A GENETIC LINKAGE MAP OF A TETRAPLOID *SALIX VIMINALIS* × *S. DASYCLADOS* HYBRID BASED ON AFLP MARKERS

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ABSTRACT

In order to study the inheritance of frost resistance as well as growth traits genetic maps specific for two tetraploid *Salix dasyclados* × *S. viminalis* hybrids based on a three-generation pedigree have been constructed. The F_1 generation was created by an interspecific cross between a high producing, commercial diploid *S. viminalis* clone (Jorunn) and a natural, frost resistant, hexaploid *S. dasyclados* clone (SW901290). Single dose AFLP markers segregating in 1:1, 3:1 or 1:2:1 ratios in 92 F_2 individuals were used. In the map of the male F_1 parent 269 markers were distributed over 37 major linkage groups and thirty-one minor groups covering 1820 cM of the genome. The map of the female F_1 parent consists of 248 markers assigned to 35 major linkage groups and twenty-two minor groups covering 1640 cM of the genome. For eight linkage groups, integration between male and female groups was possible due to 3:1 markers that linked both to male and female markers segregating in a 1:1 ratio. Seventy-four percent of the markers originate from the male grandparent (SW901290), which could be expected due to the hexaploid level in the *S. dasyclados* grandparent. In average eleven percent of the markers on the maps showed a distorted segregation.

Keywords: genetic linkage map, single-dose markers, tetraploidy, AFLP-markers, *Salix viminalis, Salix dasyclados*.

INTRODUCTION

Biomass plantations have a large potential for producing renewable energy. 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 (GULLBERG 1993; ZSUFFA *et al.* 1993). The shrub species *Salix viminalis* (L.) and *S. dasyclados* (Wimm.) and their hybrids are among the most broadly used species in SRIC systems.

Due to the commercial significance of *Salix* for biomass production, breeding programs using traditional methods of phenotypic screening and recurrent selection has been developed (GULLBERG 1993; ÅHMAN & LARSSON 1995). The introduction and use of DNA markers such as RFLP, and AFLP detected many loci and allowed the construction of linkage maps in forest tree species (BRADSHAW *et al.* 1994; GRATTAPAGLIA & SEDEROFF 1994; PAGLIA *et al.* 1998; ARCADE *et al.* 2000; CERVERA *et al.* 2001). In *S. viminalis,* the hybrid *S. viminalis* × *S. schwerinii, S. alba* as well as in *S. fragilis* linkage maps using AFLP, RFLP and microsatellite markers have recently been developed (HANLEY *et al.* 2002, TSAROUHAS *et al.* 2002, BARACACCIA *et al.* 2003). *Salix viminalis* and *S. schwerinii* are diploid species while *S. alba* is tetraploid and several ploidy levels have been identified in *S. fragilis* and *S. dasyclados* (LARSSON & BREMER 1991). Mapping in polyploid species is less advanced compared to diploid species mainly due to the complexity of the polysomic inheritance. A large number of different genotypes are expected in the segregating population and to detect some of these segregation types, very large population sizes are needed. The genome constitution of the species is often unknown (i. e. allo- versus autopolyploid) which also complicates the mapping. Despite this, linkage maps for polyploid species have been constructed in e. g., sugarcane (DA SILVA *et al.* 1993), sweetpotato (KRIEGNER *et al.* 2000), potato (MEYER *et al.* 1998) and roses (RAJAPAKSE *et al.* 2001). The general approach to avoid the difficulties in mapping poplyploids has been to use only fragments that are present in single dose, segregating 1:1 (WU *et al.* 1992; RIPOL *et al.* 1999; LUO *et al.* 2001, BARACCIA *et al.* 2003).

Selection and breeding in *Salix* have been oriented mainly toward increased growth, frost and disease resistance (LARSSON 1998). While tree breeding is time-consuming mainly due to long generation intervals, molecular knowledge of the inheritance of economical important traits will permit the production of improved

phenotypes through more effective and less time-consuming breeding and parental selection. *Salix* species offer good model systems for biological studies due to small genome sizes (2C = 0.76–0.89pg; THIBAULT 1998) and short time to mature for flowering (two years). Linkage disequilibrium generated by hybridisation as well as the possibility to capture non-additive genetic variance through clonal propagation further increases the potential for using molecular assisted breeding in *Salix* species.

With molecular-linkage maps, it is possible to locate Quantitative Trait Loci (QTL) affecting economically important traits. QTL 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 favourable QTL alleles, and the relationships between QTL underlying different physiological processes. QTL affecting growth related traits i.e. shoot height, shoot diameter and number of shoots, have been mapped in *Salix* (TSAROUHAS *et al.* 2002).

To increase frost resistance in SRIC systems, *S. dasyclados* clones from Russia have been introduced into the breeding population (LARSSON 1998). In the present study we report a *Salix* genetic linkage map based on a three-generation pedigree. The F_1 generation was produced by an interspecific cross between a diploid, high yield producing *S. viminalis* clone and a frost resistant hexaploid *S. dasyclados* clone. The F_2 generation was made by crossing a male and a female clone within the tetraploid F_1 family. Single dose AFLP markers in coupling phase segregating in 1:1 ratio and a few double dose markers segregating in 5:1 in the F_2 were used to compose two parental maps according to the pseudotestcross strategy (GRATTAPAGLIA & SEDEROFF 1994), while single-dose markers segregating in a 3:1 ratio were utilised to align homologous groups between the two parental maps.

MATERIAL AND METHODS

DNA content measurements

To estimate the ploidy level of SW901290, the DNA content of the clones was compared to that of *S. viminalis* and *S.dasyclados*. DNA content was estimated on isolated nuclei in a flow cytometer. To isolate nuclei, buds were crushed in liquid nitrogen. The material was transferred to a chopping buffer and processed as described by WAARA *et al.* (1998). Similarly, the ploidy level was estimated for the F_1 parents and 23 F_2 individuals. The DNA content is given as values relative to *S. viminalis*.

Mapping pedigree

The mapping pedigree was initiated in 1995 by crossing the female diploid *S. viminalis* (clone: Jorunn) to the male hexaploid *Salix dasyclados* (SW901290). Two of the tetraploid F_1 progenies were crossed in 1998 to produce the F_2 mapping pedigree used in the present study. The selection of the parents was based on freeze-test results. Frost susceptible was Jorunn while SW901290 was frost resistant. Following seed germination in a greenhouse, ninety-two individuals were selected and planted in 2 litres pots containing soil medium (20 % clay, 80 % peat) for further use in DNA analysis.

DNA isolation and AFLP analysis

Genomic DNA was isolated from frozen young leaves according to the Fast Prep protocol and kits (BIO 101, Vista CA) modified by adding 1.25 % polyvinylpyrrolidone (avg. mol wt 40,000), 0.2 % 2-mercaptoethanol and 0.5 % ascorbic acid to the extraction buffer.

AFLP (VOS *et al.* 1995) analysis was performed as described by TSAROUHAS *et al.* (2002). Sixty-three EcoRI-MseI primer combinations with +3 selective bases were utilised (Table 1). The primers complementary to the EcoRI adapter (selective amplification) were endlabeled with γ-^{33}P-ATP. The gels were scored visually by two reviewers independently for minimising scoring errors. AFLP loci are indicated as F or M (when segregating in either the female or the male F_1 parent) or with no indication (when segregating in both parents), followed by a code denoting the primer combination (Table 1) and their position on the gel progressing sequentially in the cathodal direction.

Linkage analysis and map construction

Based on the two-way pseudotestcross strategy (GRATTAPAGLIA & SEDEROFF 1994) two separate parental maps were generated using single-dose markers but also a few double dose markers. Each of the two maps are based on markers present in one F_1 parent and segregating 1:1 and in a few cases 5:1 (double dose markers), and on markers present in both parents and segregating 3:1 or a few markers 1:2:1. The scored markers were tested for segregation distortion and linkage using the JOINMAP 2.0 program (STAM & VAN OOIJEN 1995). JOINMAP allows the use of raw data with different type of segregation. The markers were tested for deviation from the expected 1:1, 3:1 and 1:2:1 Mendelian segregation ratios by χ^2 analysis in the

JOINMAP module "JMSLA". Markers with p-value less than 0.05 were classified as distorted and given asterisks ("*") according to *p*-value in Figure 2. Distorted 1:1 markers were checked for 5:1 segregation. Markers not significantly different from 5:1 segregation have "D" in front of the labelling in Figure 2. Using the JOINMAP module "JMGRP", linkage groups were assigned by increasing the LOD score for grouping with steps of 0.5 LOD unit. At LOD 3.5 a preliminary grouping of markers was taken. The JOINMAP module "JMGRP" was then used for each preliminary group separately to further divide the groups until grouping was consistent at higher LOD. The calculations of the linkage maps for each group (module "JMMAP") were done by using all pairwise recombination estimates smaller than 0.49 and LOD scores higher than 0.1 but with a "JUMP" command of 4.0. The "JUMP" command sets a threshold for the increase in the χ^2-value when a marker is added to the map. If a marker is causing a considerable jump in the χ^2-value, exceeding the threshold value, the marker is put aside or the positioning of the marker is postponed. A ripple was performed after the addition of every three markers. Kosambi's mapping function (KOSAMBI 1944) was used to convert recombination values to map distances (in cM). Only markers in coupling phase were included in the map construction due to larger error variance in the estimate of recombination frequency for markers in repulsion phase (MAYER *et al.* 1998, WU *et al.* 1992).

The genotypic data was crosschecked for linkage with the MAPMAKER (LANDER *et al.* 1987) and MAPMANAGER QTXb 14.0 (MANLY *et al.* 2001) programs. Marker loci grouping and order corresponded well with results obtained from JOINMAP.

Estimated and observed 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) and estimated with the formula

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

where G is the estimated genome length, N is number of markers, X is the maximum distance between two adjacent markers in cM at a certain LOD threshold and K is the number of marker pair at or above the same LOD threshold. A LOD threshold of 4.0 was chosen for this estimate.

The observed length of the genome was estimated adding the length of all groups in the map including triplets and doublets.

RESULTS

Characterisation of parental clones and progeny

SW901290 was morphologically similar to *S. dasyclados*, and a phylogenetic tree based on AFLP data strongly support that SW901290 should be classified as *S. dasyclados* (V. Semerikov, A. C. Rönnberg-Wästljung, U. Lagercrantz, in preparation). Different ploidy-levels have been reported in *S. dasyclados* (LARSSON & BREMER 1991). The DNA content of SW901290 was similar to the other analysed *S. dasyclados* clones, and approximately 2.7 times that of *S. viminalis* (Table 2). The estimates for the F_1 and the F_2 plants were similar and intermediate between the parental clones. These data suggests that SW901290 is hexaploid, and that the F_1 and F_2 plants are all tetraploid.

Inheritance of AFLP-markers

From the 63 different primer combinations (Table 1) 781 polymorphic AFLP-markers were scored. On average each primer combination yielded twelve polymorphic amplification products. Four hundred and five (52 %) markers revealed a 1:1 segregation ratio while 217 (28 %) markers exhibited a 3:1 segregation pattern. Fifteen markers (2 %) showed a 5:1 segregation as expected from a double dose marker appearing in one of the parents (Table 3). The distribution of markers present in one or the other of the F_1 parents is shown in Figure 1.

The experimental design in the present study allows an analysis of the inheritance of markers in three generations. Sixty-six per cent of the markers were present only in the *S. dasyclados* (SW901290) male grandparent (Table 4), while 15 % of the markers were derived from the *S. viminalis* female grandparent, which was similar to the proportion (13 %) of markers present in both grandparents (Table 4). The inheritance of the markers from the F_1 generation to the F_2 generation showed a more even distribution. Twentyfive per cent and 27 per cent of the markers originated from the female and male F_1 parent, respectively, while 28 per cent of the markers were found in both F_1 parents (Table 4).

Genetic linkage map

A total of 781 AFLP loci were tested for linkage. Four hundred and thirty two (55 %) of the AFLP-markers formed groups (at LOD = 4.0 and above) (Table 3). Seventy-two percent of all markers segregating 1:1 in

Table 1. Primer combination used in the AFLP analyses. The last three selective nucleotides of the primers are shown. The numberes correspond to the number of the gel.

MseI-primer	EcoRI-primer					
	AAA	AAG	ACC	AGC	ATC	ATG
CAA			25	21		
CAC		75	37	20	76	
CAG			22			
CAT			28	29		
CCA	65	64	38	31		66
CCC	62	43	10	12	61	63
CCG	47	48	9	11	46	45
CCT	51	49	34	33	50	
CGA	73	71	26	16	72	74
CGC	69	67	36	27	68	70
CGG	55	53	13	14	54	56
CGT	59	57	24	15	58	60
CTA			32	18		
CTC			39	30		
CTG			7	8		
CTT			35	19		

Table 2. DNA content of parental, F_1 and F_2 plants.

Plants	Nc[1]	Nr[2]	Mean[3]	SE
S. viminalis	6	1	1.0	0.01
S.dasyclados	5	1	2.7	0.03
SW901290	1	2	2.7	0.14
F_1	2	2	2.0	0.08
F_2	23	1	1.9	0.04

[1)] Number of analysed clones.
[2)] Number of replicate measurements of each clone.
[3)] Mean relative to *S. viminalis*.

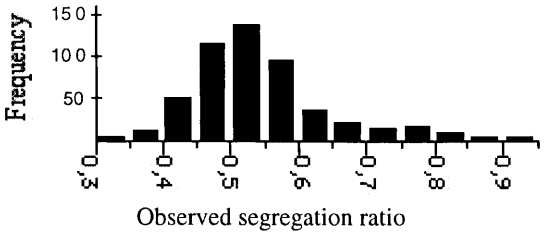


Figure 1. Observed segregation ratio for fragments present in one or the other of the F_1 parents.

the F_2 population were placed on the map, while only 42 % of the markers with a 3:1 segregation were mapped (Table 4). Two of the 5:1 markers were placed in linkage group Y on the map (Fig. 2). These markers could not be connected to any other groups on the map as expected for double dose markers. The two markers are probably distorted 1:1 markers even though they could not statistically be separated from a 5:1 segregation. The male map consists of 37 major linkage groups, each containing from four to ten markers (Fig. 2), 12 triplets and 19 doublets. The female map has 36 major groups, each with four to nine markers (Fig. 2), eleven triplets and ten doublets. In eight groups, integration between male and female markers was possible due to 3:1 markers that linked both to male and female markers segregating in a 1:1 ratio. One to five 3:1 markers were present on each group for the integration of male and female markers. Eight groups are composed of only 3:1 markers and are in common between maps (Fig. 2). The length of the linkage groups containing more than three markers varied between 6 to 66 cM in the male map and between 8 to 66 cM in the female map. The length of the triplets and doublets ranged from 3 to 42 cM and from 1 to 32 cM respectively for both maps. Taking all linkage groups into consideration the average length between markers was 6.8 and 6.6 cM for the male and female map respectively. The observed genome length including both triplets and doublets was 1820 cM for the male map and 1640 cM for the female map. Estimated length of the genome was 4274 cM for the male map and 3286 cM for the female map, indicating 43 and 50 percent coverage of the observed groups for the two maps.

Most of the major groups originate from the *S. dasyclados* male parent, while five groups in the female

Table 3. Number of AFLP markers with different segregation pattern scored on the gels and placed on the *Salix* maps.

	F 1:1[1]	M 1:1[1]	F 5:1[1]	M 5:1[1]	3:1	1:2:1	distorted ($p < 0.05$)	Sum
Scored markers	198	207	5	10	217	3	141	781
Markers placed on the maps[2]	138	152	0	2	91	<	46	432

[1] M and F in the 1:1 and 5:1 markers refer to male and female in the F_1 generation.
[2] includes linkage groups with three and two markers.

Table 4. Number of AFLP markers segregating 1:1, 5:1 or 3:1 (% of markers placed on the *Salix* map, within parentheses) originating from the P_1, P_2 and from both grandparents ($P_1 + P_2$), and from F_{11} and F_{12} and both parents ($F_{11} + F_{12}$).

F1 generation	P generation				Sum
	P_1	P_2	$P_1 + P_2$	-[1]	
F_{11} (female 1:1)	147 (0)	15 (67)	20 (65)	16 (69)	198 (70)
F_{12} (male 1:1)	139 (79)	31 (55)	21 (81)	16 (50)	207 (73)
F_{11} (female 5:1)	4 (0)	0 (0)	1 (0)	0 (0)	5 (0)
F_{12} (male 5:1)	2 (0)	0 (0)	8 (25)	0 (0)	10 (20)
$F_{11} + F_{12}$ (3:1)	142 (39)	38 (50)	26 (42)	11 (45)	217 (42)
distorted $p < 0.05$	77 (26)	31 (39)	25 (48)	7 (28)	140 (33)
Sum	511 (57)	115 (50)	101 (54)	50 (52)	777 (55)

[1] "-" indicate either that the markers were not found in the grandparents or missing data.

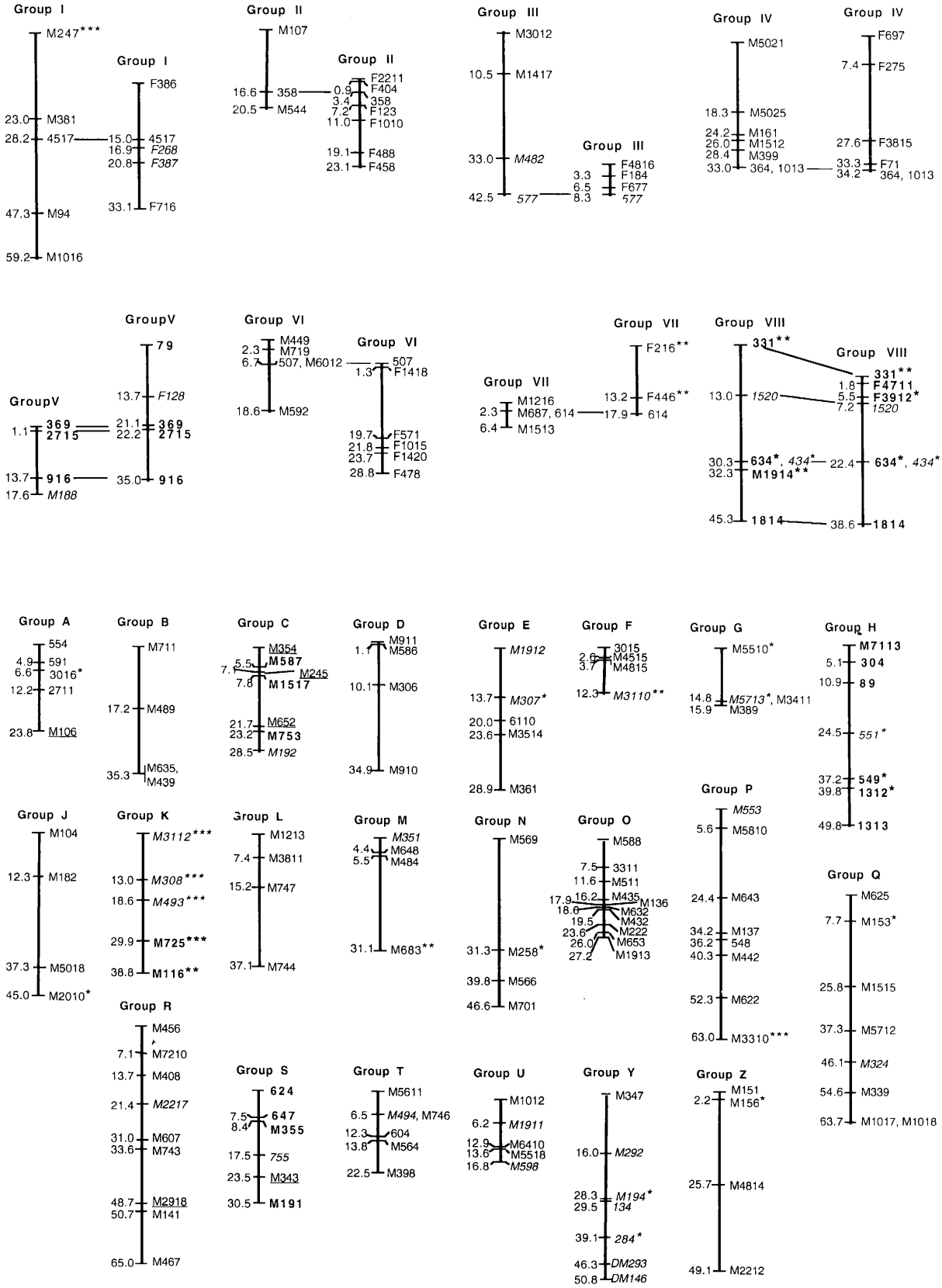
map and seven groups in the male map originates from the *S. viminalis* female parent (Fig. 2; V, VIII, C, H, K, S, m, q, 1). Taking both doublets and triplets into consideration, eleven out of 57 groups are of *S. viminalis* origin in the female map while in the male map 17 of 68 linkage groups are inherited from the *S. viminalis* grandparent. To assign a group as inherited from one of the grandparents, markers in the group, present in both or non of the grandparents, were assumed to be inherited as the rest of the markers (see for ex. groups K and q; assumed to be inherited from *S. viminalis*). Three of the major groups and one triplet (Fig. 2; a, c, s) have markers that are present both in the *S. dasyclados* female grandparent and in the *S. viminalis* male grandparent.

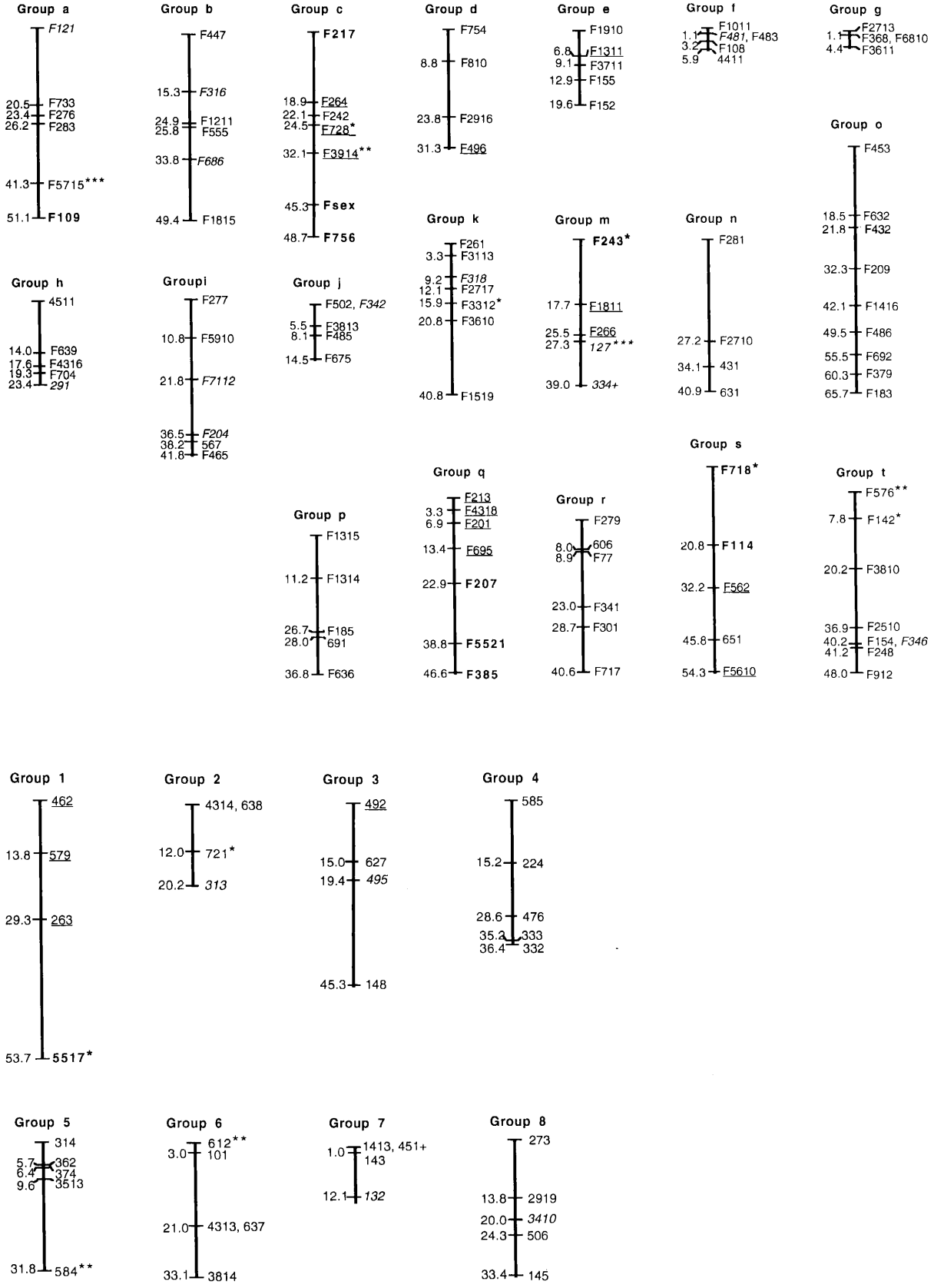
DISCUSSION

Inheritance of AFLP-markers

A significant proportion of the generated AFLP markers (74 %) originated from the *S. dasyclados* parental clone (taking 1:1 and 3:1 markers into consideration). This is in accordance with the flow cytometry study which showed that the *S. dasyclados* clone most likely is hexaploid so 3/4 of the total amount of markers would be expected to originate from the *S. dasyclados* clone. If the hexaploid *S. dasyclados* grandparent clone is of allo- or of autopolyploid origin is not known.

Although the F_1 parent clones are species hybrids there was high seed germination (98 %) in the F_2 population (data not shown) and most of the markers segregate as single-dose 1:1 or 3:1 markers. This suggests a high level of preferential or bivalent pairing of chromosomes during meioses. In early studies by NILSSON (1918) hybrids between diploid *S. viminalis* and hexaploid *S. phylicifolia* were made. These hybrids exhibited normal meioses with formation of 38 bivalents (NILSSON 1918). In addition to bivalent pairing a high level of heterozygosity in the parents could also result in a high number of single-dose segregation of 1:1 and 3:1. High heterozygosity level could be expected in the *S. dasyclados* grandparent since it originates from a natural population. Heterozygosity level in outbred forest trees have been estimated to vary around 20–30 % in *Populus* depending on species and marker system used (BRADSHAW *et al.* 1994; CERVERA *et al.* 2001). Similar values have also been reported for *Eucalyptus* species (MARQUES *et al.* 1998).


Group I
M247***
23.0 M381
28.2 4517
47.3 M94
59.2 M1016
Group I
F386
15.0 4517
16.9 F268
20.8 F387
33.1 F716
Group II
M107
16.6 358
20.5 M544
Group II
F2211
0.9 F404
3.4 358
7.2 F123
11.0 F1010
19.1 F488
23.1 F458
Group III
M3012
10.5 M1417
33.0 M482
42.5 577
Group III
F4816
3.3 F184
6.5 F677
8.3 577
Group IV
M5021
18.3 M5025
24.2 M161
26.0 M1512
28.4 M399
33.0 364, 1013
Group IV
F697
7.4 F275
27.6 F3815
33.3 F71
34.2 364, 1013
GroupV
79
13.7 F128
21.1 369
22.2 2715
35.0 916
GroupV
1.1 369
2715
13.7 916
17.6 M188
Group VI
M449
2.3 M719
6.7 507, M6012
18.6 M592
Group VI
507
1.3 F1418
19.7 F571
21.8 F1015
23.7 F1420
28.8 F478
Group VII
F216**
13.2 F446**
17.9 614
Group VII
M1216
2.3 M687, 614
6.4 M1513
Group VIII
331**
13.0 1520
30.3 634*, 434*
32.3 M1914**
45.3 1814
Group VIII
331**
1.8 F4711
5.5 F3912*
7.2 1520
22.4 634*, 434*
38.6 1814
Group A
554
4.9 591
6.6 3016*
12.2 2711
23.8 M106
Group B
M711
17.2 M489
35.3 M635, M439
Group C
M354
5.5 M587
7.1 M245
7.8 M1517
21.7 M652
23.2 M753
28.5 M192
Group D
M911
1.1 M586
10.1 M306
34.9 M910
Group E
M1912
13.7 M307*
20.0 6110
23.6 M3514
28.9 M361
Group F
3015
2.6 M4515
3.7 M4815
12.3 M3110**
Group G
M5510*
14.8 M5713*, M3411
15.9 M389
Group H
M7113
5.1 304
10.9 89
24.5 551*
37.2 549*
39.8 1312*
49.8 1313
Group J
M104
12.3 M182
37.3 M5018
45.0 M2010*
Group K
M3112***
13.0 M308***
18.6 M493***
29.9 M725***
38.8 M116**
Group L
M1213
7.4 M3811
15.2 M747
37.1 M744
Group M
M351
4.4 M648
5.5 M484
31.1 M683**
Group N
M569
31.3 M258*
39.8 M566
46.6 M701
Group O
M588
7.5 3311
11.6 M511
16.2 M435
17.9 M136
18.6 M632
19.5 M432
23.6 M222
26.0 M653
27.2 M1913
Group P
M553
5.6 M5810
24.4 M643
34.2 M137
36.2 548
40.3 M442
52.3 M622
63.0 M3310***
Group Q
M625
7.7 M153*
25.8 M1515
37.3 M5712
46.1 M324
54.6 M339
63.7 M1017, M1018
Group R
M456
7.1 M7210
13.7 M408
21.4 M2217
31.0 M607
33.6 M743
48.7 M2918
50.7 M141
65.0 M467
Group S
624
7.5 647
8.4 M355
17.5 755
23.5 M343
30.5 M191
Group T
M5611
6.5 M494, M746
12.3 604
13.8 M564
22.5 M398
Group U
M1012
6.2 M1911
12.9 M6410
13.6 M5518
16.8 M598
Group Y
M347
16.0 M292
28.3 M194*
29.5 134
39.1 284*
46.3 DM293
50.8 DM146
Group Z
M151
2.2 M156*
25.7 M4814
49.1 M2212


Group a
F121
20.5 F733
23.4 F276
26.2 F283
41.3 F5715***
51.1 F109
Group b
F447
15.3 F316
24.9 F1211
25.8 F555
33.8 F686
49.4 F1815
Group c
F217
18.9 F264
22.1 F242
24.5 F728*
32.1 F3914**
45.3 Fsex
48.7 F756
Group d
F754
8.8 F810
23.8 F2916
31.3 F496
Group e
F1910
6.8 F1311
9.1 F3711
12.9 F155
19.6 F152
Group f
1.1 F1011
F481, F483
3.2 F108
5.9 4411
Group g
1.1 F2713
F368, F6810
4.4 F3611
Group h
4511
14.0 F639
17.6 F4316
19.3 F704
23.4 291
Groupi
F277
10.8 F5910
21.8 F7112
36.5 F204
38.2 567
41.8 F465
Group j
F502, F342
5.5 F3813
8.1 F485
14.5 F675
Group k
F261
3.3 F3113
9.2 F318
12.1 F2717
15.9 F3312*
20.8 F3610
40.8 F1519
Group m
F243*
17.7 F1811
25.5 F266
27.3 127***
39.0 334+
Group n
F281
27.2 F2710
34.1 431
40.9 631
Group o
F453
18.5 F632
21.8 F432
32.3 F209
42.1 F1416
49.5 F486
55.5 F692
60.3 F379
65.7 F183
Group p
F1315
11.2 F1314
26.7 F185
28.0 691
36.8 F636
Group q
F213
3.3 F4318
6.9 F201
13.4 F695
22.9 F207
38.8 F5521
46.6 F385
Group r
F279
8.0 606
8.9 F77
23.0 F341
28.7 F301
40.6 F717
Group s
F718*
20.8 F114
32.2 F562
45.8 651
54.3 F5610
Group t
F576**
7.8 F142*
20.2 F3810
36.9 F2510
40.2 F154, F346
41.2 F248
48.0 F912
Group 1
462
13.8 579
29.3 263
53.7 5517*
Group 2
4314, 638
12.0 721*
20.2 313
Group 3
492
15.0 627
19.4 495
45.3 148
Group 4
585
15.2 224
28.6 476
35.2 333
36.4 332
Group 5
314
5.7 362
6.4 374
9.6 3513
31.8 584**
Group 6
612**
3.0 101
21.0 4313, 637
33.1 3814
Group 7
1.0 1413, 451+
143
12.1 132
Group 8
273
13.8 2919
20.0 3410
24.3 506
33.4 145

←← Figure 2. Genetic linkage map of a tetraploid *Salix* hybrid. Markers starting with F indicate origin from the F_1 female parent, markers starting with M indicate origin from the F_1 male parent, no letter indicate a 3-1 segregation and + indicate a 1:2:1 segregation in the F_2. Markers in normal style show that the markers are inherited from the P male grandparent (SW901290), markers in bold show that the markers are inherited from the P female grandparent (Jorunn). Markers in italics indicate that both P parents had the marker. Markers underscored indicate either that none of the P parents had the markers or missing data for the P parents. An *, **, *** indicate a distorted segregation at $0.01 < p < 0.05$, $0.001 < p < 0.01$, $p < 0.001$ respectively. An "D" indicate a double dose marker segregating 5:1 in the F_2. Roman labelling of the groups indicate that the groups are aligned between the two F_1 maps, capital letter show groups from the F_1 male map, small letter indicate groups from the F_1 female map, number labelling indicate groups with only 3:1 markers.

Skewed segregation ratios were found in 83 (11 %) of the markers at $p < 0.01$ and in 140 (18 %) of the markers at $p < 0.05$, which is higher than expected. Segregation distortion of markers has been found in other *Salix* maps. In a backcross map of *S. viminalis* × *S. schwerinii* 18 % of the AFLP markers were distorted ($p < 0.05$) (TSAROUHAS *et al.* 2002) as well as in 15 % of the AFLP/microsatellite markers in a *S. viminalis* map (Hanley *et al.* 2002). In tetraploid maps of *S. alba* and *S. fragilis* 14 and 11 % ($p < 0.05$) of the polymorphic markers were distorted (BARCACCIA *et al.* 2003). High level of AFLP-marker distortion has also been reported in *Eucalyptus* (MARQUES *et al.* 1998) and in *Populus* (CERVERA *et al.* 2001). BRADSHAW and STETTLER (1994) showed that segregation distortion found in an F_2 interspecific cross between *Populus trichocarpa* and *P. deltoides* was due to a recessive lethal allele, tightly linked to a RFLP marker, causing embryo and seedling mortality. Several other mechanisms such as chromosome loss or chromosomal rearrangements during speciation as well as alleles for pollen lethality have been suggested as explanation for distorted segregation ratios (BRADSHAW & STETTLER 1994; MARQUES *et al.* 1998). Quadrivalent pairing in tetraploids result in some extent to double reduction in the gametes and thus distorted segregation ratios (WU *et al.* 2001). Despite the tetraploid genome constitution of the F_1 parents in the present study, the proportion of markers with distorted segregations in the F_2 do not differ from other studies with diploid *Salix* (HANLEY *et al.* 2002, TSAROUHAS *et al.* 2002). A high level of preferential pairing, could be the explanation.

Genetic linkage map

The expected number of groups in the two parental maps of this apparently tetraploid hybrid is 38, since the basic chromosome number in *Salix* is 19 (LARSSON & BREMER 1991). The high number of small groups suggests that there are unmapped gaps on the *Salix* chromosomes. This is also indicated by the estimated 43 and 50 % coverage (observed map length/estimated map length) of the constructed maps. TSAROUHAS *et al.* (2002) studying a backcross population between the *S. viminalis* × *S. schwerinii* hybrid and *S. viminalis* also found an excess of groups. Non-random sampling of AFLP-markers over the genome (PAGLIA *et al.* 1998) as well as hot spots for recombination (KESSELLI *et al.* 1994) has been suggested as explanations to gaps. Many small groups and many unmapped markers have also been reported in an AFLP map study of *Populus deltoides* (WU *et al.* 2000). Other marker systems where segregation of all alleles could be followed are needed for an alignment of the groups into the expected 38. Codominant markers, such as microsatellites, are also needed to align homologous groups within and between the female and male maps.

The inheritance of the markers could be followed in three generations and the presence of the markers in the different generations is indicated in the labelling of markers on the map (Fig. 1). Most of the linkage groups are inherited intact from either of the grandparents to F_1, with a few exceptions (Fig. 2, groups; a, c, s). One fourth of the linkage groups would be expected to be inherited from the *S. viminalis* grandparent. This was true for the male map where exactly 1/4 of the linkage groups was inherited from *S. viminalis*. In the female map number of linkage groups with *S. viminalis* origin are lower than expected. Three groups containing markers present in one grandparents, as well as one marker from the other grandparent, was found in the female map. One possible explanation could be that a band is missing in the second grandparent and that these markers actually should be found in both of the grandparents. Markers with no band in any of the grandparents have been recognised for other markers indicating some error in the AFLP procedure. Similar problems might have arisen in the three groups with markers of different grandparental origin.

Although the high number of polymorphic loci per reaction generated by the AFLP procedure can facilitate the efficient and rapid construction of a genetic linkage map, the type of markers that are generated are typical dominant. This may have limited an extensive integration of the two parental maps in the present study. The 3:1 markers are less informative due to the low information content between marker pairs segregating 1:1 and 3:1 (RITTER *et al.* 1990). This is also reflected in the map where the 3:1 markers were difficult to place.

Only 42 % of the 3:1 markers showing sufficient linkage to other markers on the map compared to 72 % of the 1:1 markers. In Eucalyptus maps based on RAPD markers only 25 % of the 3:1 markers showed strong linkage to markers segregating 1:1 (VERHAGEN & PLOMION 1996). Furthermore, simulation studies have shown that dominant markers that segregate in both parents (3:1) may provide limited information for an unbiased and accurate recombination frequency estimate (MALIEPAARD *et al.* 1998; WU *et al.* 2000).

Eleven percent (at $p < 0.05$) of the markers on the map show a distorted segregation, a smaller value than the total percentage of distorted markers indicating that markers with skewed segregation are difficult to place on the map. There is one group composed only of distorted markers (group K) showing five distorted markers within 39 cM. All markers in the group have a segregation distortion in the same direction with more individuals sharing band presence. This might be an indication of an irregular pairing during meioses. Small clusters of markers were found in groups VIII and H, while other markers with skewed segregation ratios were scattered across the linkage groups. Clusters of distorted markers were found in *Eucalyptus* maps (VERHAGEN & PLOMION 1996) as well as in *Populus* maps (Bradshaw and Stettler 1994; Cervera *et al.* 2001). Distorted markers that appear in clusters suggest that these areas contain genes that affect viability (STRAUSS & CONKLE 1996; CHENG *et al.* 1996; VERHAGEN & PLOMION 1996). To verify the connection between distorted markers in clusters and genes affecting viability in this pedigree, further studies are needed.

Many polyploid species does not only show preferential pairing of chromosomes (extreme allopolyploids) or only multivalent parings (extreme autopolyploids), rather they represent intermediate stages displaying a combination of both allo- and autopolyploid pairing behaviour (JACKSON & JACKSON 1996, FJELLSTRÖM *et al.* 2001). It has also been suggested that a mosaic of disomy and tetrasomy at various loci might be a general mechanism underlying the inheritance of many tetraploids (FJELLSTRÖM *et al.* 2001, WU *et al.* 2001). A combination of disomic and tetrasomic pairing behaviour was discusssed for the tetraploid maps of *S. alba* and *S. fragilis* even though their results indicated a high degree of disomic inheritance (BARCACCIA *et al.* 2003). In the present study a combination of disomic pairing for some chromosomes and tetrasomic pairing in other chromosomes, here indicated by linkage group K, might also be the case in this mapping population.

This study presents a map of a tetraploid hybrid *Salix*, although not complete, it opens new possibilities for using molecular markers in the breeding of polyploid *Salix*. Additional markers (SSRs) will give a better coverage of the genome in addition to alignment of homologous groups in the map. This map will be the base for studies of inheritance for different adaptive trait along with growth traits.

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REFERENCES

ARCADE, A., ANSELIN, F., FAIVRE RAMPANT, P., LESAGE, M. C., PAQUES, L. E. & PRAT, D. 2000: Application of AFLP, RAPD and ISSR markers to genetic mapping of European and Japanese larch. *Theor. Appl. Genet.* **100**:299–307.

BARCACCIA, G., MENEGHETTI, S., ALBERTINI, E., TRIEST, L. & LUCCHIN, M. 2003: Linkage mapping in tetraploid willows: segregation of molecular markers and estimation of linkage phases support an allotetraploid stucture for *Salix alba* × *Salix fragilis* interspecific hybrids. *Heredity* **90**: 169–180.

BRADSHAW, H. D. & STETTLER, R. F. 1994: Molecular genetics of growth and development in *Populus*. II. Segregation distortion due to genetic load. *Theor. Appl. Genet.* **89**: 551–558.

BRADSHAW, H. D., VILLAR, M., WATSON, B. D., OTTO, K. G., STEWART, S. & STETTLER, R. F. 1994: Molecular genetics of growth and development in *Populus*. III. A genetic linkage map of hybrid poplar composed of RFLP, STS, and RAPD markers. *Theor. Appl. Genet.* **89**: 167–178.

CERVERA, M-T., STORME, V., IVENS, B., GUSMAO, J., LIY, B. H., HOSTYN, V., VAN SLYCKEN, J., VAN MONTAGU, M. & BOERJAN, W. 2001: Dense genetic linkage maps of three *Populus* species (*Populus deltoides*, *P. nigra* and *P. trichocarpa*) based on AFLP and microsatellite markers. *Genetics* **158**: 787–809.

CHAKRAVARTI, A., LASHER, L. K. & REFEER, J. E. 1991: A maximum likelihood method for estimating genome length using genetic linkage data. *Genetics* **128**: 175–182.

CHENG, R., SAITO, A., TAKANO, Y. & UKAI, Y. 1996: Estimation of the position and the effect of a lethal factor locus on a molecular marker linkage map. *Theor. Appl. Genet.* **93**: 494–502.

DA SILVA, J. A. G, SORRELLS, E., BURNQUIST, L. & TANKSLEY, S. D. 1993: RFLP linkage map and genome analysis of *Saccharum spontaneum*. *Genome* **36**: 782–791.

FJELLSTRÖM, R. G., BEUSELINCK, P. R. & STEINER, J. J. 2001: RFLP marker analyses supports tetrasomic inheritance in *Lotus corniculatus* L. *Theor. Appl. Genet.* **102**: 718–725.

GRATTAPAGLIA, D. & SEDEROFF, R. 1994: Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. *Genetics* **137**: 1121–1137.

GULLBERG, U. 1993: Towards making willows pilot species for coppicing production. *For. Chron.* **69**: 721–726.

HANLEY, S., BARKER, J. H. A., VAN OOIJEN, J. W., ALDAM, C., HARRIS, S. L., ÅHMAN, I., LARSSON, S. & KARP, A. 2002: A genetic linkage map of willow (*Salix viminalis*) based on AFLP and microsatellite markers. *Theor. Appl. Genet.* **105**: 1087–1096.

HULBERT, S. H., ILOTT, T. W., LINCOLN S. E., LANDER E. S. & MICHELMORE, R. W. 1988: Genetic analysis of the fungus, *Bremia lactucae*, using restriction fragment length polymorphisms. *Genetics* **120**: 947–958.

JACKSON, R. C., JACKSON, J. W. 1996: Gene segregation in autotetraploids: Prediction from meiotic configurations. *Am. J. Bot.* **83**(6): 673–678.

KESSELLI, R. V., PARAN, I. & MICHELMORE, R. W. 1994: Analysis of a detailed genetic linkage map of *Lattuca sativa* (Lettuce) constructed from RELP and RAPD markers. *Genetics* **136**: 1435–1446.

KOSAMBI, D. D. 1944: The estimation of map distance from recombination values. *Ann. Eugen.* **12**: 172–175.

KRIEGNER, A., CERVANTES, J. C., BURG, K., MWANGA, R. O. & ZHANG, D. P. 2000: A genetic linkage map of Sweet-potato (*Ipomoea batatas* (L.) Lam.) based on AFLP markers. CIP Program Report 1999–2000.

LANDER, E. S., GREEN, P., ABRAHAMSON, J., BARLOW, A., DALY, M. J., LINCOLN, S. E. & NEWBURY, L. 1987: MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* **1**: 174–181.

LARSSON, S. 1998: Genetic improvement of willow for short-rotation coppice. *Biomass and Bioenergy* **15**:23–26.

LARSSON, G. & BREMER, B. 1991: Korgviden – nyttoväxter förr och nu. (The *Salix viminalis* group – useful plants then and now.) *Svensk Bot. Tidskr.* **85**: 185–200.

LUO, Z. W., HACKETT, C. A., BRADSHAW, J. E., MCNICOL, J. W. & MILBOURNE, D. 2001: Construction of a genetic linkage map in tetraploid species using molecular markers. *Genetics* **157**: 1369–1385.

MALIEPAARD, C., ALSTON, F. H., VAN ARKEL, G., BROWN, L. M., CHEVREAU, E., DUNEMANN, F., EVANS, K. M., GARDINER, S., GUILFORD, P., VAN HEUSDEN, A. W., JANSE, J., LAURENS, F., LYNN, J. R., MANGANARIS, A. G., DEN NIJS, A. P. M., PERIAM, N., RIKKERINK, E., ROCHE, P., RYDER, C., SANSAVINI, S., SCHMIDT, H., TARTARINI, S., VERHAEGH, J. J., VRIELINK VAN, GINKEL, M. & KING, G. J. 1998: Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers. *Theor. Appl. Genet.* **97**: 60–7.

MANLY, K. F., CUDMORE, JR, R. H. & MEER, J. M. 2001: Map Manager QTX, cross-platform software for genetic mapping. *Mammalian Genome* **12**: 930–932.

MARQUES, C. M., ARAUJO, J. A., FERREIRA J. G., WHETTEN R., O'MALLEY, D. M., LIU B-H. & SEDEROFF, R. 1998: AFLP genetic maps of *Eucalyptus globulus* and *E. tereticornis*. *Theor. Appl. Genet.* **96**: 727–737.

MEYER, R. C., MILBOURNE, D., HACKETT, C. A., BRADSHAW, J. E., MCNICHOL, J. W. & WAUGHT, R. 1998: Linkage analysis in tetraploid potato and association of markers with quantitative resistance to late blight (*Phytophtora infestans*). *Mol. Gen. Genet.* **259**: 150–160.

NILSSON, H. 1918: Experimentelle Studien uber Variabilitat, Spaltung, Artbildung und Evoluation in der Gattung *Salix*. Lunds Universitats Årsskrift. N. F. Avd. 2, Bil. 14, Nr. 28. 144 pp.

PAGLIA, G. P., OLIVERI, A. M. & MORGANTE, M. 1998: Towards second-generation STS (sequence-tagged sites) linkage maps in conifers: a genetic linkage map of Norway spruce (*Picea abies* K). *Mol. Gen. Genet.* **258**: 466 –478.

RAJAPAKSE, S., BYRNE, D. H., ZHANG, L. ANDERSON, N., ARUMUGANATHAN, K. & BALLARD, R. E. 2001: Two genetic linkage maps of tetraploid roses. *Theor. Appl. Genet.* **103**: 575–583.

RIPOL, M. I., CHURCHILL, G. A., DA SILVA, J. A. G. & SORRELLS, M. 1999: Statistical aspects of genetic mapping in autopolyploids. *Gene* **235**: 31–41.

RITTER, E., GEBHARDT, C. & SALAMINI, F. 1990: Estimation of recombination frequencies and construction of RFLP linkage maps in plants from crosses between heterozygous parents. *Genetics* **125**: 645–654.

STAM, P. & VAN OOIJEN, J. W. 1995: JoinMap ™ version 2.0: Software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen.

STRAUSS, S. H. & CONKLE, M. T. 1986: Segregation, linkage, and diversity of allozymes in knobcone pine. *Theor. Appl. Genet.* **72**: 483–493.

THIBAULT, .J 1998: Nuclear DNA amount in pure species and hybrid willows (*Salix*): a flow cytometric investigation. *Can. J. Bot.* **76**: 157–165.

TSAROUHAS, V., GULLBERG, U. & LAGERCRANTZ, U. 2002: An AFLP and RFLP linkage map and quantitative trait loci (QTL) analysis of growth traits in *Salix*. *Theor. Appl. Genet.* **105**: 277–288.

VERHAEGEN, D. & PLOMION, C. 1996: Genetic mapping in *Eucalyptus urophylla* and *Eucalyptus grandis* using RAPD markers. *Genome* **39**: 1051–1061.

VOS, P., HOGERS, R., BLEEKER, M., REIJANS M., LEE, T., HORNES, M., FRIJTERS, A., POT, J., PELEMAN, J., KUIPER, M. & ZABEAU, M. 1995: AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**: 4407–4414.

WAARA, S., NYMAN, M. & JOHANNISSON, A. 1998: Efficient selection of potato heterokaryons by flow cytometric sorting and the regeneration of hybrid plants. *Euphytica* **101**: 293–299.

WU, K. K., BURNQUIST, W., SORRELLS, M. E., TEW, T. L., MOORE, P. H., & TANKSLEY, S. D. 1992: The detection and estimation of linkage in polyploids using single-dose restriction fragments. *Theor. Appl. Genet.* **83**: 294–300.

WU, R. L., HAN, T. F., HU, J. J., FANG J. J., LI, L., LI, M. L., & ZENG, Z-B. 2000: An integrated genetic map of *Populus deltoides* based on amplified fragment length polymorphisms. *Theor. Appl. Genet.* **100**: 1249–1256.

WU, R. L., GALLO-MEAGHER, M., LITTELL, R.C., ZENG, Z-B. 2001: A general polyploid model for analyzing gene segregattion in outcrossing tetraploid species. *Genetics* **159**: 869–882.

ZSUFFA, L., SENNERBY-FORSSE, L., WEISGERDER, H. & HALL, R. B., 1993: Strategies for clonal forestry with poplars, aspens, and willows. *In:* Clonal Forestry II: Conservation and Application (Eds) Ahuja M. R., Libby W. J., Springer – Ve rlag Berlin Heidelberg.

ÅHMAN, I. & LARSON, S. 1994: Genetic improvement of willows (*Salix*) as a source of bioenergy. *Norw. J. Agric. Sci.* Suppl. **18**: 47–56.