Genetic mapping of sex-linked markers in *Salix viminalis* L.

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A total of 88 selective primer combinations were screened using bulked males and females sampled from four families of *Salix viminalis*. A total of more than 1000 polymorphic fragments was obtained, of which only four cosegregated with sex. These four sex-linked markers were subsequently scored in individuals that were used for bulked sample preparation in additional individuals of the same families, and in individuals in other families. A pair of primers that amplified the sex-linked fragments was constructed from one of the sex-linked amplified fragment length polymorphism (AFLP) fragments. In hybridization of Southern blot filters with the sex-linked DNA fragments, the band was present in females and absent in males, but the opposite pattern of band segregation (a band found in males and no band in females) was never observed in either the AFLP or RFLP experiments. Two of the sex-linked markers were placed on a linkage map. They both map at the same location in a linkage group comprising other markers not segregating with sex. Our data suggest that a single locus governs the sex determination and that nonrecombining sex chromosomes are absent in *S. viminalis*. A close association was found between skewed sex ratio and segregation distortion at this locus.
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Introduction

In most dioecious plant species, the presence of heteromorphic sex chromosomes is still disputed (Parker, 1990), and with the exception of a handful of species (eg *Silene latifolia*; Monéger *et al*, 2000) little is known about sex determination mechanisms. With the advent of the molecular age, the situation is changing rapidly but we are still far from a global picture of plant sex determination mechanisms and their evolution (Grant *et al*, 1994; see Ainsworth, 1999, 2000). The favored approach of most recent studies on sex determination in plants is reverse genetics: (i) identify molecular markers tightly linked to sex; (ii) screen a genomic library with the identified markers; (iii) isolate candidate gene(s) responsible for sex determination from the genomic library; and (iv) confirm gene function by complementation analysis (Terauchi and Kahl, 1999). Most of these studies have so far only reached the first stage. Sex-linked markers have recently been found in white campion (*S. latifolia*; Mulcahy *et al*, 1992), asparagus (*Asparagus officinalis* L.; Jiang and Sink, 1997; Reamon-Büttner *et al*, 1998; Reamon-Büttner and Jung, 2000), kiwi plant (*Actinidia chinensis*; Harvey *et al*, 1997), pistacio (*Pistacia*; Hormaza *et al*, 1994), hemp (*Cannabis sativa* L.; Mandolino *et al*, 1999), hop (*Humulus lupulus*; Polley *et al*, 1997), *Atriplex garrettii* (Ruas *et al*, 1998), grapevine (*Vitis*; Dalbó *et al*, 2000) and the basket willow (*Salix viminalis*; Alström-Rapaport *et al*, 1998).

The *Salicaceae* (300 willow species and 30 poplar species) are noteworthy in that all but two, *Populus lasiocarpa* and *Salix martiana*, are dioecious. Only 4–10% of all flowering plants are dioecious, and dioecy is sporadic, with occasional dioecious species in otherwise hermaphrodite genera, suggesting recurrent evolution of dioecy in plants (Parker, 1990). Another intriguing feature of many Salicaceae is their biased sex ratio. For instance, the sex ratio of the basket willow (*S. viminalis* L., $2n = 38$) populations is usually strongly female-biased, and controlled crosses suggest that sex biases are not only the result of environmental effects (Alström-Rapaport *et al*, 1997). Interestingly, the same features, predominance of dioecy and biased sex ratios, are also found in the dioecious genus *Dioscorea* (Terauchi and Kahl, 1999). Another feature of both *Salix* and *Dioscorea* is the large number and small sizes of their chromosomes, making it difficult to test for the presence of heteromorphic chromosomes.

Unfortunately, little is known about the sex determination system of Salicaceae. Two previous studies (Alström-Rapaport *et al*, 1997, 1998) indicated that the presence of sex chromosomes is unlikely, and suggested a multilocus autosomal sex-determination system. The absence of heteromorphic sex chromosomes or chromosome-mediated sex determination in a plant species might not in itself be very surprising, as these are apparently very rare in flowering plants (Parker (1990),

but see Guttman and Charlesworth, 1998), although one might have expected them to evolve in a genus where dioecy appeared relatively early.

The main goal of the present study was to identify and map sex-linked markers and, more specifically, to test the hypothesis that sex-determining loci were on different chromosomes as suggested by our previous studies. Bulked segregant analysis with amplified fragment length polymorphism (AFLP) was used since the AFLP technique has been successfully used to identify sex-linked markers in *A. officinalis* (Reamon-Büttner *et al*, 1998; Marziani *et al*, 1999; Reamon-Büttner and Jung, 2000), *Dioscorea tokoro* (Terauchi and Kahl, 1999) and *Rumex acetosa* (Ainsworth *et al*, 1999). The fragments segregating with sex were then converted to RFLP markers and placed on an independently generated AFLP-based map.

Materials and methods

Plant material
The F$_2$ progeny of a factorial mating design between four male clones (X5–X8) and four female clones (X1–X4) were analyzed in the present study (Table 1). These eight clones were initially retained for studies on sex determination and sex ratio because crosses among them led to different sex ratios (Alström-Rapaport *et al*, 1997). The material used here is further described in Alström-Rapaport *et al* (1997, 1998). The general strategy of the analysis is given in Figure 1.

AFLPs
DNA was extracted from leaves using the FastDNA kit (BIO 101, Inc.) according to the manufacturer's protocol with small modifications: 1% PVP-40, 0.1% of ascorbic acid and 0.1% of 2-mercaptoethanol were added to the extraction buffer before use. The AFLP procedure (VOS *et al*, 1995) includes five steps: (1) restriction of DNA by two enzymes, *Eco*RI and *Mse*I, and simultaneous ligation of adapters; (2) amplification of the resulting fragments
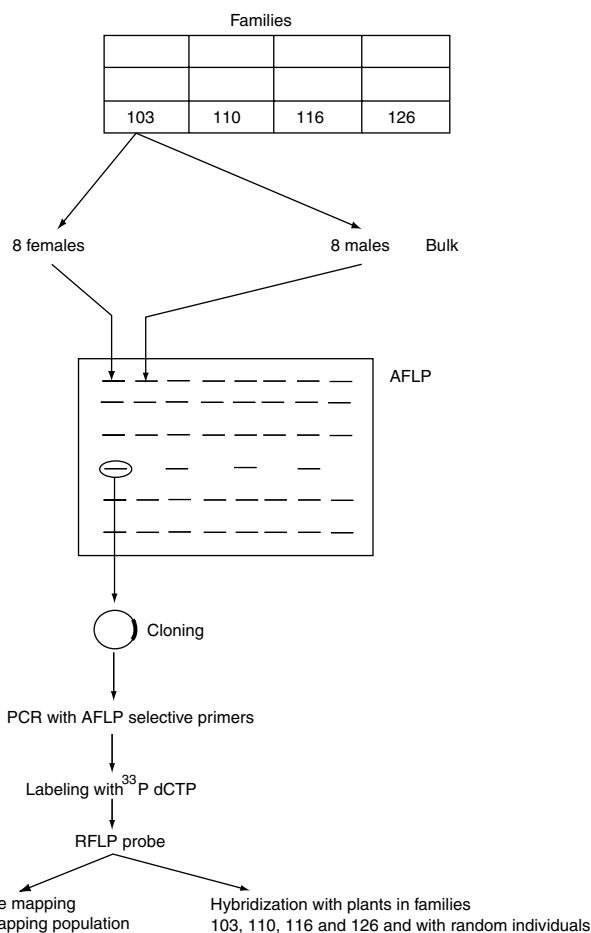

Figure 1 General experimental strategy.

with preselective primers; (3) amplification of the diluted products with selective primers (two selective nucleotides were used with the *Eco* primer, and three with the *Mse* primer, and the *Eco* primer was labeled with γ^{33}P-ATP); (4) electrophoretic separation of the fragments in denaturing 4.5% PAAG; (5) radioautography of the gel.

Bulked samples of eight males and eight females were prepared from preamplification products in each of families 103, 110, 126 and 116. These four pairs of bulked samples were investigated by screening 88 selective primer combinations. A band was suspected to be sex linked if it was present in one sex and absent from the other in at least two families. To verify the sex-linked pattern of these bands, their distributions were checked in individual plants of the same families and of other families in the mating scheme.

Band isolation and cloning
Bands were cut from the dried gel, DNA extracted by the 'crush and soak' method of Sambrook *et al* (1989) and amplified using the same PCR conditions as in the selective amplification step. The PCR product was then cleaned (QIAQUICK PCR purification kit, QIAGEN) and cloned into p-GEM T easy plasmid (Promega Co.). Positive colonies were selected according to the electrophoretic mobility of the inserted fragment. For this purpose, PCRs with the selective primers were con-

Table 1 *S. viminalis* analyzed for sex-linked markers

Family	Mother	Mother parents	Father	Father parents	Female/male sex ratio	Number of individuals used in the present study
115	970-48	X1, X8	970-89	X1, X8	1.47	18
103	971-82	X1, X7	960-83	X3, X5	2.75*	36
126	971-82	X1, X7	972-13	X4, X5	4.93*	32
116	971-64	X1, X7	970-89	X1, X8	1.65	33
132	971-64	X1, X7	968-14	X1, X5	2.36*	10
118	969-72	X1, X6	970-89	X1, X8	1.58*	10
133	969-72	X1, X6	968-14	X1, X5	2.17*	10
106	962-41	X3, X8	960-83	X3, X5	3.35*	10
113	962-87	X3, X5	975-26	X4, X7	1.40	10
112	960-99	X3, X5	975-26	X4, X7	1.23	10
110	973-99	X4, X6	975-26	X4, X7	1.24	33
111	975-80	X4, X7	975-26	X4, X7	2.87*	17
107	958-74	X2, X8	975-26	X4, X7	2.67*	10
129	957-34	X2, X6	968-14	X1, X5	0.71	10

Families 103, 110, 126 and 116 were used in the bulked segregant analysis. *Departs significantly from 1:1 (χ^2 test); 55–93 individuals per family were used when estimating the sex ratio.

ducted using the colonies as template. The mobility of the PCR product was compared with the corresponding AFLP fragment on the sequencing gel. At least two clones were selected and sequenced for each sex-linked AFLP marker. For one of these fragments, it was possible to design a pair of primers, allowing its amplification. The PCR conditions were 1X DynaZyme buffer (Finnzymes F-500), 0.2 mM dNTPs, 0.2 µM primer sTA-CCG1 (CAC TTC TTG GAT TTC TTC CCA CC), 0.2 µM primer sTA-CCG2 (CAC CGA GGC ATT GGA GAT AAA C), 0.04 U/µl *Taq* polymerase and ∼1 ng/µl DNA.

RFLPs

In order to look at the differences between sexes in the pattern of the hybridization of sex-linked probes, a first set of RFLP experiments was conducted using 16 randomly chosen *S. viminalis* clones and some of the clones in the AFLP experiment. Plants were analyzed by Southern blot of RFLPs and hybridization with radioactively labeled cloned sex-linked AFLP fragments. Total DNA was extracted from fresh leaves according to Doyle and Doyle (1990) or from autumn buds using the protocol of Devey *et al* (1996); the latter gave better results than the former. A measure of 10 µg of DNA was digested with *Eco*RV and the resulting fragments were separated at 1.5 V/cm on 0.8% agarose gel in 1 × TAE buffer. After electrophoresis, DNA was transferred to a nylon HybondN+ (Pharmacia) membrane using the alkali-transfer method. The sex-linked DNA fragments used for the hybridization experiments were amplified from the corresponding plasmids using pairs of selective AFLP primers under the same PCR conditions as for the selective amplification. The resulting PCR product was then purified using the QIAquick PCR purification kit (QIAGEN). The radioactive probe was prepared from 100–200 ng of cleaned PCR product using Oligolabeling Kit (Pharmacia). The labeled mixture was then passed through Sepharose CL-6B spin column to clean the labeled probe. Prehybridization was conducted for 4 h at 65°C in Hybaid oven in a solution containing 1% dextran sulfate, 4 × SETS, 10 × Denhardts and 0.1% SDS. A volume of 100 µl of freshly boiled herring testes DNA (10 mg/µl) was added to 30 ml prehybridization solution before prehybridization. Hybridization was conducted overnight at 65°C. The hybridization solution is the same as the prehybridization solution, but 10% dextran sulfate was used instead of 1%. Hybridized filters were then washed in 2 × SSC, 0.1% SDS for 5 min at 65°C, then for 15 min at room temperature and 1–2 times for 15 min at 65°C. After that, filters were exposed to BIOMAX Kodak film with intensifying screen 1–7 days at −70°C.

Genetic linkage map

S. viminalis clone Jorunn and the *S. dasyclados* clone 901290 were crossed to produce F$_1$ seeds. Two F$_1$ plants were then crossed to produce the F$_2$ mapping population. The 92 F$_2$ individuals were scored for 756 AFLP markers. In addition, two cloned sex-linked AFLP fragments (TA-CCG and AA-CCG) were used as RFLP probes and hybridized to Southern blot filters with *Eco*RV- and *Bam*HI-digested DNA from 66 individuals in the mapping population. The sex of these 66 individuals was also recorded.

A linkage map based on markers segregating in one or the other F$_1$ parent (1:1 segregation ratios) was constructed using MAPMAKER (version 3.0; Lincoln *et al*, 1992). Each resulting linkage group was thus derived from segregation in either the male or the female F$_1$ parent. AFLP markers segregating in both parents (3:1 segregation ratios) were then used with the program package JoinMap (Stam, 1993; Stam and Van Ooijen, 1995) to merge homologous linkage groups from the two parents. In total, 16 linkage groups are the result of segregation in either the male or the female parent. At a LOD score of 3.0, 371 markers were assigned to 49 major linkage groups (larger than 15 cM).

Results

Bulked segregant analysis

A total of 88 selective primer combinations were screened using bulked males and females sampled from four families. On average, 53 AFLP bands per primer combination were found to be monomorphic, 19 bands were polymorphic between families and two bands were polymorphic within some families and monomorphic in the rest of the families. Four clear and strong bands that were polymorphic in at least two families were found using four different primer combinations: (i) *Eco* primer bearing selective nucleotides 'AA' and *Mse* primer having 'CAT' selective nucleotides; (ii) *Eco* primer with 'AA' selective nucleotides and *Mse* primer with 'CAG' nucleotides; (iii) *Eco* primer with 'TA' and *Mse* primer with 'CCG'; and (iv) *Eco* with 'TG' and *Mse* with 'CAA'. The fragment sizes are 485, 192, 528 and 302 bp, and are called aAA-CAT, aAA-CAG, aTA-CCG and aTG-CAA, respectively.

The analysis of individual samples confirmed that they were cosegregating with sex. Another 11 weaker bands that were also polymorphic within some families, but monomorphic among the rest of them, were also tested in individual samples, but none was found to cosegregate with sex. The four sex-linked markers were scored in individuals that were used for bulked sample preparation, in additional individuals from the same families and in individuals from other families (Table 2). None of the AFLP fragments was completely associated with sex in the families investigated. However, for those associated with sex, the association between markers and sex was strong in most families. A pair of primers that amplified the sex-linked fragments was constructed from the sex-linked AFLP fragment aTA-CCG. The presence or absence of the PCR products obtained with these primers corresponds to the presence or absence of the AFLP fragment in most cases. This marker was designated 'sTA-CCG'. Most of the individuals were scored with this pair of primers (Table 2). Since some of the families (eg families 133 and 113, and 133 and 107) are not related to each other in the parent and grandparent generations, it is reasonable to conclude that these sex-linked markers are not specific for this experimental group, but are common to natural populations of *S. viminalis*. In general, the four AFLP fragments and the PCR marker sTA-CCG were present in females and absent in males. The discrepancies reported in Table 2 could be attributed to various causes. First, they could result from misclassifications in sex or family. However,

Table 2 Number of individuals in each family with (first number) and without (second number) the AFLP fragments associated with sex

Family	Individual	Sex	AFLP fragments				sTA-CCG
			aAA-CAT 485 bp	aAA-CAG 192 bp	aTA-CCG 528 bp	aTG-CAA 302 bp	PCR marker
103	Males		4/11	0/15	3/12	0/15	1/14
	Females		14/3	17/0	14/4	17/0	16/3
110	Males		1/7	0/1	1/7	2/11	0/16
	Females		8/1		7/2	11/2	14/3
111	Males						0/6
	Females						11/0
115	Males						(0/10)
	Females						(0/8)
126	Males		0/8	0/8	0/8	0/3	0/14
	Females		8/0	16/1	8/0	6/0	16/2
116	Males		1/7				1/15
	Females		6/0				15/2
106	Males		0/5	0/5	0/5	0/5	(0/5)
	Females		4/0	5/0	5/0	5/0	(1/4)
107	Males		3/2	2/3	2/1	$\frac{1}{4}$	0/5
	Females		3/0	0/3	2/0	5/0	5/0
112	Males		0/3	0/4	0/4	0/4	0/4
	Females		6/0	0/6	6/0	6/0	6/0
113	Males		2/3	1/4	0/5	0/5	0/5
	Females		5/0	2/3	5/0	5/0	5/0
118	Males		2/2	1/3	0/4	0/4	0/4
	Females		5/1	4/2	4/1	5/1	5/1
132	Males		0/5	0/4	0/3	0/3	0/5
	Females		4/1	3/1	4/0	5/0	5/0
133	Males		0/5	0/5	0/5	0/4	0/5
	Females		5/0	5/0	5/0	4/1	5/0
129	Males		1/4	1/4	1/3	0/5	0/5
	Females		3/2	1/4	5/0	5/0	4/1

The results in parentheses probably correspond to experimental artifacts.

Table 3 Presence or absence of sex-specific RFLPs in males (m) and females (f) of families 103, 126, 110 and 116 and in the randomly chosen individuals; Probe used, size of fragment (kb) and its presence (1) or absence (0) in the individual plant

Individual	pAA-CAG								pTA-CCG
	19	16	15.5	14.8	11.6	9.8	5.0	3.7	19
m 103-49	0	0	0	0	0	0	1	0	0
m 103-19	0	0	0	0	0	0	1	0	0
m 103-89	0	0	0	0	0	0	1	0	0
f 103-78									1
f 103-88	0	1	1	0	1	1	1	1	1
f 103-77	0	1	1	0	1	1	0	1	1
f 103-87	0	1	1	0	1	1	0	1	1
m 126-24	0	0	0	0	0	0	0	0	0
f 126-14	0	1	1	0	1	1	0	1	1
f 126-23	0	1	1	0	1	1	0	1	1
m 110-43	0	0	0	0	0	0	0	0	0
m 110-38	0	0	0	0	0	0	0	0	0
f 110-07	0	0	0	1	0	0	0	0	1
m 116-31	0	1	0	0	0	0	0	0	0
m 116-10	0	1	0	0	0	0	0	0	0
f 116-35	0	1	1	0	1	1	0	1	1
Prob.		0.02	0.00		0.00	0.00		0.00	0.00
M	0	0	0	0	0	0	0	0	0
M	0	0	0	0	0	0	0	0	0
M	0	0	0	0	0	0	1	0	0
M	0	0	0	0	0	0	0	0	0
M	0	1	0	0	0	0	0	0	0
M	0	1	0	0	0	0	0	0	0
M	0	0	0	0	0	0	1	0	0
M	0	0	0	0	0	0	1	0	0
F	1	0	0	0	1	1	0	0	1
F	1	0	0	0	1	1	1	0	1
F	0	0	0	0	1	1	0	0	1
F	0	0	0	0	1	1	1	0	1
F	0	1	0	0	0	0	0	0	0
F	1	1	0	0	0	0	0	0	0
F	1	0	0	1	0	0	1	0	1
F	0	0	0	1	0	0	1	0	1
Prob.	0.08			0.08	0.08				0.00

Probability (*P*) of independence of the band and sex was calculated using χ^2 distribution separately for individuals from families (103, 126, 110) and for random individuals.

if this were the case, one would expect all the markers to show the same pattern. Second, poor quality of DNA preparation could lead to artifacts or to inconsistent amplification. Third, experimental errors, such as contamination of the PCR mix with PCR product or plasmid DNA or sample leakage between neighboring wells in sequencing gel, cannot be completely ruled out, although all necessary precautions were taken to minimize their occurrence. Discrepancies could also be due to comigrating AFLP fragments or to restriction site mutation. The latter is most likely responsible for the absence of the aAA-CAG AFLP fragment in both males and females in the family 110. Finally, recombination among marker loci and the locus responsible for sex cannot be ruled out.

RFLP experiments

Results of hybridization of the Southern blot filters with the cloned sex-linked AFLP fragments are shown in Table 3. Only two of the tested probes, pTA-CCG and pAA-CAG, produced RFLP bands associated with sex. The pTG-CAA probe showed no polymorphism, while the pAA-CAT probe was polymorphic but this polymorphism was not associated with sex; the failure of the last two probes is probably the result of cloning the wrong comigrating AFLP fragment. Selective amplification produced many weak bands, some of which could comigrate in the acrylamide gel with the targeted fragment and be excised out of the gel and cloned together with the targeted fragment. The pTA-CCG probe produced a single band that was linked to sex, while the pAA-CAG probe produced many bands some of which were linked to sex, and others not. The 19 kb RFLP band seems to be allelic to bands 15.5 and 3.7 kb (pAA-CAG probe). Indeed, the 19 kb band was absent in the progenies of families 103, 126, 110 and 116, but present in the random sample. Conversely, bands 15.5 and 3.7 kb were absent in the random sample but were present in the progenies, and they both show exactly the same segregation pattern. Obviously, larger samples of individuals will have to be tested to confirm the allelic state of the 15.5 and 3.7 kb bands to the 19 kb band. In most cases, the band was present in females and absent in males, but it is noteworthy that the opposite pattern of band segregation (a band found in males and not in

females) was never observed in either AFLP or RFLP experiments.

Segregation of markers

In each family, we sampled 10–36 individuals with equal proportions of males and females (Table 2). The strong female association of the markers, particularly for the sTA-CCG sex primer fragment, therefore resulted in a ratio of individuals with and without the band in the samples close to 1.0 (Table 4). However, the sex ratios in the samples used in this study and in the families from which these samples were drawn are significantly different in eight of the 14 families (103, 126, 118, 132, 133, 106, 111 and 107). If this is taken into account, the number of individuals carrying the marker in these five families exceeds what would be expected for a 1:1 segregation. Conversely, in family 129, there is a deficit of individuals carrying the marker. The apparent segregation distortion observed in families with an excess of individuals carrying the marker can have two explanations: (1) both the mother and the father carry the marker for these five families and we get a 3:1 segregation of the marker(s); or (2) only one of the parents carries the marker, and some other genetic phenomenon causes the distortion. If both parents carried the marker, then we would expect a 1:1 segregation of the marker in the male progeny, which was not observed for any of the crosses (Table 2). Assuming that only one parent in each family, in this case the mother, carries the marker, the skewed marker segregation ratios and sex ratios observed in some families must be explained by some other factor. For example, an excess of individual plants carrying the marker could be caused by reduced germination of seeds in which the marker is absent. However, germination tests show that the proportion of germinated seeds was high in all families (Table 4). Reduced germination therefore fails to explain the skewed segregation in family 126, 132 and 129. The segregation distortion must have occurred prior to or at the time of fertilization. Segregation of a recessive lethal allele at a linked locus, differential gametic viability or unequal gamete segregation at meiosis could all explain our data.

Mapping of the sex-linked fragments

The female-to-male ratio of the mapping population was 1.6. The probe pTA-CCG revealed a single polymorphic locus (hereafter designated as 'pTA-CCGa') that segregated perfectly with sex. The pAA-CAG probe detected two polymorphic loci, one of which segregated perfectly with sex (hereafter designated as 'pAA-CAGb'), while the second showed no linkage to sex. At a LOD score above seven, the two cosegregating sex-linked loci (and sex itself) mapped to a linkage group derived exclusively from markers segregating in the female parent (Figure 2). The length of the linkage group was 60 cM, indicating frequent recombination along the chromosome containing the sex-determining locus.

Discussion

Female-associated markers

Bulked segregant analysis revealed four AFLP fragments associated with sex in *S. viminalis*. The four fragments showed the same pattern in all families studied; they were present in almost all females and absent in nearly all males. This is also true for the sex-linked RAPD marker that was found previously (Alström-Rapaport *et al*, 1998) and it suggests that males differ from females by the absence of some chromosomal fragments. Support for this is provided by the fact that RFLPs derived from two of the AFLPs associated with sex, namely the single 19 kb pTA-CCG restriction fragment and five pAA-CAG fragments, are specific to females. RFLP markers based on two out of the four sex-linked fragments (probe pAA-CAG and pTA-CCG) mapped to the same location (Figure 2). The segregation patterns of the two AFLP fragments aAA-CAT (485 bp) and aTG-

Table 4 Marker segregation and sex ratio

Family	Presence/absence ratio of the sTA-CCG sex primer fragment	Female/male ratio of individuals used in this study	Female/male ratio of all progeny	Estimated presence/absence ratio of fragment for all progeny	Prop. Germinated seeds
115	—	0.80	1.47	—	0.87
103	1.00	1.27	2.75*	1.75	—
126	1.00	1.29	4.93*	2.83	0.99
116	0.94	1.06	1.65	1.35	0.94
132	1.00	1.00	2.36*	2.36	0.93
118	1.00	1.50	1.58*	1.05	0.89
133	1.00	1.00	2.17*	2.17	0.97
106	—	1.00	3.35*	0.23	0.84
113	1.00	1.00	1.40	1.40	0.83
112	1.50	1.5	1.23	1.23	0.91
110	0.74	1.06	1.24	0.84	—
111	1.83	1.83	3.87*	3.87	—
107	1.00	1.00	2.67*	2.67	—
129	0.67	1.00	0.71	0.5	0.86

The second and third columns give the observed ratio of individuals with/without the sTA-CCG sex primer marker and sex ratio in the studied samples, respectively. The third and fourth columns give the sex ratio and the estimated ratio of individuals with/without the sTA-CCG sex primer marker in the whole family. The last column gives the proportion of germinated seeds for each family. *Departs significantly from 1:1 (χ^2 test). — denotes nonavailable data.
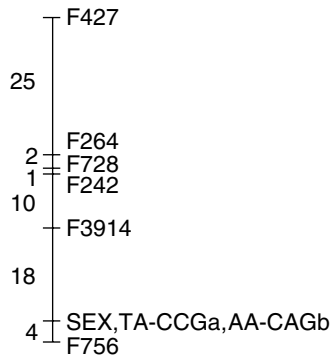
Figure 2 Linkage group carrying sex-linked markers. The linkage group is derived from AFLP markers segregating in the female parent and two sex-linked RFLP markers (pTA-CCGa, pAA-CAGb). Both RFLP markers cosegregated with sex. Recombination distances are given in Kosambi centimorgans.

CAA (302 bp), which could not be mapped because they were not successfully cloned, are almost identical to the two mapped fragments in 13 families (Table 2). We therefore conclude that the four fragments are closely located physically or are physically separated but reside in an area where recombination is limited.

The pattern in *S. viminalis* is exactly the opposite to that observed in *D. tokoro* (Terauchi and Kahl, 1999), *Asparagus* (Jiang and Sink, 1997; Reamon-Büttner *et al*, 1998), *A. garrettii* (Ruas *et al*, 1998), *C. sativa* (Mandolino *et al*, 1999) and *H. lupulus* (Polley *et al*, 1997), where the sex-linked AFLP/RAPD fragment was present in males and absent in females. In all these cases, it was already known or could be inferred that the male was the heterogametic sex and the female homogametic. This can apparently be ruled out in *S. viminalis*. The pattern observed in *S. viminalis* is similar to that observed in *Pistacia vera* (Hormaza *et al*, 1994).

Sex chromosomes versus single sex-determination locus

The large number of primer pairs used and polymorphic fragments, more than 1000, resulted in only four sex-linked fragments that appear to be closely linked. Assuming that the 1000 fragments are evenly distributed over the genome, we expect around 27 segregating fragments per chromosome. Likewise, a large number of RAPD primers were tested in a previous study that resulted in a single sex-associated marker. In comparison, using 60 RAPDs primer pairs, four sex-linked markers were found for a single family of *S. latifolia* which has distinct heteromorphic sex chromosomes (Mulcahy *et al*, 1992). Clearly, the presence of a sex determination system similar to that of *S. latifolia* is not likely for *S. viminalis*. On the contrary, our results suggest that a very restricted region of the chromosome is involved in sex determination.

Why have not sex chromosomes evolved?

As concluded by Alström-Rapaport *et al* (1998), there are apparently no sex chromosomes in *S. viminalis*. Before discussing the absence of nonrecombining sex chromosomes, a few words on the genetic map are necessary. The linkage map has not yet coalesced to the 19 groups one would expect from the known chromosome number

in *S. viminalis* and it is likely that some of the minor linkage groups will cluster with major ones when more markers are added and/or an extended pedigree population is used. However, a better map will not change our conclusions regarding the absence of sex chromosomes, although it will probably change the structure of the linkage group(s) carrying sex-determining loci. The linkage group was derived from markers segregating exclusively in the female parent. A more comprehensive map will identify the homologous linkage group derived from segregation in the male parent. A comparison of these two linkage groups could reveal structural differences between chromosomes carrying male- and female-determining genes. As stated previously, our data suggest that males differ from females by the absence of some chromosomal segments.

So, why have not sex chromosomes evolved in *S. viminalis*, a species belonging to a genus in which almost all species are dioecious? Preliminary results suggest that other willow species differ from *S. viminalis*. The sex-linked markers developed in *S. viminalis* failed to cosegregate with sex in *S. caprea* (V Semerikov, unpublished data) and SCAR markers developed from the RAPD band found by Alström-Rapaport *et al* (1998) in *S. viminalis* have so far given mixed results in *S. eriocephala* (LE Gunter, personal communication). If confirmed, these results would indicate that there is no ancient sex-determination system shared by all willow species, or at least that the area around it has not been conserved. In *Silene*, dioecy has probably evolved twice within a period estimated to be between 8 and 24 million years (Desfeux *et al*, 1996). Hence, sex chromosomes can evolve in a relatively short period of time. A recent molecular phylogeny of the Salicaceae (Leskinen and Alström-Rapaport, 1999) using ribosomal DNA sequences (5.8S, ITS1 and ITS2) found very low sequence divergence between species. *Salix* fossils have been retrieved from the Eocene, 55–65 MYA (Collinson, 1992). This suggests a very low rate of sequence evolution with substitution rates of 0.4×10^{-9} substitutions per year, assuming a divergence of *Salix* species 40 MYA (Leskinen and Alström-Rapaport, 1999). Perhaps the same low rate of evolution also applies to sex-determination systems, explaining the maintenance of a single or multilocus sex determination system in some if not all species of *Salix*. This however supposes that slow sequence evolution is coupled to slow structural genome evolution, a relationship that, to the best of our knowledge, is not established.

Acknowledgements

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