

Identification of a RAPD Marker Linked to Sex Determination in the Basket Willow (*Salix viminalis* L.)

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In many dioecious plants, gender influences economic value, breeding schemes, and/or opportunities for commercial use of genetically transformed materials. The objective of this study was to identify molecular markers linked to sex determination loci in the dioecious plant *Salix viminalis* L. A 4 × 4 factorial mating design was used to identify sex ratios in full-sibling progeny, to generate a working genetics model for segregating sex ratios, and to search for molecular markers linked to sex determination genes. Bulked segregant analysis, utilizing 380 arbitrary decamer primers to generate randomly amplified polymorphic DNA (RAPD) products was initially applied to progeny sets from 3 of the 16 full-sibling families. Of the 1080 RAPD bands examined, only a single 560 bp band was shown to be linked to a sex determination locus. The same 560 bp band occurred in three additional full-sibling families and was present in one female parent and one male parent involved in the factorial mating design. This marker, UBC354₅₆₀, is biparentally inherited, is associated with femaleness in certain genetic backgrounds, and is linked to allele A1 in the proposed two-locus epistatic genetic model of sex determination for *S. viminalis*. Southern blots confirmed marker homology among progeny and parents used in this study.

Sex determination (i.e., the expression of femaleness or maleness) in plants is not well understood. Neither the genetic nor the physiological basis of gender has been completely resolved in any plant species, in spite of the striking progress made over the last decade in the understanding of floral development (Durand and Durand 1990; Dellaporta and Calderon-Urrea 1993; Grant et al. 1994; Weigel and Nilsson 1995). Recent studies suggest that all plant species possess the necessary genes to develop perfect flowers (e.g., homeotic genes—MADS box genes; Coen and Meyerowitz 1991) and that sex determination genes acting downstream or independently of homeotic genes arrest the development of either stamen or carpels at various stages, leading to the diversity of floral types and sexual modes observed among flowering plants (Dellaporta and Calderon-Urrea 1993; Hardenack et al. 1994). Our current understanding of the genetic basis of floral development has been achieved by focusing on a few model organisms for which large collections of natural or artificially induced mutations exist (Chattopadhyay and Sharma 1991). Among those well-studied species are *Antirrhinum majus* L. and *Arabidopsis thaliana*

(L.) Heynhold, which have perfect flowers; *Zea mays* L., which is monoecious; and *Mercurialis annua* L., which is dioecious (Durand and Durand 1991). In the dioecious Salicaceae family (willows, poplars), the mechanisms of sex determination have not been resolved, though McLetchie and Tuskan (1994) reported no evidence of sex chromosomes in *Populus trichocarpa* × *P. deltoides* hybrids, despite testing a large number of potentially linked markers. Likewise, studying the sex ratios in a factorial mating scheme, Alstrom-Rapaport et al. (1997) concluded that it is unlikely that *Salix viminalis* L. possesses sex chromosomes and proposed a multilocus sex determination system.

The genetic basis of sex determination in dioecious plants may be extremely diverse. Some dioecious species have heteromorphic sex chromosomes (e.g., *Silene latifolia* Poiret), whereas in other species sex is determined by one or several autosomal nuclear loci, possibly influenced by cytoplasmic genes (Durand and Durand 1991). Similarly, hormonal regulation of gender does not appear to follow any well-established pattern. In *Mercurialis*, cytokinins cause male-to-female conversion (Louis 1989), whereas in *Asparagus officin-*

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alis L., the opposite effect has been observed (Bracale et al. 1991). Moreover, Yin and Quinn (1995) have recently proposed a one hormone model for sex determination that includes the genetic control of hormone receptors as well as endogenous hormone concentrations.

Besides its obvious relevance to the understanding of evolution of mating systems, the unraveling of sex determination mechanisms would also be extremely useful in plant breeding and improvement. In dioecious species it is often beneficial to select one gender over another; for example, in *Asparagus*, female clones are preferred over male clones, and in *Populus* short-rotation energy plantations, male clones typically achieve higher dry weight yields than female clones (Tschaplinski and Tuskan 1994). However, in most perennial species it is impossible to determine gender until the organisms are reproductively mature (~1–20 years old). The inability to determine gender at early ages can create problems in advanced-generation breeding schemes, particularly when all superior parental selections or all progeny are unknowingly composed of one gender. Furthermore, for genetically engineered plants, commercial release may require sterility (Strauss et al. 1995). Thus further characterization of sex determination through the identification of linked markers or the isolation of expressed DNA sequences would advance many plant improvement programs.

Bulked segregant analysis involving randomly amplified polymorphic DNA (RAPD) products has been used successfully to identify molecular markers linked to sex determination in several dioecious plant species. Mulcahy et al. (1992) identified four primers which produced markers associated with gender in *Silene latifolia*, a dioecious species possessing heteromorphic sex chromosomes; Hormaza et al. (1994) detected a single marker associated with females and absent in males in *Pistacia vera* L.; and Sakamoto et al. (1995) recently reported on a 730 bp RAPD marker that was associated with male *Cannabis sativa* L. In the present study we use bulked segregant analysis of RAPD products to identify markers linked to sex determination in *Salix viminalis*. Segregation sex ratios within full-sibling families and marker occurrence are then used to construct a genetic model for sex determination in *S. viminalis*.

Table 1. Observed sex ratios among full-sibling progeny from 13 families of 4 × 4 factorial mating design used to identify randomly amplified polymorphic DNA (RAPD) markers linked to gender in *S. viminalis*

Maternal parent	Paternal parent							
	X5		X6		X7		X8	
	Females	Males	Females	Males	Females	Males	Females	Males
X1	68	(968) 26	98	(969) 2	58	(971) 31	63	(970) 20
X2		— ^a	32	(957) 71		—	58	(958) 21
X3	37	(960) 47	59	(961) 24		—	39	(962) 43
X4	46	(972) 43	20	(973) 93	51	(975) 36	54	(974) 48

Values within columns indicate the number of female or male progeny per family. Arbitrary family identification numbers are presented within parentheses for each female:male parental combination.

^a — indicates no viable seeds were obtained from these parental crosses.

Materials and Methods

Plant Material and DNA Extraction

Four male clones of *S. viminalis* were crossed to four female clones according to a complete factorial mating design (Alstrom-Rapaport et al. 1996). Of these 16 crosses, three crosses did not produce any offspring; the remaining 13 crosses produced progeny that varied in sex ratio from female bias to male bias (Table 1). The phenotypic sex ratios were used to construct a working genetics model of gender determination as a means of improving our ability to detect linked DNA markers. The simplest model (i.e., the model with the fewest number of loci and alleles) that did not significantly ($\alpha \leq 0.05$) deviate from the phenotype data was comprised of two loci, each with four alleles, interacting in an epistatic manner. In this model, the first locus contained alleles *m1* and *m2* for maleness and *f1* and *f2* for femaleness, where the degree of dominance is *f1* > *m1* > *f2* > *m2*. The second locus was incompletely epistatic to the first, where genotypes *-S1S1*, *-S1S2*, or *-S1S3* are recessive to the first locus; genotypes *-S1S4*, *-S2S2*, *-S2S3*, *-S3S3*, or *-S2S4* result in female progeny only, regardless of the allelic arrangement at the first locus; and genotypes *-S3S4* or *-S4S4* result in male progeny only. It should be noted that this is one of several alternative models which fit the phenotypic data. From this working model parental genotypes were inferred. Although the expected phenotypic sex ratios ranged from 15:1 to 1:3 female:male ratio, the above model predicted that three full-sib families—960, 962, and 972—were each segregating at a single locus for an allele that determined gender. Progeny from these three families were then used in a bulked segregant

analysis to identify a marker associated with gender.

Genomic DNA was extracted based on a modified CTAB procedure (Doyle and Doyle 1987). A single leaf minus the midrib was hand-macerated under liquid nitrogen and immediately suspended in 600 μ l of a 2× CTAB extraction buffer containing 1% (w/v) polyvinylpyrrolidone (PVP) in a 1.5 ml microcentrifuge tube. The macerate/buffer mixture was gently agitated at room temperature for 1 min and then incubated at 65°C for 30 min, followed by two 1× volume extractions with chloroform:isoamyl alcohol (24:1). The final aqueous phase was transferred to a new microcentrifuge tube, DNase-free RNase A (final concentration 70 μ g/ml) was added to the supernatant and the entire volume was incubated at 37°C for 30 min. DNA was precipitated with 2/3 volume of 0°C isopropanol. Following centrifugation at 5200×g for 15 min, the isopropanol was removed and the DNA pellet was rinsed with 75% (v/v) ethanol containing 10 mM ammonium acetate. The pellet was dried under vacuum and desiccant for 1 h and then suspended in 50 μ l TE buffer (pH 8.0) for storage at 4°C. The DNA concentration was determined for each sample using a Hoefer TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, California).

Polymerase Chain Reaction and Bulk Segregant Analysis

DNA characterization relied on a polymerase chain reaction (PCR) procedure for generating RAPD bands (Welsh and McClelland 1990; Williams et al. 1990). The RAPD reactions were completed in an MJ Research PTC-100 thermocycler (MJ Research Inc., Chatham, New Jersey) with a 35 cycle protocol involving 5 s at 94°C denaturation, 30 s at 36°C annealing, and 1

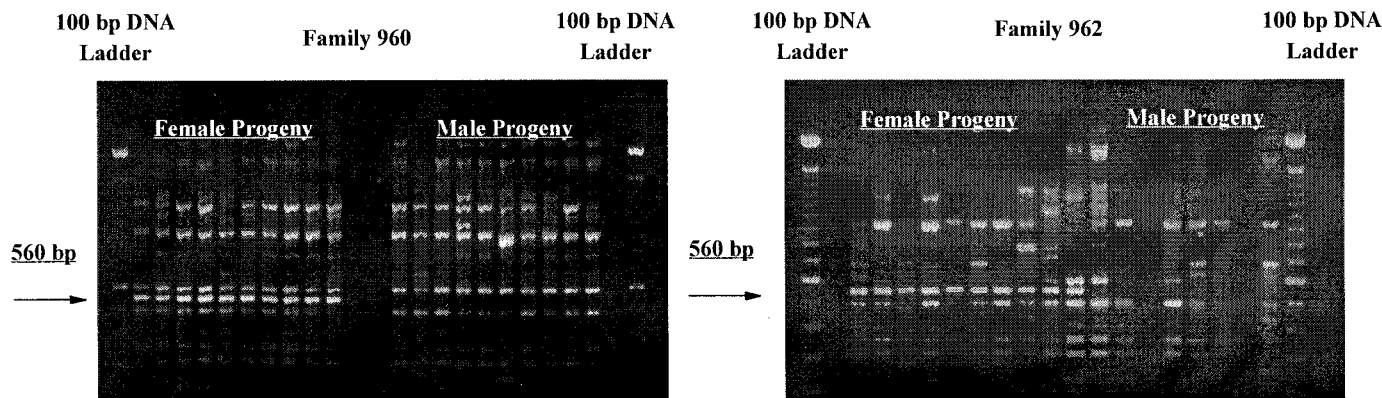


Figure 1. Gender differences among female and male progeny for the occurrence of a RAPD band at 560 bp produced using primer UBC354 in full-sibling family 960 and 962 of *S. viminalis*.

min at 72°C polymerization steps within each cycle, followed by a 5 min at 72°C final polymerization step. Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, 0.1% (v/v) Triton X-100, 1.0 µg bovine serum albumin (Sigma, St. Louis, Missouri, catalog no. A-4503), 0.5 units *Taq* polymerase (Promega, Madison, Wisconsin), 200 µM each dNTP, 10 ng primer, and 1.0 ng DNA template, to a final volume of 10 µl. A control reaction containing all PCR components except the DNA template was run with each set of reactions to verify the lack of contaminant DNA. Amplified PCR products (i.e., RAPD bands) were resolved using a 1.5% (w/v) agarose 0.5× TBE gel containing 0.1 µg/ml ethidium bromide electrophoresed under constant voltage (60 V) for 2.5 h. A 100 bp DNA standard (BRL, Grand Island, New York) was used to assign molecular weights to individual RAPD bands. Photographs of each gel were taken under a UV light source (254 nm) and used to score bands for data analysis. Bulked segregant analysis was used to identify polymorphic DNA markers (Michelmore et al. 1991). A total of 380 decamer primers (Operon Technologies Inc., Alameda, California, and University of British Columbia, Vancouver, British Columbia) were evaluated initially using three separate families. Each family consisted of 10 female and 10 male bulked-DNA samples. DNA samples were bulked based on equal nucleotide concentration (1 ng/1 µl) per genotype once at the beginning of the study.

Southern Blot Analysis

Following standard RAPD procedures and electrophoresis, DNA was transferred to positively charged nylon membranes (Boehringer Mannheim, Indianapolis, Indiana) and fixed by baking in a 80°C oven for 1 h. A hybridization probe derived

from a 560 bp RAPD band amplified with primer UBC354 was isolated from maternal parent X3 and purified from a 3% (w/v) Meta-Phore agarose (FMC, Rockland, Maine) gel containing 0.5× TAE buffer using the Gene-Clean kit (BioLab, La Jolla, California). The isolated DNA fragment was labeled by random priming using a nonradioactive DIG-11-dUTP labeling system (Boehringer Mannheim). The labeling reaction was accomplished by a PCR procedure in the following fashion: 94°C for 5 min initial denaturation, 35 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min, final polymerization for 5 min at 72°C. Each 50 µl reaction contained 1× PCR buffer (BRL), 2.5 mM MgCl₂, 0.1% (v/v) Triton X-100, 0.2 µg bovine serum albumin, 0.2 units *Taq* polymerase, 200 µM each dNTP in which 20% (v/v) TTP was substituted by DIG-11-dUTP, and 50 ng primer and 200 ng DNA template. Hybridization was carried out in 5× SSC, 2% (v/v) blocking reagent (Boehringer Mannheim), 0.1% (w/v) N-lauroylsarcosine and 0.02% (w/v) SDS for 16 h hybridization at 72°C. Filters were washed three times in 2× SSC and 0.1% (w/v) SDS at room temperature. Immunological detection was performed with the DIG DNA detection kit (Boehringer Mannheim) according to manufacturer's instruction.

Results

Of the 380 random decamer primers used in the bulked segregant analysis, 354 revealed amplified DNA products in the three tested families; 285 of these primers produced an average of 3.8 scorable bands per primer or approximately 1080 bands. In all, 984 bands were monomorphic within and between the female and male bulks in all three families, indicating that these bands are not tightly linked to

sex-determining loci. Ninety-six bands displayed polymorphisms between female and male bulks in one or more of the tested families. Individual female and male genomic DNA templates were then subjected to PCR amplification using the primers that revealed polymorphisms. Ninety-five of these polymorphic bands displayed a high degree of recombination between marker presence or absence and gender for individual female or male DNA templates, indicating that such markers are not tightly linked to regions of the genome controlling gender. Only one primer, UBC354, with sequence 5'-CTAGAGCCG-3', displayed a band at 560 bp that expressed complete 1:1 segregation between female and male progeny in two of the tested families (Figure 1). The 560 bp band was found among amplified products from DNA of all 10 female progeny and was absent in all 10 male progeny in families 960 and 962. The 560 bp band was absent in all progeny of family 972. Again, all other polymorphic bands were only partially absent or partially present in one or both genders in the three tested families. Interestingly progeny from families 960 and 962 share a common maternal parent. Thus, to verify inheritance of the 560 bp band, the UBC354 primer was used in a RAPD reaction in combination with genomic DNA from each of the parents in the full factorial mating design. The marker, UBC354₅₆₀, was present in only one maternal parent—X3—the common parent of families 960 and 962, and one paternal parent—X6 (Table 2). As expected, neither parent from family 972 contained the UBC354₅₆₀ marker.

Because the X6 paternal parent was used in a single cross with the X3 maternal parent, as well as in crosses with other maternal parents that did not contain

Table 2. Observed UBC354₅₆₀ frequency among full-sibling progeny from selected families of *S. viminalis* and marker occurrence among the parental genotypes

Maternal parent	Marker occurrence ^a	Paternal parent							
		X5 (-)		X6 (+)		X7 (-)		X8 (-)	
		Females	Males	Females	Males	Females	Males	Females	Males
X1	-	(968)		(969)		(971)		(970)	
X2	-	—		(957)		—		(958)	
X3	+	(960)		1:15	14:6	—		(962)	
X4	-	21:0	0:24	21:0	1:18	—		19:2	0:20
		(972)	0:20	(973)	32:5	(975)		(974)	—

The ratios within each set of female and male progeny indicate the number of offspring carrying UBC354₅₆₀ versus the number of offspring not carrying the marker. A fraction of the total number of offspring per family (see Table 1) were analyzed with respect to marker occurrence.

^a + indicates that the marker UBC354₅₆₀ was present in the parental genotype and - indicates that the marker was not present.

^b — indicates progeny sets that were not tested for marker occurrence.

UBC354₅₆₀, bulked segregant analysis was applied to 10 female and 10 male bulked-progeny sets from each of three additional families—957, 961, and 973 (Table 1). Family 961 was derived from the maternal and paternal parents that contain UBC354₅₆₀. Families 957 and 973 each have different maternal parents that lack the marker and share the paternal parent that contains UBC354₅₆₀. The bulked female progeny from family 961 produced the UBC354₅₆₀ marker, while the bulked male progeny did not. Both the female and male progenies in families 957 and 973 produced the UBC354₅₆₀ marker. (These points are addressed further in the discussion.) As expected, the UBC354₅₆₀ marker was inherited in a simple Mendelian fashion in all families where one or both of the parents contain the marker, with the exception of family 973. In family 973, the observed ratio of marker presence to marker absence was approximately 3:1, a significant deviation from the expected 1:1 ratio.

Within those families where UBC354₅₆₀ occurred, a larger set of female and male progeny (i.e., a minimum of 20 progeny per gender per family) were individually tested to confirm the relationship between marker occurrence and gender. UBC354₅₆₀ occurred in 100% of the amplification reactions involving the female progeny in family 960, 100% of the female progeny in family 961, and 90% of the female progeny in family 962 (Table 2). UBC354₅₆₀ occurred in none of the amplification reactions involving the male progeny in family 960, 5% of the male progeny in family 961, and none of the male progeny in family 962. Based on a binomial distribution, the probability that UBC354₅₆₀ is not linked to a sex-determining locus in families 960, 961, and 962 is less than 1×10^{-13} . Alternatively, in families 957 and 973, the marker occurred in 6% and 20% of the female progeny, respectively, and in 68% and 86% of the male progeny, respectively.

To verify homology of the UBC354₅₆₀

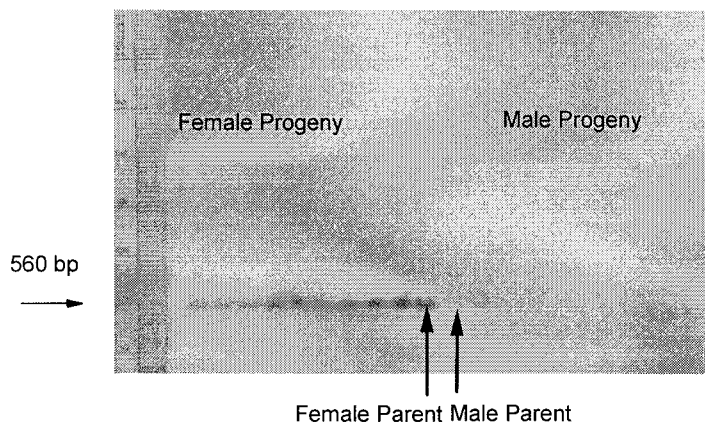


Figure 2. Southern blot analysis of RAPD profiles for progeny and parents of family 962 with the 560 bp fragment from maternal parent X3. The 560 bp fragment was labeled using a DIG-11-UTP. Size of the marker is indicated in base pairs on the left side.

marker among the surveyed parents and progeny, a DIG-labeled probe for UBC354₅₆₀ was recovered from the amplified RAPD profile of the X3 maternal parent. This probe was then hybridized to individual RAPD profiles from all tested progeny and parents within the factorial mating design. Southern blot analysis indicated that the 560 bp marker from the X3 maternal parent is homologous to the markers displayed by the X6 paternal parent and by all progeny expressing the UBC354₅₆₀ marker resulting from crosses involving either the original X3 maternal parent or the alternate X6 paternal parent (Figure 2). The probe did not hybridize to any other tested progeny or parents (data not shown). These results indicate that the 560 bp RAPD band produced with primer UBC354 in the X3 maternal parent is identical in state to bands produced in its progeny and either identical or highly homologous to the 560 bp RAPD produced in other nonrelated parents and progeny.

Within families 960, 961, and 962 estimates of crossing over frequency were used to calculate mapping distance between the marker and a sex-determining locus in *S. viminalis*. These calculations indicate that UBC354₅₆₀ is approximately 1.6 ± 1.1 cM away from such a locus or linked loci. Assuming that a similar base chromosome number ($1x = 1n = 19$) and close taxonomic relationship between *Salix* and *Populus* will correspond to a high degree of synteny, and knowing that mapping distance in *Populus* averages 180 kb/cM (Bradshaw et al. 1994), we estimate that UBC354₅₆₀ is 260 kb away from one of at least two sex-determining loci in *S. viminalis*. Because more than 1000 RAPD bands, representing an average of 57 bands per chromosome, have been scored using bulked segregant analysis and only one linked marker has been identified, it is likely that the chromosome region controlling gender in *S. viminalis* contains relatively few genes and/or is relatively small. These data also strongly support the previous conclusions by Alstrom-Rapaport et al. (1996) and McLetchie and Tuskan (1994) that sex chromosomes do not exist in *S. viminalis* in particular and possibly in Salicaceae in general. Accordingly we propose that UBC354₅₆₀ is tightly linked to an autosomal locus that partially determines femaleness in *S. viminalis*.

Discussion

The data indicates that the gender marker UBC354₅₆₀ is biparentally inherited, is

linked to a nuclear gene, and in certain genetic backgrounds is associated with femaleness in *S. viminalis*. In those families where the marker is present but is not strictly associated with femaleness or maleness, a multilocus epistatic gene model provides a reasonable explanation for the lack of relationship between marker occurrence and gender. That is, in several families female:male phenotypic ratios are not related to the presence or absence of the marker, indicating that an alternate locus is obstructing the expression of the sex determining locus associated with UBC354₅₆₀. To be able to consistently fit the phenotypic sex ratio data for all segregating progeny as well as the marker data for all female and male progeny in each of the tested families, the two-locus epistatic working model was modified. The modified model is as follows: The four alleles at the two loci, *A* and *B*, are denoted as *A1*, *A2*, *A3*, and *A4*, and *B1*, *B2*, *B3*, and *B4*, respectively. Furthermore, allele *A1* corresponds to the allele found in this study that is linked to marker UBC354₅₆₀. This model fits both the observed phenotypic sex ratios and the observed marker segregation ratios for all tested families except 973 (compare Tables 1 and 2), assuming the following interactions between the two loci: genotypes *--B1B1* or *--B3B3* confer female progeny, while genotypes *--B1B3* or *--B4B4* confer male progeny. Genotype *--B1B2* gives female progeny, except for *A2A4B1B2* and *A3A4B1B2*, which results in male progeny. Individuals with the genotype *--B1B4* are female, except for *A1A3B1B4*, which are male. Genotype *--B2B3* produce female progeny, with the exception of *A2A4B2B3* and *A3A3B2B3*, which produce male progeny. Genotype *--B2B4* results in female progeny, except for *A3A4B2B4*, which results in male progeny. Genotypes *--B2B2*, *A1B2B2*, and *A2B2B2* result in female progeny, while all other genotypic combinations yield male progeny. Individuals with genotypes *A1A2B3B4* and *A1A3B3B4* are male, while genotypes *A2A3B3B4* and *A3A3B3B4* are female. The resulting expected sex ratio and segregation pattern at the marker locus for this model are summarized in Table 3. These data support the conclusions of Alstrom-Rapaport et al. (1996) that gender in *S. viminalis* is controlled by several loci interacting in an epistatic manner.

For family 973, the modified two-locus epistatic model accurately predicts the phenotypic sex ratio given in Table 1, yet the segregation ratio per gender for the

Table 3. Expected sex ratios for a two-locus epistatic model with four alleles at each locus

Maternal parent	Genotype	Paternal parent							
		X5 <i>A3A3B2B2</i>		X6 <i>A1A3B1B4</i>		X7 <i>A3A3B2B2</i>		X8 <i>A2A2B1B3</i>	
		Females	Males	Females	Males	Females	Males	Females	Males
X1	<i>A2A3B1B2</i>	3 (0:1)	1 (0:1)	15 (7:8)	1 (1:0)	3 (0:1)	1 (0:1)	3 (0:1)	1 (0:1)
X2	<i>A3A3B3B4</i>	1 (0:1)	1 (0:1)	1 (0:1)	3 (2:1)	1 (0:1)	1 (0:1)	3 (0:1)	1 (0:1)
X3	<i>A1A4B2B2</i>	1 (1:0)	1 (0:1)	3 (1:0)	1 (0:1)	1 (0:1)	1 (0:1)	1 (1:0)	1 (0:1)
X4	<i>A2A3B3B3</i>	1 (0:1)	1 (0:1)	1 (0:1)	3 (2:1)	1 (0:1)	1 (0:1)	1 (0:1)	1 (0:1)

The expected marker segregation ratios (presence:absence) are given in parentheses for each tested family/gender combination. The allele corresponding to the gene closely linked to marker UBC354₅₆₀ is given in **bold letters**. The observed sex and marker segregation ratios shown in Table 1 and 2, respectively, are not significantly different ($\alpha \leq 0.05$) from the expected ratios shown here.

UBC354₅₆₀ marker does not fit the observed data for the male progeny, that is, the marker is overrepresented in the male progeny. It may be that the tested progeny do not represent a random set of all possible progeny from this cross. Sampling error among gametes could account for the lack of fit (Westergaard 1958). Furthermore, cytoplasmic sex ratio distortion may be overriding the expression of a nuclear gene. Thus the proposed two-locus epistatic model may need to be revised to include cytoplasmic gene interactions. The marker UBC354₅₆₀ is certainly linked to a nuclear locus, as it segregates in progeny from the X3 female parent. Still it may be that the predicted second locus is in fact cytoplasmic, thus changing the expected phenotypic sex ratios and marker ratios among the progeny of the tested families. It may also be possible that the paternal parent for family 973 is polyploid. Not only do the marker ratios vary from the expected 1:1 ratio in family 973, but progeny from paternal parent X6 also express an unusual gender ratio in family 969, suggesting that paternal parent X6 accounts for the aberrant results in family 973. Changes in chromosome number are common in members of the Salicaceae family (Bradshaw and Stettler 1993) and such a change would distort the expected phenotypic sex and marker segregation ratios.

In summary, the results from this study support the proposition that several loci, possibly interacting in an epistatic manner, are involved in sex determination in *S. viminalis*. Still the combination of genetic modeling and molecular genetics assays has led to the successful identification of a DNA marker that is associated with sex determination in *S. viminalis*. The identified linked marker UBC354₅₆₀ will be useful in distinguishing female progeny re-

sulting from specific crosses. Additional markers will need to be identified for the second locus predicted by the genetics model before expected sex ratios within all progeny could be described for all crosses. Nevertheless, UBC354₅₆₀ is located close enough to the first sex-determination locus to be used in a chromosome walking experiment in an effort to isolate and characterize the genes that partially determine gender in *Salix* and possibly in *Populus*. We are continuing to screen bulked-DNA samples from families 960 and 962 in an effort to identify additional flanking markers to UBC354₅₆₀ and to identify markers associated with the other sex-determination locus in *S. viminalis*. The genetic model will continue to be revised as more information becomes available on parental karyotypes and/or from additional markers.

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