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An AFLP and RFLP linkage map and quantitative trait locus (QTL) analysis of growth traits in *Salix*

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Abstract A genetic linkage map of *Salix* (2n = 38), composed of 325 AFLP and 38 RFLP markers has been constructed. The map was based on a population (n = 87) derived from a cross between the male hybrid clone "Björn" (*Salix viminalis* × *Salix schwerinii*) and the female clone "78183" (*S. viminalis*). Three hundred fifty seven AFLPs corresponding to DNA polymorphisms heterozygous in one parent and null in the other were scored. A total of 87 RFLP probes, most (83) derived from the *Populus* genome, yielded 39 and 11 polymorphic loci segregating in a 1:1 and 1:2:1 ratio respectively. Two maps, one for each parent, were constructed according to the "two-way pseudo-testcross" mapping strategy. The *S. viminalis* × *S. schwerinii* map (2,404 cM) included 217 markers and formed 26 major linkage groups while *S. viminalis* (1,844 cM) consisted of 146 markers placed on 18 major groups. In addition, eight and 14 additional minor linkage groups composed of less than four markers (doubles and triplets) were obtained in the *S. viminalis* × *S. schwerinii* and the *S. viminalis* maps, respectively. Both maps provided 70–80% genome coverage with an average density of markers of 14 cM. To investigate possible homologies between the parental maps, 20 AFLPs and 11 RFLPs segregating in 3:1 or 1:2:1 ratios were included in the linkage analysis. Eight linkage groups homologous between the two maps were detected. The present genetic map was used to identify quantitative trait loci (QTLs) affecting growth-related traits. Eleven QTLs were identified; seven QTLs for height growth, one QTL for stem diameter, one QTL for the height: diameter ratio, one QTL for the number of vegetative buds during flowering time and one QTL for the number of shoots. The estimated magnitude of the QTL effect ranged from 14 to 22% of the total phenotypic variance. One QTL associated with height growth and one affecting the height: diameter ratio were overlapping in the same marker interval with the QTL affecting stem diameter. QTL stability over years was estimated for traits measured in multiple years. Generally, QTLs were only significant in a single year although two QTLs for height growth were close to reaching the significance level in 2 consecutive years.

Keywords AFLP · RFLP · Genetic map ·*Salix* · QTL for growth-related traits

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Introduction

The genus *Salix*, family *Salicaceae*, comprises more than 300 species including trees, shrubs and creeping shrublets (Larsson and Bremer 1991). *Salix* are widespread in both Northern and Southern hemisphere, excluding Australasia and New Guinea. Owing to their fast growth, ease of establishment and wide range of adaptability, *Salix* spp. are the most-widely used species for biomass production in short-rotation intensive culture (SRIC) systems (Andersson et al. 1983; Gullberg 1993; Zsuffa et al. 1993). The shrub species, *Salix viminalis*, *Salix dasyclados*, *Salix schwerinii* and their hybrids, are among the most-broadly used species in SRIC systems.

The application of quantitative genetics, based on phenotypic data, is the most-common strategy in forest-tree breeding. While phenotypic assessment provides reliable figures for genotypic evaluations, it is time consuming. Molecular markers and genetic maps offer new opportunities to study the genetics of quantitative traits. The advent of molecular markers directly based on DNA sequences, such as RFLPs, recovered many loci and allowed the construction of linkage maps in forest trees (*Populus*: Bradshaw et al. 1994; *Pinus taeda* L.: Devey et al. 1994). In the last decade, the development of arbitrarily primed markers based on PCR and RAPD strategies (Williams et al. 1990), significantly aided the con-

struction of high-density genetic maps. In addition, the invention of the AFLP (amplified fragment length polymorphism) technique (Vos et al. 1995) provided a new class of highly polymorphic markers combining both RFLP and PCR strategies. The AFLP technique has been recently used to rapidly create genetic maps in several agricultural plants (Becker et al. 1995; Van Eck et al. 1995; Keim et al. 1997; Wang et al. 1997; De Riek et al. 1999) and forest-tree species *Eucalyptus globulus*, *Eucalyptus tereticornis*: Marques et al. 1998; *Larix*: Arcade et al. 2000; *Populus*: Frewen et al. 2000; Wu et al. 2000; Cervera et al. 2001).

Using molecular-linkage maps, it is possible to locate quantitative trait loci (QTLs) affecting economically important traits. QTLs can be further studied in terms of the magnitude of their effects on the phenotype, the mode of their gene action, the parental origins of the favorable QTL alleles, and the relationships between QTLs underlying different physiological processes.

Earlier genetic studies have shown that it is possible to map QTLs for growth and adaptive traits in forest trees (Bradshaw and Stettler 1995; Grattapaglia et al. 1995, 1996; Kaya et al. 1999; Frewen et al. 2000). Although the potential of QTL mapping has been examined in several forest-tree species, there are no such studies on species in the genus *Salix*. This would be valuable, however, since *Salix* species offer good model systems for biological studies due to their small genome sizes (2C = 0.76–0.98 pg; Thibault 1998) and short time to flowering (second year). Owing to their well-developed asexual propagation systems, *Salix* offers the possibility to reduce non-genetic variance, and increase the power and accuracy of QTL mapping. Growth traits, i.e. annual height growth, diameter and number of shoots, are important components of the biomass production in SRIC systems. The presence of additive genetic variance in growth traits has been well-demonstrated in several factorial crossings of *S. viminalis* (Rönnberg-Wästljund and Gullberg 1999; Rönnberg-Wästljund 2001). Molecular knowledge on the inheritance of such economically important traits will permit the production of improved phenotypes through more-effective and less time-consuming selection and breeding.

In the present paper we report a genetic map of *Salix* (2n = 38) based on a two-way pseudo-testcross strategy, where each parent of the cross is mapped (Grattapaglia and Sederoff 1994). The two maps (male, female) were composed of AFLP and RFLP markers, with particular emphasis on AFLPs, using a cross of a (*S. viminalis* × *S. schwerinii*) hybrid to *S. viminalis*. Results from our QTL mapping for growth-related traits, measured over 4 consecutive years, are presented.

Materials and methods

The mapping pedigree

A full-sib family, produced by Svalöf Weibull AB, was used as our mapping population. This family was derived from a cross between the male hybrid clone "Björn" (*S. viminalis* × *S. schwerinii*) and the female clone "78183" (*S. viminalis*). More than 100 progenies were planted outdoors at the experimental station of Pustnäs, Uppsala, Sweden. A set of 94 progenies was included in the mapping population.

DNA-isolation

Genomic DNA was isolated from fresh non-expanded leaves taken from the apical stem of growing plants. The isolation of DNA for RFLP analysis was performed essentially as described by Sharpe et al. (1995). For the AFLP procedure the DNA was isolated according to the Fast Prep protocol and kits (BIO 101, Vista Calif.) modified by adding 1.25% polyvinylpyrrolidone (average mol wt 40,000), 0.2% 2-mercaptoethanol and 0.5% ascorbic acid to the extraction buffer. The DNA concentration was measured by a fluorometer.

RFLP analysis

Anonymous DNA probes (mostly 2–3 kb) from the *Populus* genome were utilized for RFLP analysis. The preparation and characteristics of those probes have been described previously (Bradshaw et al. 1994). DNA digestion with restriction enzymes (*Eco*RI, *Eco*RV and *Bam*III), electrophoretic separation, blotting onto hybond N^+ nylon membranes and hybridization were performed essentially as described by Sharpe et al. (1995). Probes were labelled with ^{32}P *d*CTP using the Amersham-Pharmaciabiotech oligolabelling kit. Membranes were washed three times for 15 min (two times at room temperature and one at 60 °C) with 2 × SSC, 0.1% SDS and wrapped in plastic foil before exposure to Kodak (BIOMAX MS) films for 1–3 days at –70 °C. Membranes were re-used after 10-min incubation in boiled 0.1% SDS and 0.1 × SSC stripping solution. The stripping procedure was repeated 1–2 times to ensure complete removal of the probe.

AFLP analysis

Amplified fragment length polymorphism (AFLP) marker analysis was performed using fluorescent labeled primers essentially as described by Schwarz et al. (2000), with the following modifications. The adaptor sequences were: 5′-*CTCGTAGACTGCGTACC*-3′, 3′-*CTGACGCATGGTTAA*-5′ (*Eco*RI adaptors), 5′-*GACGATG-AGTCCTGA*-3′, 3′-*TACTCAGGACTCAT*-5′ (*Mse*I adaptors). PCR conditions involved: preamplification, 30 cycles of 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C; selective amplification, 2 min at 94 °C; 13 cycles of 30 s at 94 °C, 30 s at 65 °C (reduced by 0.7 °C/cycle) and 2 min at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C, followed by 10 min at 72 °C. Selective amplification was performed with *Eco*RI+2 primers labeled with 5-carboxy-fluorescein (5-FAM), or N,N,N,N-tetramethyl-6-carboxyrhodamin (TAMRA) and 1.5 µl of a denaturated mixture of [0.5 µl of a FAM PCR product, 0.7 µl of a TAMRA-labeled PCR product, 0.33 µl of a 6-carboxy-X-rhodamin (ROX)-labelled internal length standard GeneScan-500 (ROX), and 1.63 µl of formamide dye (74% formamide, 26% dextran blue)] was loaded on a 4.25% denaturing polyacrylamide (4.25% acrylamide/bisacrylamide 19/1, 8.3 M urea in 1 × TBE) gel on an ABI Prism 377 DNA Sequencer (Perkin Elmer LTD). A total of 60 primer combinations were used.

The obtained data was imported to GENESCAN 2.1 (PE/Applied Biosystems). Sizing of the fragments (100–450 bp) was performed with the internal lane standard according to the local Southern algorithm (Southern 1979) following a manual lane tracking. Data from GENESCAN was exported to GENOGRAPHER software for scoring (Benham et al. 1999). Lanes were normalized based on the total signal intensity of the lane and sizing was again performed by the local Southern algorithm. The obtained scoring was cross checked by the GENOTYPER 2.0 software in which fragments were detected by seven pre-made macros similar to those described in the GENOTYPER 2.0 AFLP Demo manual (PE/Applied Biosys-

tems). Both programs yielded similar results. Polymorphic fragments with similar sizes (≤0.5 bp), and bands not showing a clear segregation pattern based on both programs, were not included in the analysis. The final genotype data was exported as a text file for further use in mapping programs.

Twelve additional *Eco*RI–*Mse*I primer combinations, with +3 selective bases each, were also utilized. For these AFLP gels, the primers complementary to the *Eco*RI adapter (selective amplification) were endlabeled with γ-^{33}P-ATP as in Vos et al. (1995). These gels were scored manually.

Linkage analysis

Initially, markers heterozygous in one parent and null in the other were selected. Thus, two sets of markers were obtained, one for each parent, and two linkage maps (*S. viminalis* × *S. schwerinii*, *S. viminalis*) were produced (Grattapaglia and Sederoff 1994). Each marker was tested for deviation from a 1:1 Mendelian segregation ratio by χ^2-analysis ($\alpha = 0.05$). The linkage analysis was primarily performed with MAP-MANAGER QTXb-10.0 (Manly and Olson 1999). Loci exhibiting 1:1 segregation ratios were assigned to linkage groups with a pair-wise test ($\alpha = 0.001$). Then, linkage in the repulsion phase for all loci was examined using the commands "flip phase" and "links". A set of the same data was tested for linkage using MAPMAKER (version 3.0, Lander et al. 1987). With MAPMAKER, loci were first grouped with a LOD threshold of 4.0 and a recombination fraction (θ) ≤ 0.30; and then by a less-strict LOD score of 3.0 and (θ) ≤ 0.35. Within linkage groups, loci were ordered with a starting LOD score of 3.0 and a minimum of 2.0. The order was refined occasionally by the "ripple" command. Loci that could not be ordered were placed as accessory loci on the map next to the closest framework locus and were not included in the estimation of the overall genetic length of the maps. Besides a few minor order differences for 12 closely linked loci (2.6%), both programs (MAPMANAGER and MAPMAKER) yielded identical ordering. For consistency, however, only the results from MAPMAKER are presented. Dominant or co-dominant (3:1 or 1:2:1 ratios) loci were placed on the obtained map with the MAP-MANAGER QTX program using the cross-type designation "arbitrary". With this program the most-likely map position (LOD at least 3.0) for each locus was initially determined. Then, MAPMAKER was employed for calculating the marker distances by using only the homozygous recessive genotypes. The error detection functions of MAPMAKER and visual re-inspection (gel or filters) were employed to check potential genotyping errors. From this examination, genotypic data from seven individuals were excluded due to an extensive amount of genotypic errors. Loci showing a skewed segregation were examined for their effects on linked markers (Hallden et al. 1996).

Estimation of genome length

The estimate of total genome length was made according the method of Hulbert et al. (1988) as modified in method ''3" of Chakravarti et al. (1991). Only framework markers were used in this calculation to avoid an overestimate of the genome coverage (Grattapaglia and Sederoff 1994). The genome length (L) was estimated as:

$$L = n(n-1)d/k,$$

where n is the total number of loci, d is the maximum observed map distance corresponding to the LOD threshold Z for declaring linkage, and k is the number of locus pairs having LOD values Z or greater. Framework loci with more than 10% missing phenotype scores were not included. The Z values tested were 3 and 4.

Growth-trait assessments

The field trial of the mapping pedigree was established in the summer of 1997. The plantation was established with a spacing of 1.5 × 1.0 m using a randomized complete block design with single plant plots and ten replications. Prior to field planting, plants were originated from 1 year-old un-rooted stem cuttings (20 cm long) grown in a greenhouse for 8 weeks. The height of the tallest shoot was measured at 4 consecutive years (1997 = ht97, 1998 = ht98, 1999 = ht99 and 2000 = ht00) in the field. At the end of the 2nd year growth (January of 1999) a stem harvest took place. Therefore, ht97 and ht98 represent 1st and 2nd year-old shoots on 1 and 2 year-old roots respectively, while ht99 and ht00 are the 1st and 2nd year-old shoots on 3 and 4 year-old roots respectively. Annual height-increments were estimated by subtracting the height of the 1-year-old shoot from the total height of the 2-year-old shoot (ht98-97, ht00-99). The number of shoots (nst) from each plant was estimated at the end of 1999 and 2000 (nst99, nst00), while the diameter (dm00) at breast height (1.3 m) of the longest shoot was assessed at the end of 2000. Stem proportion was calculated as the height: diameter ratio and estimated for the year 2000 (ht00: dm00). At the spring of 2000 the number of flower buds (nfl) and the number of vegetative flushing buds (ngr) of the longest stem of each plant were counted. The proportion of flower buds over the total number of shoot buds [nfl/(nfl+ngr) = rflgr] in each plant was estimated. The investigated traits were selected with emphasis on potential economic importance to biomass production. Besides ngr, all trait values followed a normal distribution. The ngr trait values were normalized by a natural logarithm transformation prior to the QTL analysis. Clonal means were used as phenotypic values in the QTL analysis. Clonal mean heritabilities were calculated as:

$$H^2 = \sigma_c^2/(\sigma_c^2 + \sigma_e^2/b_c),$$

where σ^2_c, σ^2_e are the *clone*, *error* variance components, respectively, and b_c is the coefficient for σ^2_c from the *clone* expected mean square calculated by the Standard Least Squares Model of the analysis of variance (JMP 3.0 1994).

QTL analysis

The two constructed maps were utilized for QTL analysis using the MAP-MANAGER QTXb-10.0 software (Manly and Olson 1999). The analysis was based on clone averages and included three steps. In the first step, the whole data was scanned for significant trait-marker association ($p < 0.01$) with single regression analysis. In the second step, simple Interval Mapping (sIM) analysis (Knott and Haley 1992) was performed. In the third step, 1,000 permutation tests were carried out to establish the significant thresholds for declaring a QTL (with overall $p < 0.05$), using the Churchill and Doerge (1994) algorithm implemented in MAP-MANAGER.

Unlinked QTLs might act as an additional environmental effect that reduces the significance of estimated marker-trait association (Zeng 1994). Therefore, Composite Interval Mapping (CIM) analysis was performed using the markers associated with the traits ($p < 0.01$) as co-factors (background markers) to reduce the residual variance (Manly and Olson 1999). While the CIM analysis was conducted separately for each map, the background markers used in these analyses were derived from both maps. One to three background markers were employed in CIM analysis.

To test for genotype * year interaction and block effects, a two-way ANOVA was conducted on height growth measured at 3 consecutive years (ht98-97, ht99 and ht00-99). The marker loci closest to significant QTLs were used as genotype factors. Significant locus pairs for traits with more than one QTL within a year (ht98-97 and ht99, see Results) were tested for epistatic interactions using a two-way ANOVA. The ANOVA tests were performed with the JMP 3.0 (1994) software.

Results

AFLP markers

A total of 72 primer combinations were analyzed mainly using the multicolor fluorescence approach (Perkin-

Table 1 Summary of results from the linkage analysis. Note: major groups are composed of 4–17 markers

Item	*S. viminalis* × *S. schwerinii* Map				*S. viminalis* Map			
	Marker number (AFLPs/RFLPs)	Number of linkage groups	Length (cM)[a]	Average distance (cM)[a]	Marker number (AFLPs/RFLPs)	Number of linkage groups	Length (cM)[a]	Average distance (cM)[a]
Mapped in major groups	197 (178/19)	26	2,286	13	111 (101/10)	18	1,577	17
Doublets and triplets	20 (15/5)	8	118	10	35 (31/4)	14	267	13
Unlinked	29				56			
Total	246	34	2,404	13	202	32	1,844	16

[a] Map units of centimorgans based on the Kosambi mapping function

Elmer 1995). The selective primers (*Eco*RI+2) labeled with 5-FAM yielded a considerably higher number of polymorphic loci compared to the TAMRA labeled primers (data not shown). Twelve out of 72 primer combinations were analyzed by the ordinary radioactive labeling method using one additional base in the *Eco*RI primer (*Eco*RI+3). The number of polymorphic loci scored per primer combination was similar in both methods (approximately six bands per primer combination) even though an extra nucleotide was added to the *Eco*RI primer (*Eco*RI+3) in the radioactive labeling method. The seemingly low polymorphism using *Eco*RI+2 primers could mainly be attributed to the limited size-range of fragments (100–450 bp) and to the conservative scoring used in the multicolor fluorescence approach. Of the 383 AFLP loci scored, 357 were heterozygous in one parent and null in the other, while 26 were segregating in both parents. The number of polymorphic (1:1) loci derived from the hybrid parent (*S. viminalis* × *S. schwerinii*) was considerably higher (18%) than those originating from the *S. viminalis* parent. A total of 70 loci (18.0%) showed significantly skewed segregation at the 5% confidence level. There were approximately equal numbers of loci with an excess of bands present as those with a deficit of bands. However, a higher proportion of loci with skewed segregation (5%) was observed in the male map.

RFLP markers

Eighty three (83) anonymous *Populus* genomic DNA probes were tested for restriction-fragment length polymorphism in the *Salix* genome. Only eight probes (10%) showed poor hybridization. Of the remaining 75 probes, 61 (81%) revealed polymorphic bands while the rest, nine (14%), were monomorphic. Five probes were tested in a low number of individuals ($n < 29$) and they were excluded from the analysis. Of the remaining 56 probes, 37 showed strong and clear bands allowing easy scoring. Of the 37 probes, six (16%) indicated clear evidence of hybridization of more than one locus; five revealed two polymorphic loci and one probe detected three. In addition to the *Populus* genomic probes, four anonymous genomic DNA probes, derived from the *Salix* genome, revealed six polymorphic loci. A 1:1 segregation ratio was observed for most of the RFLP loci. From a total of 50 (44 + 6) scored RFLP loci, 39 (78%) were segregating in a 1:1 ratio while 11 (22%) were heterozygous in both parents and segregated in a 1:2:1 ratio. Five RFLP loci (10%) showed significantly skewed segregation at the 5% significance level.

Linkage analysis and map construction

A total of 433 polymorphic loci (383 AFLPs, 50 RFLPs) were tested for linkage. Using a LOD score of at least 3.0, 363 loci (325 AFLPs and 38 RFLPs) formed linkage groups organized in to two parental maps. In the *S. viminalis* × *S. schwerinii* map, 217 markers were ordered in 34 linkage groups while 29 loci remained unlinked. Twenty six linkage groups (Fig. 1) had 4–16 markers with a length size of 44–183 cM and an average marker distance of 13 cM. Eight groups were composed of less than four markers each (doublets and triplets) with a total length of 118 cM (Table 1). Thirty one of the 217 mapped loci exhibited skewed segregation ratios. Fifteen of these mapped to three linkage groups (1, 2 and 10; Fig. 1). In the *S. viminalis* map, 146 loci were placed on 32 linkage groups while 53 loci remained unlinked. Eighteen groups (Fig. 2) consisted of 4–16 loci with a length size of 29.3–171.3 cM and an average marker distance of 16 cM. Fourteen groups included only 2–3 markers (doublets and triplets) with a total length distance of 267 cM (Table 1). Fourteen loci with skewed segregation ratios were observed in nine linkage groups. Out of 82 total unlinked markers (in both maps) 24 were skewed and 14 contained more than 20% missing data points (mostly RFLPs). The estimated lengths of the maps were 2,404 cM and 1,844 cM for *S. viminalis* × *S. schwerinii* and *S. viminalis* respectively. Doublets and triplets (small groups) are not represented on the map figures, unless they possessed a significant QTL (Figs. 2, 3) and/or displayed homologies between the parental maps (Fig. 3). Their length size, however, has been accounted in the total map distance

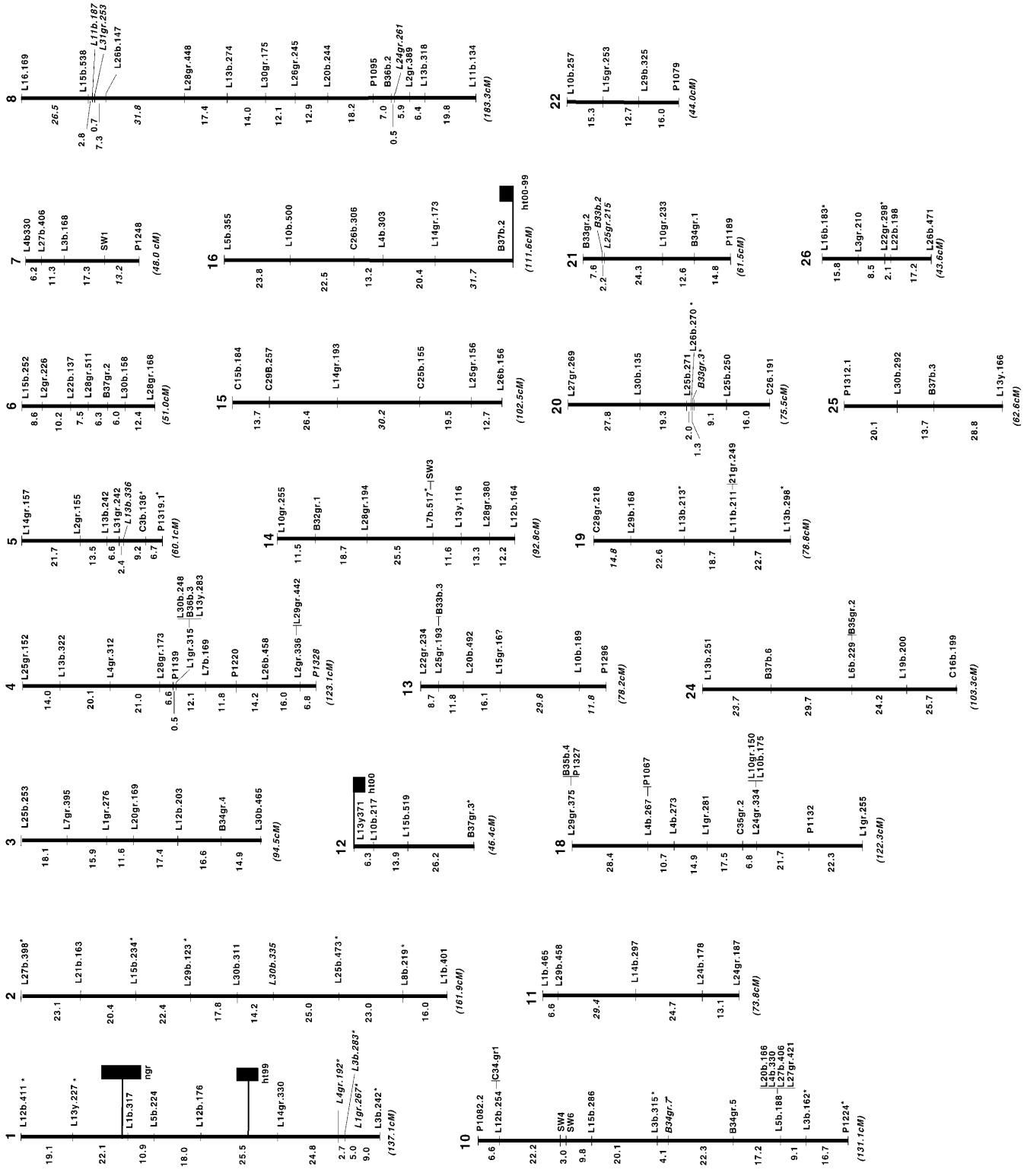
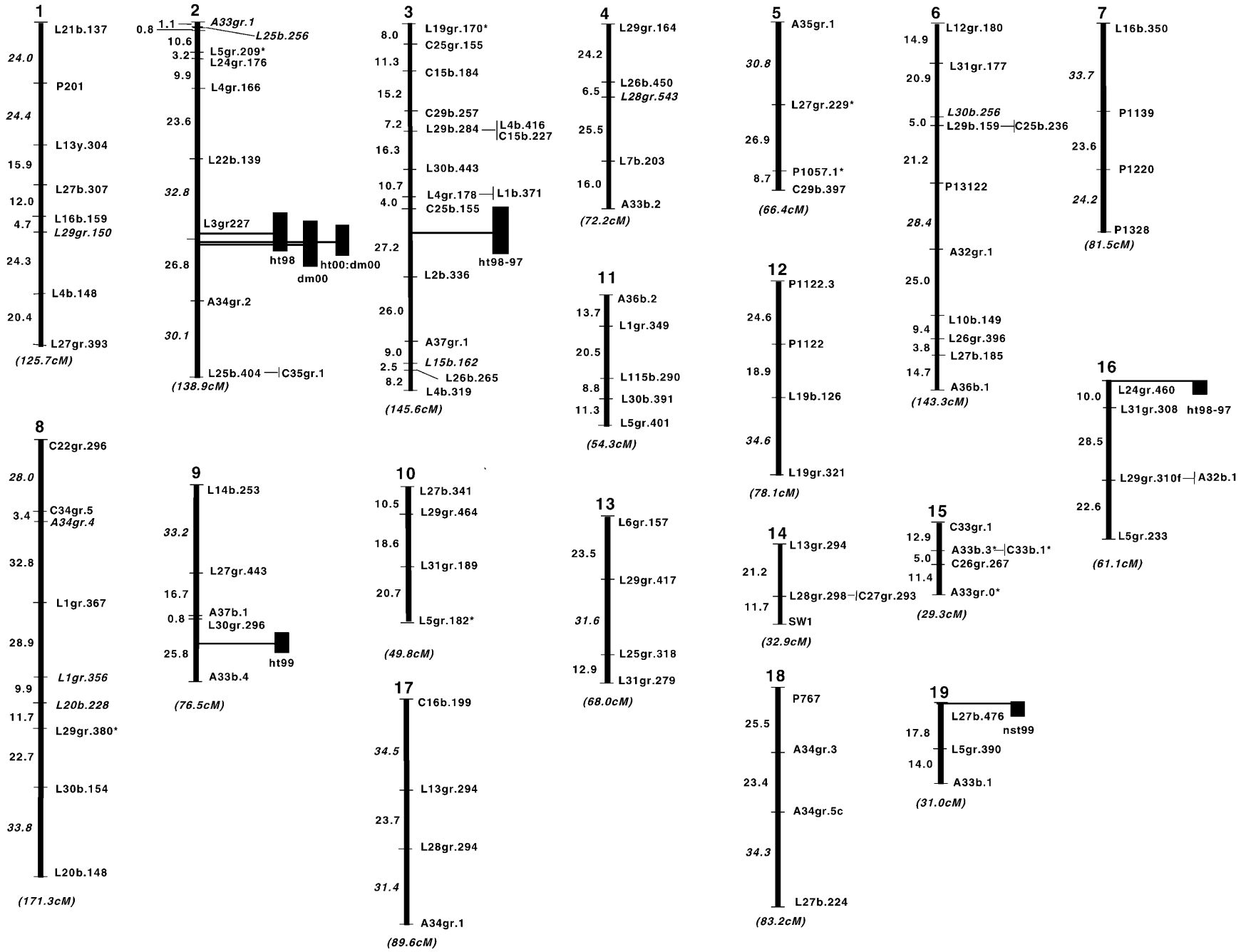
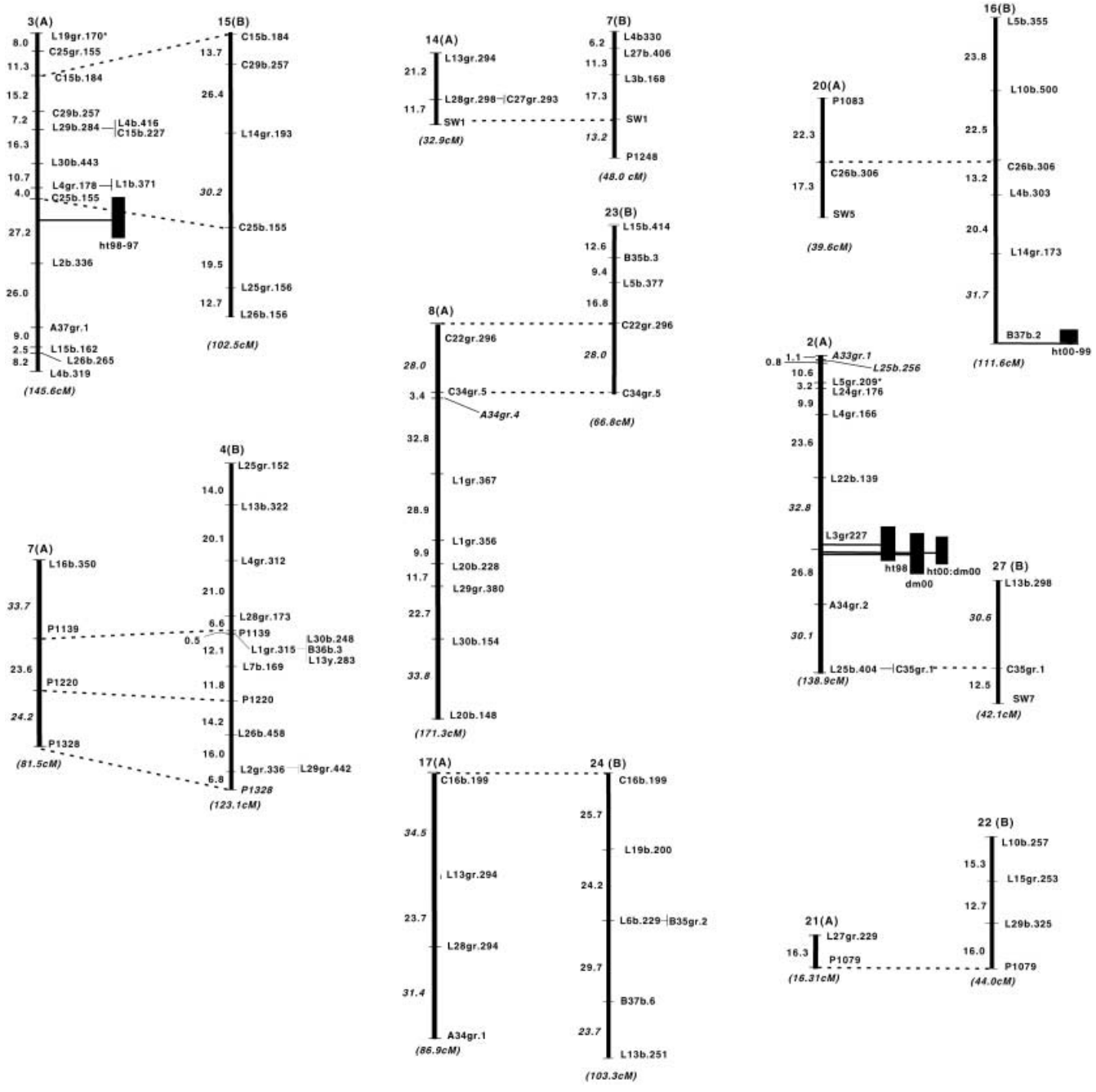


Fig. 1 The *S. viminalis* × *S. schwerinii* map (clone Björn) and the most-likely QTL positions. The estimated marker distance for pairs of loci that mapped with 3 ≤ LOD < 4 is in *italics*. AFLP loci are indicated as *L* (when segregating in one parent) or *C* (when segregating in both parents), followed by the primer combination code and the molecular weight (bp). Loci generated by the conventional radioactive AFLP are indicated as A or B (*S. viminalis* or *S. viminalis* × *S. schwerinii* map respectively). The molecular size of these loci is not presented but a number has been added showing their position on the gel (starting from the top to the bottom). RFLP loci are designated with a letter *P*, when derived from *Populus* genomic probes, or *SW*, when originated from *Salix* genomic probes Skewed loci are indicated by a *star* (*). Loci that were ordered at 3 ≤ LOD < 2 are presented in *italics*. *Bars* to the right indicate the area in which LRS exceeds significance for the QTL threshold (MAP-MANAGER QTXb-10.0). Map units (cM) shown on the left side of each linkage group were calculated by the Kosambi mapping function


1
L21b.137
24.0
P201
24.4
L13y.304
15.9
L27b.307
12.0
L16b.159
4.7
L29gr.150
24.3
L4b.148
20.4
L27gr.393
(125.7cM)
2
0.8
1.1
A33gr.1
L25b.256
10.6
L5gr.209*
3.2
L24gr.176
9.9
L4gr.166
23.6
L22b.139
32.8
L3gr227
ht98
ht00:dm00
dm00
26.8
A34gr.2
30.1
L25b.404
C35gr.1
(138.9cM)
3
8.0
L19gr.170*
C25gr.155
11.3
C15b.184
15.2
C29b.257
7.2
L29b.284
L4b.416
C15b.227
16.3
L30b.443
10.7
L4gr.178
L1b.371
4.0
C25b.155
27.2
ht98-97
L2b.336
26.0
A37gr.1
9.0
L15b.162
2.5
L26b.265
8.2
L4b.319
(145.6cM)
4
L29gr.164
24.2
L26b.450
6.5
L28gr.543
25.5
L7b.203
16.0
A33b.2
(72.2cM)
5
A35gr.1
30.8
L27gr.229*
26.9
P1057.1*
8.7
C29b.397
(66.4cM)
6
L12gr.180
14.9
L31gr.177
20.9
L30b.256
5.0
L29b.159
C25b.236
21.2
P13122
28.4
A32gr.1
25.0
L10b.149
9.4
L26gr.396
3.8
L27b.185
14.7
A36b.1
(143.3cM)
7
L16b.350
33.7
P1139
23.6
P1220
24.2
P1328
(81.5cM)
8
C22gr.296
28.0
C34gr.5
3.4
A34gr.4
32.8
L1gr.367
28.9
L1gr.356
9.9
L20b.228
11.7
L29gr.380*
22.7
L30b.154
33.8
L20b.148
(171.3cM)
9
L14b.253
33.2
L27gr.443
16.7
A37b.1
0.8
L30gr.296
25.8
ht99
A33b.4
(76.5cM)
10
L27b.341
10.5
L29gr.464
18.6
L31gr.189
20.7
L5gr.182*
(49.8cM)
11
A36b.2
13.7
L1gr.349
20.5
L115b.290
8.8
L30b.391
11.3
L5gr.401
(54.3cM)
12
P1122.3
24.6
P1122
18.9
L19b.126
34.6
L19gr.321
(78.1cM)
13
L6gr.157
23.5
L29gr.417
31.6
L25gr.318
12.9
L31gr.279
(68.0cM)
14
L13gr.294
21.2
L28gr.298
C27gr.293
11.7
SW1
(32.9cM)
15
C33gr.1
12.9
A33b.3*
C33b.1*
5.0
C26gr.267
11.4
A33gr.0*
(29.3cM)
16
L24gr.460
10.0
L31gr.308
ht98-97
28.5
L29gr.310f
A32b.1
22.6
L5gr.233
(61.1cM)
17
C16b.199
34.5
L13gr.294
23.7
L28gr.294
31.4
A34gr.1
(89.6cM)
18
P767
25.5
A34gr.3
23.4
A34gr.5c
34.3
L27b.224
(83.2cM)
19
L27b.476
nst99
17.8
L5gr.390
14.0
A33b.1
(31.0cM)



◀ **Fig. 2** The *S. viminalis* map (clone78183) and the corresponding QTL. The estimated marker distance for pairs of loci that mapped with 3 ≤ LOD < 4 is in *italics*. AFLP loci are indicated as *L* (when segregating in one parent) or *C* (when segregating in both parents), followed by the primer combination code and the molecular weight (bp). Loci generated by the conventional radioactive AFLP are indicated as A or B (the *S. viminalis* or *S. viminalis* × *S. schwerinii* map respectively). The molecular size of these loci is not presented but a number has been added showing their position on the gel (starting from the top to the bottom). RFLP loci are designated with a letter *P*, when derived from *Populus* genomic probes, or*SW*, when originated from *Salix* genomic probes Skewed loci are indicated by a *star* (*). Loci that were ordered at 3 ≤ LOD < 2 are presented in *italics*. *Bars* to the right indicate the area in which LRS exceeds significance for the QTL threshold (MAP-MANAGER QTXb-10.0). Map units (cM) shown on the left side of each linkage group were calculated by the Kosambi mapping function

Fig. 3 Homologous loci between the two maps. A and B designate the *S. viminalis* and *S. viminalis* × *S. schwerinii* map, respectively. Lines between homologous linkage groups connect the 3:1 or 1:2:1 markers used to merge the two maps

(Table 1). The average density of markers was approximately one per 14 cM for both maps.

Genome length

Using LOD scores 3 and 4, the estimated total length of the map derived from the *S. viminalis* × *S. schwerinii* parent was *L* = 2,897 cM and 3,152 cM, respectively,

Table 2 Mapped QTLs for height growth (ht98, ht99, ht00 and ht00-99), number of shoots (nst99), number of vegetative buds (ngr) at flowering time and diameter (dm00). Note: QTL analysis is based on results from MAP-MANAGER QTXb version 10; * indicates LRS value above the empirical statistical significant thresholds as determined by the permutation test (see Materials and methods)

Item	Linkage group	LRS-sIM[a] (PV%)[c]	LRS-CIM[b] (PV%)[c]	H^2 (%)	Gen. (%)[d]	Allele effect[e] (%)
Traits						
Map A *S. viminalis* (clone-78183)						
ht98	2	15.1(17%)*	14.6(16%)*	67	25	9.0
ht98-97	3	15.4(17%)*	15.5(17%)*	57	30	13.0
ht98-97	16	12.7(12%)	14.0(15%)*	57	26	12.0
ht99	9	15.3(17%)*	14.1(15%)*	78	21	11.0
nst99	19	17.6(20%)*	17.7(20%)*	51	39	20.0
dm00	2	15.8(18%)*	15.9(18%)*	81	22	14.0
ht00:dm00	2	15.7(18%)*	16.6(19%)*	54	35	8.0
Map B *S. viminalis* × *S. Schwerinii* (clone-Björn)						
ht99	1	15.5(18%)*	15.5(17%)*	78	23	11.0
ht00	12	12.6(13%)	13.7(17%)*	86	19	8.0
Ht00-99	16	14.1(15%)	15.1(16%)*	70	22	13.0
ngr	1	14.9(15%)*	16.2(22%)*	31	70	36.0

[a] LRS based on simple-interval mapping (sIM)
[b] LRS based on composite-interval mapping (CIM)
[c] The percentage of phenotypic variation accounted by each QTL
[d] The percentage of the total genotypic variance explained by the QTL (Gen=Phen/H^2)
[e] The phenotypic effect of the favorable QTL allele expressed as percentage of the mean of the population

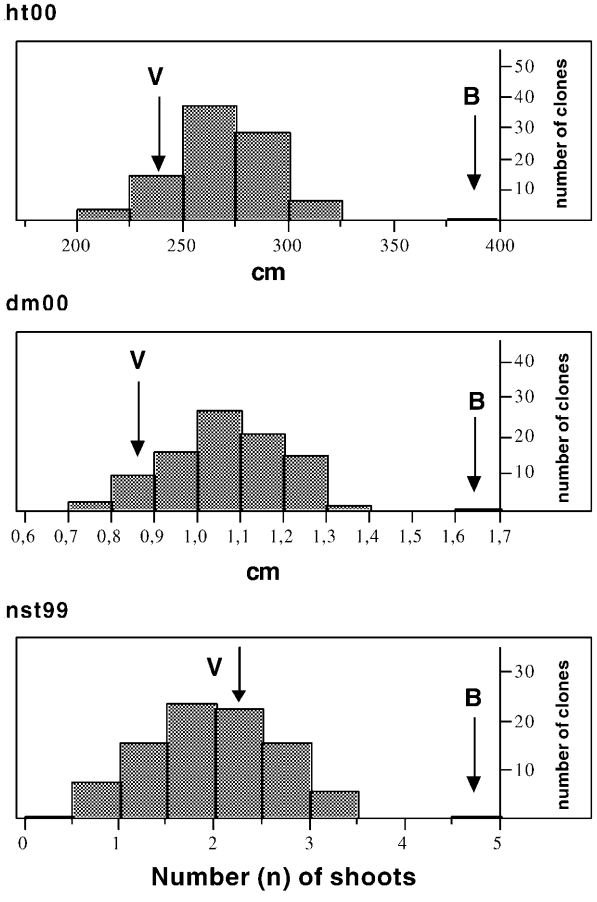


Fig. 4 Distribution of phenotypes for some of the studied traits [height in 2000 (ht00), diameter (dm00) and number of shoots in 1999 (nst99)]. The phenotypic values for the parents **B** (clone Björn: *S. viminalis* × *S. schwerinii*) and **V** (clone 78183: *S. viminalis*) are indicated by *arrows*

and from the *S. viminalis* parent L = 2,537 cM and L = 2,766 cM, respectively. These values are considerably higher than the observed genome length, indicating that the mapped loci did not provide full coverage of the *Salix* genome. According to these estimates the present maps cover about 79% (2,404/3,025) and 70% (1,844/2,652) of the *S. viminalis* × *S. schwerinii* and *S. viminalis* genome, respectively.

Homologous linkage groups between parental maps

Twelve loci segregating in both parents (seven AFLPs and five RFLPs) indicated homology between eight pairs of linkage groups from the two maps (Fig. 3). Three of the pairs had at least two loci in common, while homology for the other pairs was based on a single locus.

Quantitative traits

Figure 4 shows the frequency distributions for some of the investigated traits. For all traits the means of the hybrid parent *S. viminalis* × *S. schwerinii* (Björn) were higher than the means of the other parent, *S. viminalis* (78183), and all the progenies. A high degree of genetic variation was found for all traits. Besides ngr, clonal mean heritabilities were fairly high, ranging from 51 to 86% (Table 2).

QTL analysis

The QTL analysis following the sIM and CIM approaches showed evidence for a total of 11 QTLs affecting

growth-related traits. In the *S. viminalis* × *S. schwerinii* map (clone Björn) three QTLs for height were detected (Fig. 1). The estimated magnitude of the QTL effect in the *S. viminalis* × *S. schwerinii* map ranged from 14 to 22% of the total phenotypic variance (Table 2). Three of the QTLs (ht00, ht98-97, ht00-99) were detected following the CIM analysis but not when the IM analysis was employed. In the *S. viminalis* map (clone 78183) seven QTLs were identified (Table 2). The QTL for dm00 mapped on linkage group 2 close to the QTL for ht98 and the QTL for the ht00: dm00 ratio (Fig. 2). The proportion of the total phenotypic variance explained by the QTLs on the *S. viminalis* map ranged from 17 to 20% (Table 2). No QTLs affecting ht97, nfl, rflgr and nst00 were identified. The ANOVA test for genotype * year interaction of height growth was significant ($p < 0.0001$) while no effect was detected for genotype or block. A significant epistatic effect was found for the two QTLs associated with ht99.

Discussion

Linkage map

In the present study, 383 AFLP and 50 RFLP markers were used to construct two genetic maps of *Salix*, one for the hybrid clone Björn (*S. viminalis* × *S. schwerinii*) and one for the clone 78183 (*S. viminalis*), spanning an average 2,124 cM of the *Salix* genome. To our knowledge the *Salix* linkage maps, shown in Figs. 1 and 2, are the first reported genetic maps of the *Salix* genome. The expected number of linkage groups ($n = 19$) for a comprehensive linkage map in *Salix* is exceeded. This indicates that several gaps in the linkage map need to be bridged. The estimates of the total genome size suggest that an additional 20% and 30% of the length of the *S. viminalis* × *S. schwerinii* and *S. viminalis* maps, respectively, needs to be covered. Gaps in the linkage map may be the result of the clustering of AFLP markers. A single combination of restriction enzymes (*Eco*RI and *Mse*I) was used for AFLP analysis that could have resulted in an absence of markers in GC-rich regions of the genome. Some AFLP loci could also, despite careful checking, contain genotype errors that might obstruct linkage. However, there could also be a biological explanation for the large number of small linkage groups. Early cytological work (Blackburn and Harrison 1924; Wilkinson 1941) reported that some *Salix* species had two basic chromosome numbers, 19 and 22, and Wilkinson (1941) proposed that these differences were due to chromosome fragmentation. The extensive studies of Håkansson (1955), on the other hand, could not confirm this view, but the excessive number of small linkage groups obtained in recent genetic maps of *Populus* (Bradshaw et al. 1994; Wu et al. 2000) revive the question of a possible biological explanation for the observed high number of small linkage groups observed in *Populus* and the closely related *Salix* genus.

The high level of segregation distortion (18%) found for AFLP markers was similar to that previously reported in *Eucalyptus* (Marques et al. 1998). Skewed segregation ratios in other types of markers, i.e. RAPDs and RFLPs, have also been observed in molecular analysis of forest-tree species (Nelson et al. 1993; Bradshaw and Stettler 1994; Barreneche et al. 1998). Genetic mechanisms such as the expression of genetic load, incompatibility, genetic isolating mechanisms during speciation and preferential chromosome loss (Bradshaw and Stettler 1994), have been suggested to explain this phenomenon. In the *S. viminalis* map, the skewed loci were randomly mapped across the genome or formed small groups. However, it is interesting to highlight the localization of 15 of the skewed loci in linkage groups 1, 2 and 10 of the *S. viminalis* × *S. schwerinii* map. The number of skewed markers on these groups showed a significant excess over the Poisson expectations ($p < 0.05$) in a goodness-of-fit-test (data not shown). This indicates the existence of large areas (possibly whole chromosomes) in the hybrid *S. viminalis* × *S. schwerinii* genome that do not follow Mendelian ratios. The relatively low number of co-dominant AFLP markers (5%) was comparable to that found in *Eucalyptus* (5%) (Marques et al. 1998) and in *Populus* (3%) (Frewen et al. 2000).

Of the 83 RFLP probes derived from the *Populus* genome, 61 (81%) detected polymorphic loci in the *Salix* genome. Several of the RFLP probes revealed multiple loci, suggesting a significant level of genome duplication in *Salix*. Such duplications have earlier been reported for many diploid plant species including *Populus* (Bradshaw et al. 1994). In this study no conserved homologous regions were detected between the genomes of the two genera, most-likely due to the low number of homologous RFLP markers present on the maps from the two species.

Quantitative traits and QTL detection

The present study demonstrates that it is possible to detect loci controlling genetic variation for growth traits in *Salix* using molecular-linkage maps and, furthermore, to characterize these loci with respect to their map position and phenotypic effect. In SRIC *Salix* systems, growth traits, i.e. height growth, diameter and number of shoots, are important components of the biomass production and have been extensively evaluated in *Salix* breeding programs. It is often assumed that growth-related traits are complex with a large number of genes involved, each having a small effect on the phenotypic variance (polygenic control). In this study we were able to detect moderate to large-effect QTLs for growth-related traits. The magnitude of the phenotypic effect of individual QTLs ranged from 14 to 22%. Interestingly, 32% of the phenotypic variance for height growth in 1999 (1st year after harvest) was explained by two QTLs. Thus, our results agree with other QTL studies in trees showing that growth traits, e.g. height, diameter and number of shoots, may in part be controlled by a few genes of large effect.

Bradshaw and Stettler (1995) have reported that 30% of the phenotypic variance for 2 year stem-volume growth in *Populus* was explained by two QTLs in an F_2 family. Further, major-effect QTLs controlling vegetative propagation traits have been reported in *Eucalyptus* in a study using the pseudo-testcross strategy (Grattapaglia et al. 1995). In these QTL studies major-effect QTLs were detected in interspecific crosses. However, the present QTL dissection study was based partially on an intraspecific cross (QTLs detected on the *S. viminalis* map). This suggests that genomic regions with relatively large effect on quantitative inherited traits, i.e. growth traits, can also be detected within species.

The study of growth components, i.e. height, diameter, etc., through QTL mapping could provide a more-detailed genetic understanding of the physiology of growth. A better understanding for the molecular basis for growth-predictor traits would make *Salix* breeding more effective. Analysis of phenotypic correlations in the present study indicates that height growth and diameter are highly correlated traits ($r = 0.82$). The QTLs associated with diameter in the present study overlapped with the QTLs affecting height growth (ht99: 1st year height growth after the first harvest) and height: diameter ratio. This suggests that height and diameter growth in *Salix* share common genetic components. Similar QTL-clustering has earlier been reported in *Populus* for stem basal area and sylleptic branches (Bradshaw and Stettler 1995) and in *Eucalyptus* for height: diameter ratio and vigor (Verhaegen et al. 1997).

In general, changes in QTLs over the years are expected in forest trees (Bradshaw and Grattapaglia 1994) since stand-development takes several years. Quantitative genetic studies for growth traits in *Salix* indicate a low genetic correlation over years (Rönnberg-Wästljung and Gullberg 1999). This is in agreement with our QTL dissection results. QTLs estimated for multiple year traits were only present in a single year. This may not be surprising for the *Salix* SRIC system considering the drastic changes on the shoot-root growth relationship over the years. In our analysis, plants had equal shoot and root age in the years 1997 and 1998 (shoot:root = 1:1 and 2:2 respectively) but not in the following years when the new shoots emerged from the 2 year-old rootstocks. On the basis of our annual height-growth analysis, however, two of the significant QTLs (ht00, L. Group 12, map: *S. viminalis* × *S. schwerinii* and ht98, L. Group 2, map: *S. viminalis*) were very close to reaching significance (LOD = 2.7, 2.9) in 2 successive years. This indicates some partial QTL stability over the years for height growth QTLs which, due to environmental variation among years, relatively small population size and high threshold levels (Beavis 1994), might not have been evident in our experiment. Partial stability of QTL expression has been detected for growth traits in *Eucalyptus* (Verhaegen et al. 1997) and *Pinus radiata* (Emebiri et al. 1998).

No QTLs were detected during the 1st year (1997) of growth. This may be attributed to the fact that plants were grown in the greenhouse prior to their planting in the field. The transfer of the plants from controlled conditions to the field may have stressed or shocked the plants, resulting in changes in growth patterns. This is further supported by the low phenotypic correlation ($r = 0.40$) between ht97 and ht98.

One QTL with a relatively large phenotypic (22%) effect was detected for the number of vegetative buds (ngr) during flowering time in the spring. The ngr estimates were highly correlated with the nfl and rflgr (–0.80 and –0.92) values. Although one would expect that many flowers in the shoots during the spring would reduce the annual stem growth, the correlation between ngr (or nfl, rflgr) and height growth was poor (–0.22). This is in agreement with an earlier genetic study in a factorial cross (8 × 8) of *S. viminalis* (Rönnberg-Wästljung and Gullberg 1999), and indicates that genetic variation in ngr has a minor effect on plant-height growth. The hybrid parent *S. viminalis* × *S. schwerinii* (clone Björn) is a high yielding clone with a high economic interest (Larsson 1998). The means of the hybrid parent (*S. viminalis* × S. schwerinii) were higher than the corresponding means of the parent *S. viminalis* and all the progenies for all traits (Fig. 4). This indicates a significant contribution of a non-additive effect in the observed variation. It has to be noted that dominant effects of the QTLs could not be assessed in the present study, either due to the lack of one homozygote class or because the homozygote class could not be distinguished from the heterozygote.

Assuming that the growth traits are related to fitness we expect that selection has fixed different alleles affecting these traits in the two species (*S. viminalis* and *S. schwerinii*). Due to a high level of heterozygosity for growth traits in a hybrid, one could expect more QTLs to be identified from the hybrid parent *S. viminalis* × *S. schwerinii* (clone Björn) than from the *S. viminalis* parent. However, the *S. viminalis* parent accounted for most of the genetic variation (and most of the QTLs) for the investigated growth traits. Of the 11 QTLs that were identified only four QTLs originated from the parent *S. viminalis* × *S. schwerinii* (clone Björn), even though the *S. viminalis* × *S. schwerinii* map spans 40% more of the genome than the *S. viminalis* map. These results indicate high genetic variation for growth traits within *S. viminalis*.

In the present study the power for QTL detection was limited due to the relatively small size of the mapping population. It has been suggested that it would be necessary for a sample size of at least 400 progenies in a mapping population to be derived from a backcross (Bevis 1994). Despite the relatively small population size, an increase in power was obtained in our study by using clonal replications of individuals in the mapping population. The ease of clonal propagation in fast-growing tree species, i.e. *Salix*, is invaluable for QTL studies since it can increase the heritability of a trait (Bradshaw and Foster 1992). Still, the relatively small population size in combination with the empirical threshold corresponding to a genome-wide Type-I error probability of 0.05

(through permutation tests) only allowed the detection of QTLs with a large to moderate effect (Manly and Olson 1999). However, lowering the significance threshold will increase the chance of proclaiming QTLs where there are none.

The number of QTLs may also have been underestimated here for other reasons. The phenotypic assessment was undertaken in a single environment. Paterson et al. (1991) indicated that QTL studies conducted in a single environment are likely to underestimate the number of QTLs, which can potentially influence a trait due to the environment × QTL interaction. Although, the present study was carried out in a single site, plants were grown under natural conditions (i.e. no irrigation, no pest control) with variable environmental conditions over the 4 years (i.e. a different range of temperatures at the growing stage). Thus, this multiple-year QTL analysis may have captured some of the "environment specific" QTLs affecting growth. Expression of QTLs under certain environmental conditions is well documented in the literature, suggesting further testing of the detected QTLs under different environmental conditions.

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