Lindegaard and Barker 1997) with cultivars such as Tora and Bjorn (bred in Sweden), and Ashton Stott (bred in the UK), giving maximum biomass yields of 20–22 oven-dried tonnes/ha in trials. However, the scope for further yield increases is still enormous. Furthermore, *Melampsora* rusts (Pei et al. 1996) and insect pests, such as the blue willow beetle (*Phratora vulgatissima*) (Björkman et al. 2000), can reduce yields significantly and there is a need for a continuous supply of high yielding cultivars, with new forms of pathogen and pest resistances, for use in commercial plantations. Varieties that are able to sustain yields in less optimal growing conditions, such as drought or poor soils, or are optimised for different end-uses, are also required.

The ability of willow breeders to select promising parents for crossing and to identify progenies with favourable combinations of characters is hampered by the limited knowledge available on the genetic basis of important traits such as yield and rust resistance. Molecular techniques can contribute directly to accelerate the efficiency of willow breeding by helping to locate genes controlling agronomic traits and providing markers for marker-assisted selections and introgressions of desired traits (Mazur and Tingey 1995). However, identifying the genetic basis of agronomic traits presents a formidable challenge in willow. There are 19 chromosomes in

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the haploid set and the genus is very heterogeneous. Many species are polyploids and numerous inter-specific hybrids occur at all ploidy levels. Willows are also dioecious and highly heterozygous. Homozygous lines are not yet available from any source and crossing among available parents give F_1 that are comprised of varied genotypes from which different types of segregating families may be derived.

Given these constraints, genetic mapping in willows is best achieved through a double pseudo-testcross strategy. This approach assumes that both parents in the cross may carry a high level of heterozygosity and allows markers that are heterozygous in either parent to be detected (Grattapaglia and Sederoff 1994). The double pseudo-testcross approach has been successfully used for genetic mapping in crops of similar genetic complexity to willow, such as poplar (Bradshaw et al. 1994; Cervera et al. 2001), apple (Maliepaard et al. 1998), rubber (Lespinasse et al. 2000) and larch (Arcade et al. 2000).

Here, we report on the use of the double pseudo-testcross approach for the construction of a genetic map of *Salix viminalis*, a willow species commercially important for biomass production, using microsatellite and AFLP markers. This work forms part of a longer-term program to identify markers linked to important agronomic traits in willow.

Materials and methods

Plant material

The K3 mapping population comprises 66 siblings from a cross between two full-sib parents, known to differ in resistance to Melampsora rust, derived from two unrelated, non-inbred willows (Åhman 1997). The cross was produced in Sweden (at Svalöf-Weibull AB) and 15-cm cuttings of grandparents, parents and progeny were supplied for planting at IACR-Long Ashton Research Station, UK (IACR-LARS).

DNA extraction

Genomic DNA was extracted from fresh leaf tissue using the Nucleon Phytopure Plant DNA Extraction Kit (Amersham Biosciences) with the addition of 10 mM 2-mercaptoethanol to Reagent 1. The quantity and quality of DNA was assessed by agarose-gel electrophoresis using 0.8% agarose with known concentrations of uncut lambda DNA (Sigma). Gel images were captured using the Gel Doc 2000 Gel Documentation System (BIO-RAD) and DNA concentrations calculated using Quantity One software (BIO-RAD).

AFLP markers

Initial AFLPs were performed using the magnetic bead method for fragment selection followed by a single selective PCR amplification (Zabeau and Vos 1993) with genomic digests carried out using *Mse*I and *Pst*I restriction enzymes. Amplifications were based on *Mse*I primers with three selective nucleotides and *Pst*I primers with two selective nucleotides, as described in Barker et al. (1999). To improve throughput, AFLPs were later performed according to Vos et al. (1995) with genomic digests carried out using *Mse*I and *Eco*RI restriction enzymes. After ligation of adaptors, pre-amplification with a single selective nucleotide at the 3′-end of both the *Eco*RI and *Mse*I primers was followed by selective amplification using three selective nucleotides per primer. In total, 48 primer combinations were used, as listed in Table 1. Primers were synthesised and de-salted by Sigma-Genosys Ltd. Segregating AFLP markers were scored independently by two persons and segregation codes assigned according to the system used in JoinMap and described in Maliepaard et al. (1997) as follows: *abxaa* for markers segregating in the male parent only; *aaxab* for markers segregating in the female parent only; *a0xa0* for markers segregating in both parents.

Microsatellite markers

From a microsatellite-enriched, small-insert genomic DNA library previously developed from *Salix burjatica*, Germany (Edwards et al. 1996), 530 clones were sequenced for development of microsatellite markers. Prior to primer design all library sequence data was screened for the presence of duplicated inserts using Autoassembler software (Applied Biosystems). Forward and reverse primers (21-mers) were designed for the flanking sequences of the microsatellite loci and were synthesised and de-salted by Sigma-Genosys Ltd. Primer pairs were tested for their ability to amplify polymorphic PCR products from the genomic DNA of the parents and grandparents of the K3 mapping population. Informative pairs were then used to amplify genomic DNA from the 66 full-sib progeny of the K3 mapping population, together with the parents and grandparents for direct comparisons on the same gels. All autoradiographs were scored independently by two persons. Segregation codes were assigned according to the system used in JoinMap and described in Maliepaard et al. (1997) as follows: *abxaa* for markers segregating in the male parent only (two alleles); *aaxab* for markers segregating in the female parent only (two alleles); *abxab* for markers segregating in both parents with two alleles; *abxac* for markers segregating in both parents with three alleles; *abxcd* for markers segregating in both parents with four alleles. Details of microsatellite primer sequences can be obtained from the authors, on request.

Both radioactive and fluorescent labelling techniques were employed for visualisation of PCR products. For radioactive detection, the forward primer of each microsatellite pair was end-labelled with [γ-^{33}P]ATP using T4 polynucleotide kinase (Amersham Biosciences). PCR reaction mixtures contained 10 ng of template DNA, 25 ng of labelled forward primer, 25 ng of reverse primer, 200 µM of each dNTP (Promega), 0.5 U *Taq* DNA polymerase (GibcoBRL), 20 mM Tris–HCl pH 8.4, 50 mM KCl and 1.5 mM $MgCl_2$. Thermocycling conditions were as follows: 35 cycles of 94 °C for 40 s, 54 °C for 60 s and 72 °C for 60 s, followed by a final 72 °C extension period of 10 min. A GeneAmp PCR system 9700 (Applied Biosystems), with a 9600 ramping setting, was used for all PCRs. Amplification products were mixed with an equal volume of 2 × Gel Loading Buffer (98% formamide, 10 mM EDTA, 0.01% xylene cyanol and 0.01% bromophenol blue) and denatured at 94 °C for 3 min. Products were separated on 6% denaturing polyacrylamide gels and electrophoresed at 55 W power for approximately 2 h. Gels were dried and exposed to Kodak Biomax MR-1 film overnight. For fluorescent detection, PCR products were labelled using fluorescently tagged 2′-deoxycytidine 5′-triphosphate ([F]dCTP) coupled to [R110], [R6G] or [TAMRA] (Applied Biosystems), according to the Manufacturers' instructions. Separation of PCR products was performed on an ABI PRISM 377 DNA Sequencer using GeneScan-500 [ROX] as the size standard (Applied Biosystems). Genotyper software (Applied Biosystems) was used to analyse electropherograms.

Poplar microsatellite primers

Nineteen poplar microsatellite primer pairs, developed by Dr. T. Bradshaw (University of Washington, USA), were tested on willow DNA. Primer sequences can be retrieved from the Poplar Molecular Genetics Co-operative SSR database (http://poplar2.

Table 1 Characteristics of polymorphic AFLP markers generated from 48 primer combinations. Restriction enzymes/adaptors/primers used in AFLP production are indicated for each combination (*E* = *Eco*RI, *M* = *Mse*I and *P* = *Pst*I). Additional characters indicate selective nucleotides

Primer combination	Male markers[a]	Female markers[b]	Heterozygous markers[c]	Total polymorphic markers
EAAC/MAAG	4	1	3	8
EAAC/MAAT	0	1	0	1
EAAC/MACC	2	1	2	5
EAAC/MTTG	4	4	0	8
EACA/MACA	5	1	2	8
EACA/MACG	0	0	1	1
EACA/MCTA	2	1	4	7
EACA/MCTC	0	3	1	4
EACA/MTGT	0	1	0	1
EACA/MTTG	5	1	0	6
EACG/MCTA	3	1	2	6
EACT/MAAG	2	2	2	6
EACT/MAAT	0	1	3	4
EACT/MACA	4	3	0	7
EACT/MACC	2	2	0	4
EACT/MGAA	3	6	3	12
EACT/MTTG	0	1	0	1
EAGA/MTGT	1	3	1	5
EAGA/MTTG	2	1	4	7
EAGC/MACC	0	1	4	5
EAGC/MGAA	1	1	4	6
EAGC/MTGT	2	1	3	6
EAGC/MTTG	2	2	2	6
EATA/MTTG	2	2	2	6
MAAG/PCA	9	2	4	15
MAAT/PAC	5	4	10	19
MACA/PAA	7	14	18	39
MACA/PCA	7	6	5	18
MACA/PCC	6	8	11	25
MACC/PAC	4	3	5	12
MACC/PCC	6	4	2	12
MCCG/PAA	2	0	7	9
MCCG/PAC	2	4	0	6
MCCG/PCA	0	2	2	4
MCCG/PCC	1	2	3	6
MCTA/PCA	4	6	6	16
MCTA/PCC	4	3	5	12
MGAA/PAA	4	4	7	15
MGAA/PAC	5	2	6	13
MGAA/PCA	5	4	4	13
MGAA/PCC	4	4	5	13
MGGC/PAA	2	7	8	17
MGGC/PAC	3	1	1	5
MGGC/PCA	4	2	9	15
MGGC/PCC	5	6	2	13
MTTG/PAC	2	6	6	14
MTTG/PCA	2	4	7	13
MTTG/PCC	4	6	13	23
Total	143	145	189	477

[a] *abxaa* marker; present in the male parent only; segregating 1:1 in the progeny
[b] *aaxab* marker; present in the female parent only; segregating 1:1 in the progeny
[c] *a0xa0* marker; heterozygous in both parents; segregating 3:1 in the progeny

cfr.washington.edu). Primers were used in PCR with genomic DNA from two willows (*Salix burjatica*, Germany and *S. viminalis*, Astrid) and one poplar (hybrid *Populus deltoides* × *Populus nigra*, Ghoy). PCR conditions were the same as described for willow. PCR products were separated by agarose-gel electrophoresis using 2% agarose. Gel purification and direct sequencing of amplification products from primer pair PMGC223 was performed according to Hanley et al. (2000). Resulting sequences were aligned using Autoassembler software (Applied Biosystems).

Map construction

All data analysis was performed using JoinMap version 3.0 software (Van Ooijen and Voorrips 2001). This programme was chosen as it has the capability to perform linkage analysis on outbred progenies involving markers of different segregation type. As is the case for JoinMap 2.0, version 3.0 uses the estimation procedures for cross-pollinators as described in Maliepaard et al. (1997). This procedure uses all available information for any combination of markers, segregating from two alleles in just one parent, through two alleles in both parents, to three and four segregating alleles in both parents. The JoinMap 3.0 software was used to test markers for segregation distortion using a chi-square test. AFLP markers with highly significant levels of distortion ($P < 0.005$) were excluded from further analyses. Parental linkage maps were constructed using AFLP and microsatellite markers heterozygous in one parent only (types *abxaa* and *aaxab*). In addition, microsatellite markers of types *abxac* and *abxcd* were used but were first separated so that only alleles from either the male or female parent were employed for construction of each respective parental map. For the integrated map, all marker data, including that for AFLP markers of type

a0xa0 and microsatellite markers of type *abxab*, were utilised. For all maps, linkage groups were determined using a minimum LOD threshold of 4.0 and map construction performed using the Kosambi mapping function with the following JoinMap parameter settings: Rec = 0.45, LOD = 1.0, Jump = 5. Resulting linkage maps were drawn using MapChart software (Voorrips 2001). Robustness of the integrated linkage map was tested by comparison of marker orders with separate parental maps.

Results

AFLP markers

In total, 477 polymorphic AFLP markers were scored from 48 primer combinations (Table 1). Of these, 143 were *abxaa*, 145 were *aaxab* and 189 were *a0xa0*. The average total number of bands identified per assay was 56 for the bead selection method and 41 for the two-step amplification method. The percentages of scorable polymorphic markers for these two methods were 35% and 18%, respectively. Following analysis of genotype frequencies for each marker, approximately 15% of markers showed segregation distortion ($P < 0.1$: chi-square test). Seventeen distorted markers with P values less than 0.005 (about 4% of markers) were discarded prior to construction of the parental and consensus linkage maps.

Microsatellite markers

From the willow microsatellite-enriched library, 160 unique primer pairs were designed and screened for their ability to detect polymorphism in the mapping population. Of these, 98 (61%) amplified easy to score, single loci, of which 53 (33%) were polymorphic between the K3 parents and could be mapped. Significant levels of segregation distortion ($P < 0.1$: chi-square test) were observed for 12 (23%) of the microsatellite loci; however, all loci were included in linkage analyses.

Of the 19 poplar microsatellite primer pairs tested on willow, five successfully amplified willow DNA (PMGC93, PMGC108, PMGC223, PMGC456 and PMGC2020). To determine whether corresponding poplar microsatellite loci were amplified in willow, PCR products from PMGC223 were sequenced and alignments performed. Resulting sequences indicated that the microsatellite motifs of the two willow and poplar loci were identical, although some minor differences in the flanking regions were detected (data not shown).

Construction of parental maps

On examination of individual genotype frequencies, two offspring were shown to have missing genotype data for a large number of AFLP markers and were excluded from further analysis.

For the paternal map, a total of 132 AFLP markers and 24 microsatellite markers showed segregation in the mapping population and were included in linkage analysis. At a LOD threshold of 4.0, 79% of markers could be assigned to 21 linkage groups each containing a minimum of three markers. In addition, seven duplets were formed and 19 markers remained unlinked. Marker-ordering proved problematic for one linkage group obtained at LOD threshold 4.0 (corresponding to Group V of the consensus map). However, at a LOD threshold of 4.5, a subset of five markers split from this group allowing remaining marker orders to be readily determined. For all other groups, map construction proved straightforward, with no markers presenting problems with respect to goodness-of-fit. The resulting 21 linkage groups spanned 733.3 cM and the average interval between markers was 7.8 cM.

For the maternal map, 139 AFLP markers and 33 microsatellite markers were included in linkage analysis. Groupings performed with a LOD threshold of 4.0 resulted in the assignment of 83% of markers to 25 linkage groups, with six duplets and 17 markers remaining unlinked. Marker orders for all groups were determined without difficulty. The resulting linkage groups spanned 911.2 cM with an average interval between markers of 8.0 cM.

Construction of the K3 consensus map

The consensus map was constructed based on 132 *abxaa* AFLP markers, 139 *aaxab* AFLP markers, 189 *a0xa0* AFLP markers and 55 microsatellites of all segregation types. Marker groupings at a LOD threshold of 4.0 resulted in the formation of 38 linkage groups containing three or more markers, with 11 duplets and 41 markers remaining unlinked. Two groups (I and II) in the consensus map incorporated previously unlinked groups from the parental maps. Calculation of recombination frequencies between all marker pairs of the resulting linkage groups led to the identification of a number of ‘suspect’ linkages in three groups I, V and IX, i.e. recombination frequency estimates were greater than 0.6. In all such cases, one marker of each pair was shown to be of the *a0xa0* type. Where highly suspect linkages were observed, as defined by a recombination frequency > 0.7 and a LOD > 1, the *a0xa0* marker involved was discarded from further analyses. For group V, also problematic in linkage group resolution with the paternal map, a suspiciously large number of dubious linkages were highlighted at LOD 4.0. On closer inspection this group was found to contain subgroups of markers that showed no linkage in the parental maps. An increase in LOD threshold to 7.0 was required to split this group into statistically more robust groups with no suspect linkages.

Marker orders were determined only for those linkage groups that could be identified in one or both parental maps. For most groups, orders were determined for all assigned markers without difficulty under the relatively stringent mapping parameters selected. In cases where markers could not be easily placed, the capability of

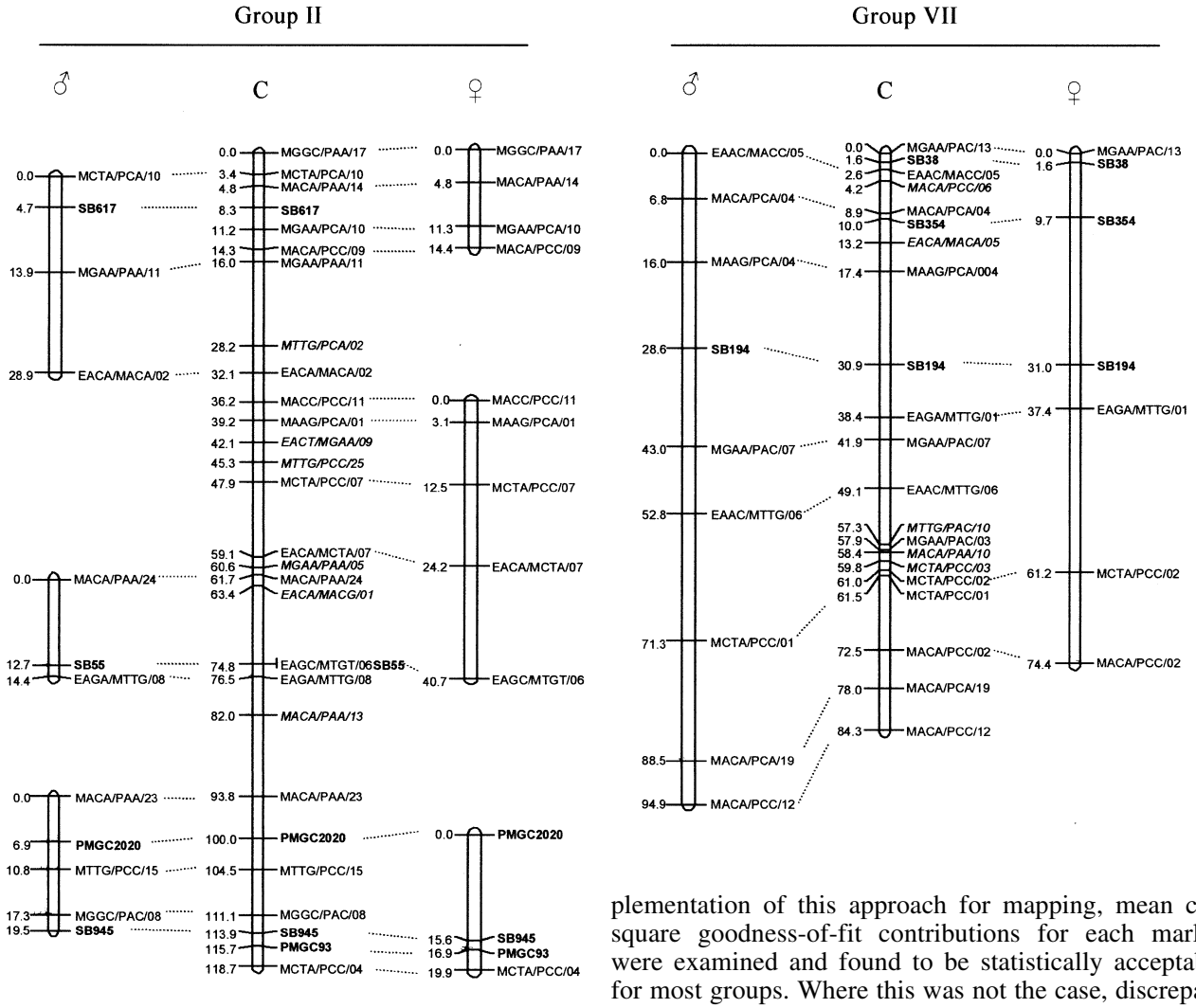


Fig. 1 Alignment of paternal (♂), maternal (♀) and consensus (C) maps for linkage groups II and VII of the K3 willow map. Marker names are shown on the right of each group with map distances (in cM) indicated on the left. For details of AFLP marker nomenclature see Table 1. AFLP markers heterozygous in both parents (*a0xa0*) are indicated in *italics*. Microsatellite markers are indicated in *bold type* with prefixes SB- for those developed from willow, and PMGC- for those developed from poplar. Corresponding marker loci are indicated by *dotted bars* between maps

JoinMap software to force problematic markers on to a map in a 'third round' of ordering was not employed. Instead, these markers were discarded.

When corresponding linkage groups of the parental and consensus maps were aligned, the map order of markers present in both a parental map and the consensus map were identical for most linkage groups. The alignments for two groups are shown in Fig. 1. However, marker orders for six groups were not in complete agreement across maps. In cases where slight differences in order were observed (groups I, III, VI, VIII, IX and XIX), the 'fixed order' feature of JoinMap was used to force the more robust parental map orders on to the corresponding groups of the consensus map. Following implementation of this approach for mapping, mean chi-square goodness-of-fit contributions for each marker were examined and found to be statistically acceptable for most groups. Where this was not the case, discrepancies in marker order were attributed to inclusion of a number of *a0xa0* AFLP markers. However, when these markers were removed, map orders were re-calculated and shown to be in agreement with those expected from the parental maps. The resulting 27 linkage groups of the K3 consensus map are shown in Fig. 2 and comprise 217 AFLP markers of types *aaxab* and *abxaa*, 74 AFLP markers of type *a0xa0* and 41 microsatellites of all types. Markers showing segregation distortion were detected on 11 of the linkage groups with obvious clusters evident on groups I, IV, VIII, X and XIX. The map distance for those groups analysed was 1,256.5 cM with an average interval between markers of 4.4 cM.

Discussion

Marker identification

The AFLP technique was used here as the procedure generates a large number of polymorphic markers per assay (Vos and Kuiper 1997) and is highly reproducible (Jones et al. 1997). Microsatellites are co-dominant and highly informative in the linkage analysis of outbreeding popu-

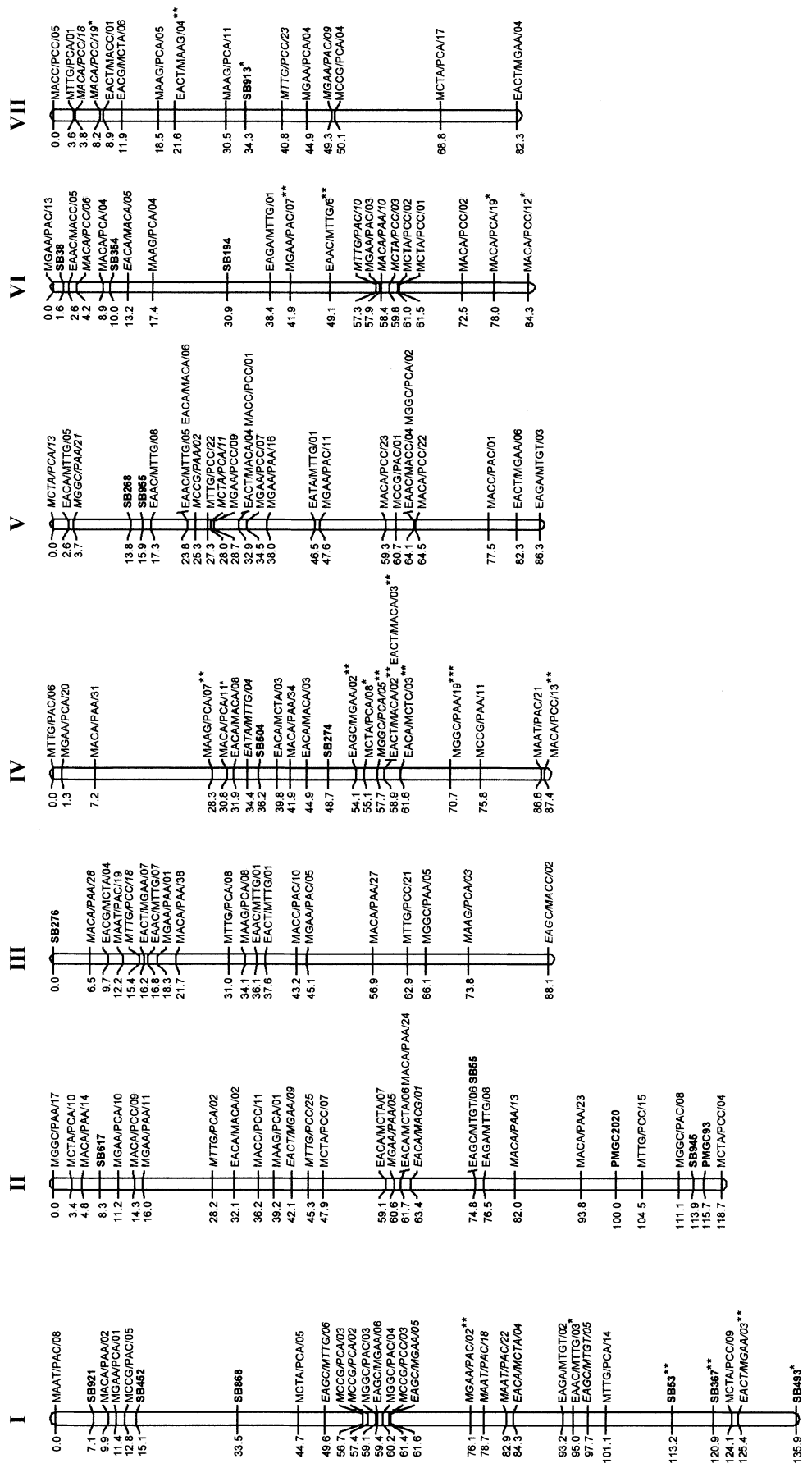
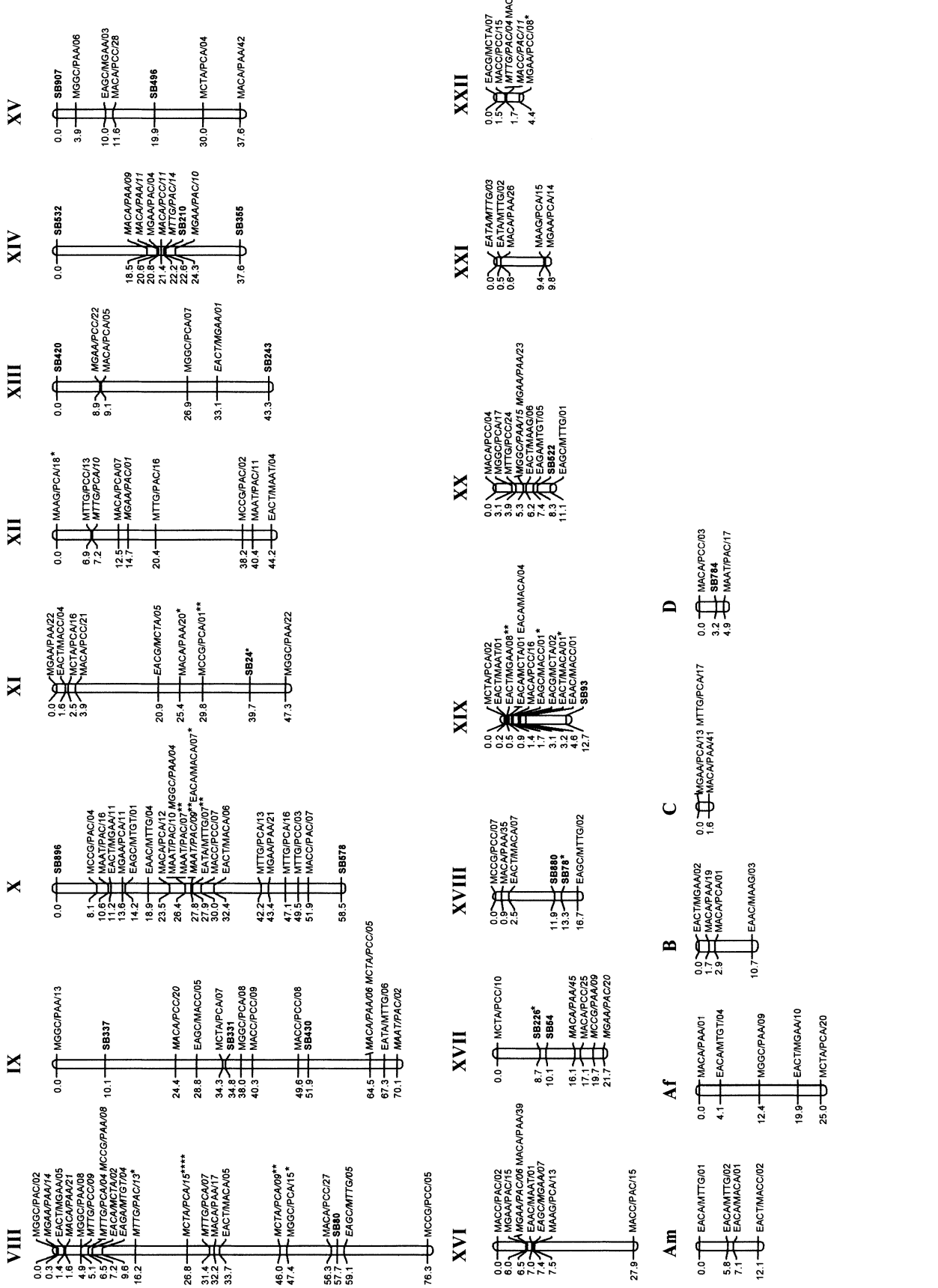


Fig. 2 The K3 consensus map of willow comprising 291 AFLP and 41 microsatellite markers. Linkage groups derived from both parents are numbered I–XXII. Also included are five linkage groups (A–D) that comprised markers from either the male or female parent exclusively and remained unlinked to other groups of the consensus map. Linkage groups Am and Af showed linkage in the presence of two *a0xa0* AFLP markers and are likely to represent a single linkage group of the consensus map. Marker names are shown on the *right* of each group with map distances (in cM) indicated on the *left*. Microsatellite markers are indicated in *bold type* with prefixes SB, for those developed from willow, and PMGC, from those developed for poplar. For details of AFLP marker nomenclature see Table 1. AFLP markers of type *a0xa0* are shown in *italics*. Markers showing significant levels of segregation distortion are indicated by *asterisks* (*: $P < 0.1$, **: $P < 0.05$, ***: $P < 0.01$, ****: $P < 0.005$, *****: $P < 0.001$, ******: $P < 0.0005$)

Fig. 2 (continued)



lations and can be used for anchoring maps derived from different populations. Both microsatellites and AFLPs can also be automated and multiplexed. Restriction fragment length polymorphism (RFLP) markers, although co-dominant, were not used in this study as our efforts are aimed at high-throughput marker analysis in willow.

Both AFLPs and microsatellites detected a large number of polymorphic loci, which is commensurate with the outbreeding/heterozygous nature of this species. Less polymorphic AFLP markers were produced using a two-step PCR (Vos et al. 1995) compared with magnetic bead selection (Zabeau and Vos 1993). This may be due to the different restriction enzymes used or the inclusion of an additional selective nucleotide on the *Eco*RI primer in the selective amplification of the two-step protocol. However, the lower polymorphism obtained was outweighed by an increase in throughput and a reduction in genomic DNA consumption. Two restriction enzyme combinations, *Mse*I/*Pst*I and *Mse*I/*Eco*RI, differing in sensitivity to cytosine methylation, were used in an attempt to achieve a wider distribution of markers across the genome (Young et al. 1999).

Poplar microsatellites were found that successfully amplified willow DNA. Sequence data obtained for PMGC223 indicated that the corresponding locus was amplified. Failure of 14 of the 19 poplar primers tested could be attributed to sub-optimal PCR conditions, although the difference in the sequence of the flanking regions suggests that prevention of specific primer annealing may also be a cause. The two mapped poplar microsatellites (PMGC93 and PMGC2020) showed linkage on Group II of the willow map although they were not reported to be linked in poplar. However, PMGC2020 mapped to a different group in the *Populus trichocarpa* map compared to the *P. deltoides* and *P. nigra* maps (Cervera et al. 2001).

Segregation analysis

The identification of markers displaying distorted segregation ratios is to be expected in a full-sib cross for an outcrossing species and has previously been described for a number of tree species (Bradshaw and Stettler 1994; Cai et al. 1994; Grattapaglia and Sederoff 1994; Lanaud et al. 1995; Krutovskii et al. 1998; Marques et al. 1998; Arcade et al. 2000). The percentage of distorted markers detected here (18%) is similar to that observed in linkage analyses of pine (14–15%; Kubisiak et al. 1995) and oak (18%; Barreneche et al. 1998). Markers can show distorted segregation due to linkage to a gene affecting viability (e.g. Lorieux et al. 2000; Cervera et al. 2001). Gametic and zygotic selection can affect linkage analysis in different ways for dominant and co-dominant markers (Lorieux et al. 1995). In willow, the genetic load has been estimated as about 1.8 lethal equivalents (Kang et al. 1994). If a marker is distorted because it is linked to a gene affecting viability then, due to linkage alone, all markers in that region should display distorted segregation ratios. Clusters of distorted markers have been observed in a number of tree species including poplar (Cervera et al. 2001), eucalyptus (Verhaegen and Plomion 1996), pine (Kubisiak et al. 1995), oak (Barreneche et al. 1998), and now willow. Highly distorted markers that do not map to such clusters should be treated with caution. AFLP markers with highly skewed segregation ratios may represent superimposed loci of equivalent electrophoretic mobility that segregate independently in the mapping population and may result in erroneous maps if included in analyses. For these reasons, the most-highly distorted AFLP markers identified in this study were discarded. However, not all distorted markers were excluded from the mapping analysis as further studies of such markers could lead to the identification of regions of interest (e.g. Lorieux et al. 2000; Cervera et al. 2001). Markers linked to sex were not found among those tested here.

Mapping strategy

Several methods have been suggested for linkage analysis of outbreeding populations (Ritter et al. 1990; Arús et al. 1994; Ritter and Salamini 1996; Maliepaard et al. 1997), of which the double pseudo-testcross strategy (Grattapaglia and Sederoff 1994; Hemmat et al. 1994; Grattapaglia et al. 1995) was employed here. For construction of each parental map, AFLP and microsatellite markers that segregated only in the respective parent and, in addition, parental alleles from microsatellites of types *abxac* and *abxcd*, were used. Inclusion of markers segregating in both parents results in recombination frequency estimates for heterozygous marker pairs averaged for both male and female meioses and may differ from parental estimates, giving rise to conflicting marker orders between parental and consensus maps.

Parental maps

For both the paternal and maternal maps, the number of resolved linkage groups exceeded the haploid chromosome number of willow (n = 19) suggesting that additional markers will be required to bridge gaps between linkage groups and improve resolution. The smaller total map length of the paternal map (733.3 cM) in comparison to the maternal map (911.2 cM) suggests that a number of regions are still under-represented by those markers available in the paternal data set.

The K3 consensus map

As with the parental maps, the number of resolved linkage groups exceeded the willow haploid chromosome number. The incorporation of multiple groups of the parental maps into single consensus groups I and II suggests that linkage-group resolution may be improved

with additional markers in the future. The inclusion of AFLP marker data of type *a0xa0* in construction of the consensus map resulted in the identification of additional linkage groups that were not represented in the parental maps. Given that the mapping population was a full-sib cross, large regions of the resulting K3 map would be expected to comprise only AFLP markers of this class. In linkage analysis of outbred populations, *a0xa0* markers can be extremely uninformative in certain configurations and very often lead to recombination frequency estimates of 0.0 (Maliepaard et al. 1997). A number of these markers were, thus, excluded in construction of the consensus map in order to achieve co-alignment with homologous linkage groups of the parental maps. However, inclusion of the *a0xa0* marker class is beneficial for determination of linkage between maternal and paternal markers (types *aaxab* and *abxaa*), which cannot be established directly. This was exemplified by groups Am and Af, which showed linkage in the presence of two *a0xa0* AFLP markers in the consensus-map data set, although these did not provide sufficient linkage information to allow map construction for this group as a whole. Suspect recombination estimates for a number of marker pairs may be a consequence of multiple homoeologous loci existing in the genome. If two homoeologous segments of the same AFLP fragment were amplified, being heterozygous in one parent at both loci and homozygous absent at both loci in the other, a band would only be observed in one parent but would be over-represented in the full-sib offspring. Such scenarios would influence recombination estimates and result in suspect linkages.

The AFLP markers gave good map coverage across all linkage groups. The identification of obvious AFLP clusters on groups I, III, VIII, XIV, XVI and XIX has also been observed in poplar (Wu et al. 2000), rose (Debener and Mattiesch 1999) and soybean (Young et al. 1999), and may be explained by reduced recombination in chromosomal regions such as centromeres (Tanksley et al. 1992; AlonsoBlanco et al. 1998). Microsatellite markers were located on 19 of the 27 linkage groups and also showed good distribution across the map.

By employing relatively stringent parameters for analysis, and by comparison of marker orders and map distances with parental maps, a statistically robust consensus map of willow has been constructed. The parental maps may prove useful in the detection of major QTLs segregating from a single parent while the consensus map will be used for the detection and allelic dissection of QTLs from both parents using an all-marker approach (Knott and Haley 1992; Maliepaard and Van Ooijen 1994). However, it should be noted that only QTLs with a large effect are likely to be detected in a small population such as K3. Consequently, larger mapping populations have been established for trait mapping and are currently under study.

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