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Sex determination and sex ratio in the dioecious shrub *Salix viminalis* L.

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Abstract Various ecological factors (e.g. herbivory, difference between males and females in colonising ability) have been invoked to explain female-biased sex ratios in populations of willow species. It was implicitly assumed that genetic factors would lead to a balanced sex ratio in the absence of ecological disturbances. In an experiment carried out in a homogeneous environment and in the absence of herbivores the progeny sex ratio of 13 crosses of basket willow (*Salix viminalis* L.) was observed to range from extreme female bias to extreme male bias. The observed sex ratio cannot be explained by the presence of sex chromosomes without assuming that additional loci are also involved in the sex determination. Alternatively, the sex ratios in this study can be explained by a sex determination mechanism governed by multiple independent loci.

Key words Sex determination sex ratio · Dioecy · *Salix viminalis*

Introduction

There are two main approaches to an understanding of the causes and maintenance of biased sex ratios. The first approach generally emphasises the optimisation and adaptive functions of sex allocation and ignores the genetics of sex determination. The second approach views sex ratio primarily, although not exclusively, as a consequence of the genetic mechanisms of sex determination (Karlin and Lessard 1986). Because genetic mechanisms can in themselves imply biased sex ratio, ignoring them can easily lead to erroneous conclusions. Furthermore, no complete understanding of the evolution of sex ratio can be claimed in the absence of a thorough knowledge of the sex determination system. Unfortunately, the sex determination mechanisms of dioecious species are still poorly characterised. The few studies available suggest a vast array of mechanisms ranging from a X/Y sex determination mechanism, as in *Silene latifolia* (Mulcahy et al. 1992), to a multi-locus system with autosomal genes, as in *Mercurialis annua* (Louis 1989).

Various ecological causes have been invoked to explain the female-biased sex ratio found in willows, thereby implicitly assuming the existence of a sex determination system that does not lead to any bias (e.g. Crawford and Balfour 1983, 1990; Danell et al. 1985; Ericsson et al. 1988; Alliende 1989; Dawson and Bliss 1989). However, as we shall show, the sex determination system itself may cause biased sex ratios. In *Salix*, early cytological studies indicated the presence of sex chromosomes, but these results were later put into question (Chattopadhyay and Sharma 1991). A recent review by Parker (1989) indicates that the presence of previously reported sex chromosomes can be unambiguously confirmed in only seven dioecious species. Consequently, it appears that classical Mendelian genetic studies are necessary as a first step in the understanding of the genetics of sex determination in *Salix*. While they may not permit confirmation of the presence of sex chromosomes, they may allow us to reject them as the sole factor in sex determination.

In the investigation presented here, crosses from the *Salix viminalis* breeding program were used to check whether biased sex ratios are obtained in the absence of ecological disturbances and to address the following questions: (1) are the observed sex ratios consistent with the existence of sex chromosomes? (2) which sex

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determination systems could explain the observed sex ratio?

Materials and methods

The four females and four males used as parents in the breeding population were selected because earlier crosses between them had led to varying sex ratios (Appendix 1). However, the results of these previous crosses could not be used directly for a sex ratio study because they derived from experiments located at different sites and therefore the environmental conditions could not be considered homogeneous. The origins of these individuals are given in Table 1.

One cutting per individual was planted in a nursery (Uppsala, Sweden, 59°48′N, 17°39′E). The eight selected parents were crossed according to a complete factorial mating scheme, a total of 16 crosses, in April 1991. Controlled crosses were done by removing male catkins and depositing large amounts of fresh pollen directly on the female flowers. Seeds were collected from each cross 1 month later. The small hairs on the seeds were removed so that seeds could be separated from each other.

Sex determination experiment

Of the 16 crosses 3 (cross 2 × 5, 2 × 7 and 3 × 7) did not produce any seeds. For each of the 13 seed-producing crosses, 1–6 seeds were sown in 100 pots made of peat (of size 8 cm × 8 cm × 8 cm). The pots were kept in the greenhouse at room temperature. When the seedlings reached a height of 1 cm, all seedlings but 1 were removed from the pot. Removal of the seedlings was done randomly to avoid selection for size, always maintaining the seedling in the middle of the pot. After 6 weeks (in the end of July 1991, the seedlings were planted outside, in a field with clay-sand soil. The plants were evenly spaced, with 2 plants per square meter) and individuals from different families were randomly distributed over the site. Plants were fertilised twice (11:5:18 NPK). In *S. Viminalis*, the sex of plants cannot be confirmed until the first time they flower. In our experiment, sex was recorded in early May 1993 and 1994.

Table 1 Sex and collection site of parental clones

Clone	Sex	Geographic position		Country
		N Lat.	E Long.	
1	Female	57°34	12°41	Sweden
2	Female	57°41	12°57	Sweden
3	Female	55°48	13°54	Sweden
4	Female	57°39	12°01	Sweden
5	Male	60°12	18°12	Sweden
6	Male	56°02	13°53	Sweden
7[a]	Male			Netherlands
8	Male	56°47	14°56	Sweden

[a] The origin of clone 7 was not known

Germination experiment

Skewed sex ratios in *Salix* may be explained by a difference in germination frequency between male and female seeds. In November 1991 a germination experiment consisting of two independent tests was conducted. Seeds from the same crosses as those in sex determination experiment were used, except for cross 2 × 8, which did not produce enough seeds for both the sex determination and germination experiments. A total of 200 seeds per cross were used in the germination experiment, 100 for each test. Seeds were placed in petri dishes containing moistened felt paper at room temperature in a greenhouse. After 24 h we recorded the number of seeds per cross that had developed a cotyledon.

Results

Sex determination experiments

In our experiment the plants produced either male or female flowers. In 1993 frost damage at the top of the shoots prevented many individuals from flowering. However, with the exception of cross 3 × 8, most individuals of each cross flowered in 1994 (Table 2). No change of sex was observed from 1993 to 1994.

Table 2 Germination frequencies, sex ratios and Chi-square ratios to test the null hypothesis of 1:1 male to female ratios among families of *Salix viminalis*

Mother	Father	Germination %		Sex ratio (no. individuals)				Chi-square
		Test 1	Test 2	Females	Males	Non-flowering	Dead	
1	5	98	96	68	26	0	2	18.77***
1	6	94	98	98	2	0	0	92.16***
1	7	98	100	58	31	1	0	8.19**
1	8	86	82	63	20	0	0	22.28***
2	6	74	80	32	71	0	0	14.77***
2	8	–	–	58	21	2	0	17.33***
3	5	100	100	37	47	7	0	1.19ns
3	6	62	64	59	24	0	0	16.41***
3	8	84	82	39	43	14	0	0.20ns
4	5	98	96	46	43	0	0	2.58ns
4	6	98	98	20	93	2	1	47.16***
4	7	96	96	51	36	2	0	2.58ns
4	8	86	96	54	48	5	0	0.35ns

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns = non-significant

Of the 13 crosses 8 showed significant deviations from a 1 : 1 sex ratio. Six crosses were female-biased, and 2 crosses were male-biased (Table 2). Each parent produced both balanced and biased sex ratios, except for female 1 with only female-biased progeny. Male 6 showed the greatest variation with a sex ratio ranging from 98% females in cross 2 × 6 to 16.5% females in cross 4 × 6. The homogeneity tests with crosses made in previous studies (Appendix 1) and the crosses made in this study (Table 2) did not show any significant difference (data not shown).

Germination experiment

In 4 crosses the germination experiment could not be carried out due to a lack of seeds (Table 2). Ten of the crosses showed high germination frequencies, varying between 86% and 100%, while seeds from 2 crosses (2 × 6 and 3 × 6) germinated more poorly. Results are consistent between the two tests. The greatest variation between the two tests was found in crosses 2 × 6 and 4 × 8.

Discussion

Are the observed sex ratios consistent with the existence of sex chromosomes

In species with sex chromosomes, fitness differences between males and females, sexual lability, gametic viability selection, meiotic drive and cytoplasmic sex ratio disorders have been suggested as possible explanations of biased sex ratios (Sandler and Novitski 1957; Westergaard 1958; Bull 1983; Werren 1987). Selective forces may act differently on male and female individuals and lead to biased sex ratios. If genes related to seed germination and plant mortality are associated with sex chromosomes, then biased sex ratios may ensue. The overall germination and survival frequencies in this study were high, and no trend in favor of one sex was observed (Table 2). A poor germination of seeds and/or high mortality of plants is therefore not likely to be the major cause of the observed deviations from balanced sex ratios. Under homogeneous environmental conditions any fitness difference between males and females distorts the sex ratios of different crosses in the same direction. This was not observed. Fitness variation between the sexes can therefore not be invoked to explain the observed biased sex ratios.

Sexual lability over time and space can also cause skewed sex ratios (Westergaard 1958). Some species of plants have been found to have solely male flowers the first flowering season and female flowers the subsequent seasons (Lovett-Doust et al. 1986). Mosseler and Zsuffa (1989) obtained unstable hermaphroditic individuals in some intra- and interspecific crosses of *Salix* species. In our experiment none of the plants appeared to be hermaphrodites and/or have labile sex expression. Moreover, no sex change, from male to female or the converse, has ever been observed since the *Salix viminalis* breeding program was initiated a decade ago. Even if sex lability exists, it is not common.

In conclusion, we have shown that our results cannot be explained by fitness variation during the diploid phase or by lability of sex expression. Therefore, the skewed sex ratios obtained in this study were not distorted during the diploid phase. If we assume the presence of sex chromosomes skewed sex ratios may be caused by meiotic drive, gametic viability selection, nuclear sex ratio distorters or cytoplasmic factors. Meiotic drive, which is the differential production of X- and Y-chromosome-bearing gametes by the heterogametic sex, has been shown to distort the sex ratio (Sandler and Novitski 1957). Although deviations from regular segregation between gametes have been reported in angiosperm trees (Gillet and Gregorius 1992), we have not found any report that connects distorted sex ratio with this mechanism. The sex ratio can also be distorted as a result of gametic selection, the differential success of X- and Y- chromosome-bearing gametes in accomplishing fertilisation. If it is assumed that females are heterogametic with respect to sex chromosomes, meiotic drive and gametic selection would both lead to variation in the sex ratio of the offspring among crosses sharing the same father, while no such variation would be expected among crosses sharing the same mother. If males are heterogametic, the opposite would be expected. Our results show that variation in the sex ratio of the offspring exists both among crosses sharing the same father and among crosses sharing the same mother (Table 2), making gametic selection unlikely in *S. viminalis*.

Cytoplasmic sex ratio distorters which override the expression of sex chromosomes are known to skew sex ratios in certain insects (Werren 1987). Although cytoplasmic sex ratio distorters have yet to be reported in plants our results may be explained by such a phenomenon, assuming the involvement of several cytoplasmic factors and nuclear restorer loci. In order to explain our data, as many as four different types of cytoplasms (a unique cytoplasm for each female parent in the experiment) would be required or, alternatively, variation in the transmission efficiency of the cytoplasmic distorter would have to be assumed. If sex ratio modifiers are nuclear a minimum of two loci independent of the sex chromosomes are necessary to explain the sex ratios of crosses 1 × 6 and 4 × 6.

In conclusion, the skewed sex ratios in this study may not be explained solely by sex chromosomes. If sex in *Salix viminalis* is determined by sex chromosomes additional loci must be present to explain our results.

In the absence of sex chromosomes, what sex determination system could explain the observed sex ratios

Environmental, nuclear-cytoplasmic or multi-locus sex determination may provide explanations to our results. Sex determination entirely depending on environmental factors has been observed in animals (Bull 1983; Karlin and Lessard 1986), while no corresponding observations have been reported in plants. However, since our experimental conditions were homogeneous it is unlikely that the great variation in sex ratios observed in our experiment can be explained by environmental causes only. This is further supported by the fact that the results from the previous studies carried out under different environmental conditions were consistent with the results in the present study.

Some gynodioecious (female and hermaphroditic individuals) plant species have a nuclear-cytoplasmic sex determination system. For instance, *Plantago* populations have several cytoplasmic male-sterility genes with specific nuclear restorers, and inter-population variation also exists (Van Damme 1986). Our data may be explained by a similar mechanism if a system of several cytoplasmic factors and nuclear restorer loci is assumed. It should be noted that male sterility caused by such a system has been reported in only one dioecious species, *Mercurialis annua*, and that the male-sterility cytoplasmic genes in this species are very rare (Durand and Durand 1990). Without further evidence, the presence of a nuclear-cytoplasmic sex determination cannot be ruled out, but its rarity among dioecious plants may make it unlikely.

Finally, sex determination based on a multilocus system has so far only been found for a single dioecious plant species, *Mercurialis annua.* In this species, sex expression is determined by genes at three independent loci (Louis 1989), and as a result the sex ratios are very variable. A similar sex determination system can explain the sex ratios obtained in our study. A minimum number of two loci, with at least four alleles each, is needed to explain our results. Alternative solutions also exist with more than two loci. Our data do not permit us to discriminate among these different alternatives, but preliminary results of a molecular study on the same material (G. Tuskan personal communication) indicates that a minimum of two loci is involved in sex determination in *S. viminalis.*

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Appendix 1 Observed sex ratios for some families in the breeding population of *Salix viminalis* and Chi-square ratios to test the null hypothesis of 1 : 1 male to female ratios

Mother	Father	Sex ratio (no. individuals)				
		Females	Males	Non flowering	Dead	Chi-square
1	5	19	5	1	0	8.17**
		26	7	1	0	10.9**
1	6	12	1	0	0	
1	7	16	7	1	0	3.52ns
		22	9	1	0	5.45*
		18	8	6	1	3.86*
2	5	9	4	0	0	1.92ns
		89	37	3	1	21.46***
2	6	1	4	0	0	
		0	5	0	0	
		8	15	3	0	2.13ns
		12	19	2	1	1.96ns
2	8	27	6	0	1	13.36***
		21	4	1	0	11.56***
3	6	3	2	0	0	
		20	3	1	0	12.56***
		27	5	0	1	15.12***
3	8	18	13	1	1	0.81ns
		15	9	2	0	1.50ns
4	5	22	23	4	0	0.02ns

$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns = non-significant

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