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Complex sex determination in the stinging nettle *Urtica dioica*

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Abstract The dioecious species *Urtica dioica* harbours wide variation in sex ratio of seeds. We conducted a series of crosses to analyse the genetic basis of sex determination in this species. Dutch populations of *U. dioica* contain low proportions of monoecious individuals beside male and female plants. Self-pollination of monoecious plants always yielded female, male and monoecious plants, generally in a ratio of one female to three male/monoecious individuals. This motivated us to write down a simple model in which gender is determined by one major sex-determination locus with four alleles. In the model males and monoecious plants have distinct genotypes but are both heterozygous at the sex-determination locus. We first made crosses among progeny obtained after self-pollination of monoecious plants. These crosses showed that the monoecious trait generally showed Mendelian inheritance and was passed on to the next generation via both pollen and seeds. Further crosses between monoecious plants and plants from dioecious system indicated that alleles from the dioecious system are often dominant. However, many exceptions to our genetic model are observed which suggest that dominance is incomplete and/or that more genes are involved in sex determination. We discuss to what extent sex determination genes explain the strongly biased seed sex ratios and argue that additional genes, for instance genes for female choice, must also be involved.

Keywords Sex ratio · Dioecy · Monoecy · Genetics · Dominance

Introduction

The occurrence of biased sex ratios in natural populations of dioecious (separate sexes) plant species is often considered to be a consequence of sex-specific life histories (e.g., sex-differential mortality, sex-differential reproductive investment). Implicit in this view is the assumption that the two sexes are produced in approximately equal numbers (Delph 1999). Interestingly, several studies on dioecious species revealed that seed sex ratios

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(SSR, fraction of males) are already biased (Webb 1992; Taylor 1996; Wolf et al. 2001, de Jong and van der Meijden 2004). Recently, Taylor (1999) demonstrated that SSR in *Silene latifolia* was highly correlated with the sex ratio of flowering plants in the populations from which the seeds were sampled. We are probably just beginning to understand the evolution of sex ratios in plants. With sex chromosomes, like XX females and XY males as in *S. latifolia*, Mendelian inheritance would produce 50% males in the offspring. Nevertheless sex ratio bias could occur in such a system through meiotic drive (Taylor 1994;Taylor and Ingvarsson 2003), pollen competition (Conn and Blum 1981) or selective abortion. When several genes are involved in sex determination, like in *Mercurialis annua* (Louis 1989), sex ratio of offspring of crosses between two different genotypes could already be biased. This would even be the case with Mendelian inheritance and without selection by the maternal parent. Sex ratios can then further be modified by meiotic drive or selection by the maternal parent. To understand the causes of sex ratio variation it is therefore a useful first step to elucidate the mechanism behind sex determination.

Typically in dioecious plant species, one of the sexes is considered heterogametic, it produces two types of gametes, while the other is homogametic, producing only one gamete type. Westergaard (1958) detailed the different ways by which the heterogametic sex can be identified.

The first, most obvious, method is to reveal the existence of heteromorphic sex chromosomes by cytological investigation. However, only in a very small number of plant species, sex chromosomes differ in size and morphology (Parker 1990). Without cytological evidence, we have to revert to other methods.

A second method involves self- or cross-pollination of monoecious individuals. Such individuals can sometimes be obtained by hormone treatment of unisexual plants (Louis 1989). In natural populations of *Asparagus officinale*, some males (putative genotype *Aa*) could be self-pollinated (Rick and Hanna 1943) to produce female (*aa*) and male (*Aa*, *AA*) offspring in the ratio 1:3. Such a ratio suggests that the male is heterogametic, the male allele (*A*) dominates the female allele (*a*) and homozygous 'super-males' (*AA*) are viable.

A third method to identify the heterogametic sex was applied by Correns (1928) in his classical study on *Bryonia dioica* and *B. alba*. In this species pair the dioecious trait was dominant over the monoecious trait. When *B. dioica* females were pollinated by monoecious (separate male and female flowers on one plant) *B. alba*, almost 100% female offspring were produced. When monoecious individuals from *B. alba* were fertilized with pollen from males of *B. dioica*, 50% male and 50% female offspring were obtained. Since the monoecious species is homozygous at the sex determination locus, these results indicate that a pollen grain of *B. dioica* carries either the male *A* or female *a*-allele, whereas the embryo sac has only the *a*-allele. More recent studies that employed crosses between dioecious and monoecious forms include Dorken and Barrett (2004) and Shannon and Holsinger (2007).

Here we study sex determination by making crosses between male, female and monoecious plants of *Urtica dioica*. Under laboratory conditions *U. dioica* flowers within 2 months from germination, making it particularly suitable for experiments. The proportion male flowering plants in the field and/or SSR vary considerably in European (Kay and Stevens 1986; de Jong et al. 2005; Glawe 2006) and North American populations (Shannon and Holsinger 2007). The mechanism of genetic sex determination has been poorly characterised in *U. dioica*. The older literature (Strasburger 1910; Meurman 1925) refers to heteromorphic sex chromosomes in male individuals, but Westergaard (1958) questioned this and Glawe (2006) could not observe any morphological differences within chromosome pairs. Low frequencies of monoecious plants occur in *U. dioica* populations in the

Netherlands (Heemskerk et al. 1998; de Jong et al. 2005) and other parts of Europe (Greig-Smith 1948; Kay and Stevens 1986). Monoecious plants sometimes occur in higher frequencies (up to 10%) in crosses between male and female plants (Shannon and Holsinger 2007). Some plants showed a stable phenotype of a single gender, even under extremely different conditions, and these were designated as male or female (Glawe and de Jong 2005). In contrast, monoecious plants were generally labile and became largely male under benign conditions. Recently, Shannon and Holsinger (2007) also used *U. dioica* by making crosses, among others between *U. dioica* spp. *dioica* and the monoecious spp. *gracilis* which were collected in different parts of the US and Canada. We will discuss their findings against our results.

In our study we address the following questions. (1) Which is the heterogametic sex? (2) What is the sex determination mechanism in dioecious and monoecious plants? (3) How is the monoecious trait transmitted? We address the first question by selfing monoecious plants and examining the sex ratio in the segregating offspring. We also cross male, female and monoecious plants, as suggested by Westergaard (1958) and Correns (1928). Because gender of males and females was entirely stable in *U. dioica* and could not be changed with, for instance, hormones, we had no other choice than to use the monoecious plants that emerged in our experiments in the crosses. For the second question we propose a one-gene-four-allele model to explain our results (Table 1). We assume the male allele to dominate the female allele in the dioecious system but not in the monoecious system. Furthermore we assume alleles from the dioecious system to dominate alleles from the monoecious system. The model serves a heuristic purpose and we will encounter several deviations from Table 1. To answer the third question we first made crosses between males/ females and monoecious plants arising in the offspring of a selfed monoecious individual, showing that the trait is passed on to the offspring following Mendel's rules. Finally, monoecious individuals are crossed with true males and females, showing that the monoecious trait is passed on through both seeds and pollen.

Table 1 Hypothetical model of sex determination in *Urtica dioica* based on one locus with four alleles and (i) dominance of male alleles over female alleles in plants from the dioecious system but not in the monoecious system, and (ii) dominance of alleles from the dioecious system over those from the monoecious system

Reproductive System	Putative Genotype[a]	Sexual Phenotype
Dioecious	$a_{\varphi}^{D}a_{\varphi}^{D}$	Female
	$a_{\varphi}^{D}a_{\sigma}^{D}$	Male
Monoecious	$a_{\varphi}^{M}a_{\varphi}^{M}$	Female
	$a_{\varphi}^{M}a_{\sigma}^{M}$	Monoecious[b]
	$a_{\sigma}^{M}a_{\sigma}^{M}$	Male
Crosses between dioecious and monoecious	$a_{\varphi}^{D}a_{\varphi}^{M}$	Female
	$a_{\varphi}^{D}a_{\sigma}^{M}$	Female
	$a_{\varphi}^{M}a_{\sigma}^{D}$	Male
	$a_{\sigma}^{M}a_{\sigma}^{D}$	Male

[a] The superscript indicates whether the allele descended from a parent from the monoecious (M) or dioecious (D) system

[b] Note that under favourable conditions plants with this genotype sometimes form 100% male flowers (Glawe and de Jong 2005), in which case they cannot be distinguished from genetic males

Material and methods

Study organism

At our study site in Meijendel (near The Hague), 6.2% of the flowering plants were monoecious (de Jong et al. 2005). *Urtica dioica* is allo-tetraploid (IPCN data base, Sitte et al. 1998) and chromosome counts of several plants from the Meijendel population confirmed tetraploidy ($2n = 4x = 52$). Allozyme data on four loci showed disomic inheritance (Mutikainen and Koskela 2002; Mutikainen, personal communication). Using 63 polymorphic markers, Glawe (2006) found that inheritance of 51 markers was consistent with Mendelian inheritance. Therefore and to keep the analysis as simple as possible, we start from the assumption of disomic inheritance of sex.

Plant material

Seeds of the parental (P) generation used in self- and cross-pollination were collected from open-pollinated females at the field site in Meijendel as described in de Jong et al. (population 2, 2005). The plants grown from each seed batch (family: M14, M16, M18, M24, M31) were therefore at least half-sibs. The lower case letters a, b, c et cetera indicate different individuals from the P generation from the same family (e.g., M31a, M31b and M18a, M18b are different individuals from family M31 and M18, respectively).

For individuals that were used repeatedly in different crosses, cuttings were obtained from the plants and cultured in vitro (MS 0 medium). Seed germination and plant growth were carried out under standard conditions (Glawe and de Jong 2005). Sometimes monoecious individuals produced few female flowers and seeds, and this set a limit to the number of seedlings planted. Gender was determined approximately 4 weeks after flowering. Germination and survival rate of the progeny of each single cross always exceeded 82% and 88%, respectively.

The heterogametic sex

(a) *Self-pollination of monoecious individuals*. To establish the heterogametic sex, plants were selfed and gender of the offspring was examined. Because the gender of male and female individuals could not be changed in *U. dioica*, our only option was to self various monoecious individuals. Twenty monoecious plants which all came from open-pollinated female plants at Meijendel were selfed (M14, M18, M24, M31; family of the other individuals is not known). Flower sex ratios (FSR, proportion male flowers) of monoecious individuals were found to vary considerably between treatments, even for clones from the same plant (Glawe and de Jong 2005). To increase the possibility to detect different sex determination genotypes that might exist we selected individuals that differed dramatically in FSR. Some monoecious plants obtained in the F_1 were selfed, in order to compare the segregation of male, female and monoecious individuals between F_1 and F_2.

(b) *Crosses between true females/true males and monoecious individuals*. Monoecious individuals that were obtained after self-pollination of monoecious M31a were used in crosses with true females and true males from several families (both are assumed to originate from crosses between dioecious individuals). When monoecious individuals were the maternal parent, these plants were emasculated prior to cross-pollination.

Also, the maternal parents were monitored throughout the crossing period and any anew-appearing male flowers were removed.

Seed sex ratios in crosses between true females and true males

Biparental crosses between true males and true females were performed to assess whether such crosses yielded biased SSR's. Seed batches were taken from five females with open pollination in the field and we estimated the SSR (proportion males plus monoecious) of at least 100 seeds. The SSR and percentage monoecious offspring of these seed batches were: M14 [0.14; 6.1%], M16 [0.44; 8.7%] M18 [0.72, 10.5%], M24 [0.5; 9.6%], and M31 [0.64; 6.0%] (see also de Jong et al. 2005). From each seed batch a single male and female were selected and crossed.

The monoecious trait and its transmission

We wanted to know whether the monoecious trait is passed on to the next generation through pollen and/or seeds. For that purpose we (i) performed full-sib crosses among progeny that were obtained after self-pollination of the monoecious plant M31a, and (ii) carried out crosses among dioecious plants and individuals that were obtained after self-pollination of M31a. The SSR of the offspring was again notes. For comparison we included members of other families as well.

Sex types

We assume one single major locus for sex determination. Furthermore we assume dominance of the male allele ($a_{\male}$) over the female allele ($a_{\female}$) in the dioecious system as is commonly found in dioecious plant species. In the monoecious system, the male and female alleles are co-dominant. We further assume that alleles from the dioecious system dominate those from the monoecious system. We develop a model with one locus and four alleles (Table 1), which is close to the ideas of Westergaard (1958). The scheme in Table 1 is useful for organising the presentation of results but, as we will see, there are several exceptions to the rule. Several alternative genetic models can be proposed and these will be discussed.

In the dioecious system we refer to true male plants, to distinguish them from male plants from the monoecious system. The putative true male genotype at the sex-determining locus is $a_{\female}\, a_{\male}$ or $a^D_{\female} a^D_{\male}$, with the superscript indicating that the allele is derived from the dioecious system (Table 1). 'True female' plants have female flowers only and are denoted as $a^D_{\female} a^D_{\female}$. We have shown previously (Glawe and de Jong 2005) that sex of these plants is stable and cannot be altered by changing environmental conditions.

Monoecious plants have genotype $a^M_{\female} a^M_{\male}$. These plants generally produce both male and female flowers. However, under favourable conditions monoecious plants produce up to 100% male flowers (Glawe and de Jong 2005). In this paper we scored the gender of plants on one occasion and we did not test for all progeny, whether gender was stable under different conditions. Thus there is a probability that some of the plants with the male phenotype were unstable and should be classified as having the monoecious genotype. To avoid this problem, we will sometimes pool monoecious and male offspring. In the model of Table 1 selfing of monoecious plants produces 25% offspring with two male

alleles ($a^M_♂a^M_♂$). These plants are designated as "super males", or, when they are obtained from selfing the genotype M31, as 'M31a male' plants.

We assume that alleles from the dioecious system always dominate those from the monoecious system so that, for instance, an $a^D_♀a^M_♂$ genotype would be female rather than male. The hypothetical genotypes and phenotypes from crosses between plants from the dioecious and monoecious system are given in Table 1.

Results

The heterogametic sex

(a) *Self-pollination of monoecious individuals.* According to Table 1 selfing of monoecious individuals ($a^M_♀a^M_♂$) results in 25% females ($a^M_♀a^M_♀$), 50% monoecious ($a^M_♀a^M_♂$) and 25% super males ($a^M_♂a^M_♂$) in the offspring. Regardless of their FSR, the offspring of all 20 monoecious individuals segregated in female, monoecious, and male individuals (Table 2a). This clearly shows that all monoecious plants examined were heterozygous at a major sex-determination locus. In Table 2a, data are shown for five individual selfings to represent a selection of the different sex ratios that were obtained; data from 15 further selfings are pooled (G-test for heterogeneity, $G_{28} = 36.58$, $P = 0.13$). For only four of the 20 selfings, the progeny conformed to a 1:2:1 female: monoecious: male ratio. Because some plants of the monoecious genotype can produce up to 100% male flowers, we pooled monoecious plants and males here. After pooling, 18 out of 20 progenies from selfing conformed to 25%

Table 2 Self pollination of monoecious plants (a) and of monoecious progeny obtained after selfing M31a (b) in *U. dioica*

Family and FSR	Number of progeny	Progeny in % (sexual phenotype)			χ^2 for 1:2:1 ratio	χ^2 for 1:3 ratio
		Females	Monoecious	Males		
(a) Generation 1						
M31a/FSR[a] = 0.78	71	23.9	47.9	28.2	0.38 ns	0.30 ns
M31b/FSR = 0.16	39	18.2	49.1	32.7	1.87 ns	1.03 ns
M18a/FSR = 0.45	71	40.8	35.2	23.9	10.27**	9.5**
M14a/FSR = 0.66	54	29.8	33.3	36.9	6.59*	0.62 ns
M24a/FSR = 0.29	58	60.3	20.7	19.0	39.79***	38.64***
Other 15 plants	804	27.9	41.8	30.3	22.67***	3.51 ns
Pooled	968	27.3	42.0	30.7	26.75***	2.67 ns
(M18a, M24a excl.)						
(b) Generation 2 (progeny from M31a)						
M31-1/FSR = 0.19	48	27.0	41.7	31.3	1.50 ns	0.11 ns
M31-2/FSR = 0.23	55	23.6	38.2	38.2	5.40 ns	0.05 ns
M31-3/FSR = 0.78	61	41.0	50.8	8.2	13.13**	8.13**
M31-4/FSR = 0.64	43	32.5	41.9	25.6	1.56 ns	1.31 ns
Pooled	207	31.4	43.5	25.1	5.15 ns	4.52*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant

[a] FSR = fraction male flowers

Table 3 Crosses between (*a*) true females and monoecious plants, and (*b*) true males and monoecious plants. Monoecious individuals were obtained after self-pollination of monoecious M31a

Parents		*N*	Progeny % (sexual phenotype)		
Mother	Father		Female	Monoecious	Male
(a) True female × M31a monoecious					
M31c	M31-4	67	83.6	4.4	12.0
M14b	M31-4	52	88.8	6.9	4.3
M18b	M31-4	61	90.3	9.7	–
		G_{het}	df = 4	11.59	$P = 0.02$
		G_{het} (monoecious and males pooled)	df = 2	1.28	$P = 0.53$
(b) M31a monoecious × true male					
M31-2	M31e	41	29.3	2.2	68.5
M31-2	M14c	56	31.1	5.3	63.6
M31-2	M16c	39	33.3	5.1	61.5
M31-1	M18c	67	31.3	10.5	58.2
		G_{het}	df = 6	3.39	$P = 0.76$
		G_{het} (monoecious and males pooled)	df = 3	0.18	$P = 0.98$

females and 75% male and monoecious individuals (Table 2a, G-test for heterogeneity, $G_{34} = 41.66$, $P = 0.17$). In the two exceptional progenies (M18a, M24a; Table 2a) too many females and too few monoecious individuals were recovered.

After self-pollination of selected monoecious F_1 plants from family M31a, all F_2 progenies segregated again in three sex phenotypes, showing the heterozygosity of the monoecious F_1 plants (Table 2b). For F_1's (M31-1, M31-2, and M31-4), the progeny conformed to a 1:2:1 female: monoecious: male distribution, while for one progeny (M31-3) it did not (Table 2b). Upon selfing M31-3, too many female and too few male offspring were obtained. Because after self-pollination, monoecious plants recurred in high frequencies in the offspring, the monoecious state is clearly genetically based.

(b) *Crosses between true females/true males and monoecious plants.* According to Table 1 a cross between a true female ($a^D_♀a^D_♀$) and a monoecious plant ($a^M_♀a^M_♂$) should result in 50% $a^D_♀a^M_♀$ (female) and 50% $a^D_♀a^M_♂$ (female), i.e. in 100% female plants. We find that, on average, 87.5% females were produced (Table 3a). While this high percentage females supports the hypothesis of dominance of alleles from the dioecious system, 12.5% of the plants do no fit the expectation. Our results suggest that the dominance is not complete so that 75.0% of the plants of genotype $a^D_♀a^M_♂$ are female but 25% has a monoecious or male phenotype.

The cross between a true male ($a^D_♀a^D_♂$) and monoecious plant ($a^M_♀a^M_♂$) should produce 25% $a^M_♀a^D_♀$ (females), 25% $a^M_♂a^D_♂$ (males), 25% $a^D_♀a^M_♂$ (females) and 25% $a^M_♀a^D_♂$ (males). In total this amounts to 50% females and 50% males. Crosses resulted, on average, in 31.2% females (Table 3b) and results were homogeneous for the four families used in the experiment. The surplus of males and occurrence of some monoecious plants in the offspring (Table 3b) suggests, that genotype $a^D_♀a^M_♂$ is not strictly female but could also be male or monoecious. Again we obtained similar results for the four families used in the crosses.

Correns (1928) obtained 100% females and 50% females, respectively, when he performed the crosses of Table 3a and b in *Bryonia*. Although not as clear cut as his results,

Table 4 Crosses between true female and true male plants from the same family

Parents		*N*	Progeny % (sexual phenotype)		
Mother	Father		Female	Monoecious	Male
True female × true male					
M14b	M14c (SSR = 0.14)[a]	64	84.4	3.1	12.5
M16b	M16c (SSR = 0.44)	64	62.5	4.7	32.8
M24b	M24c (SSR = 0.50)	67	50.7	–	49.3
M31c	M31e (SSR = 0.64)	61	24.6	1.6	73.8
M18b	M18c (SSR = 0.72)	63	34.9	–	65.1
		G_{het}	df = 8	66.38	$P < .0001$
		$G_{het\ (monoecious\ and\ males\ pooled)}$	df = 4	58.71	$P < .0001$

[a] SSR (fraction males) of the seed batch (field) from which the two parents were selected

our results (87.5% and 31.2% females, respectively) point in the same direction and suggest the male to be the heterogametic sex.

Seed sex ratios in crosses between true females and true males

With Mendelian inheritance the cross between a true male ($a^D_♀a^D_♂$) and true female ($a^D_♀a^D_♀$) should produce 50% male and 50% female offspring. Crosses between true female and true male individuals from the same family resulted, however, often in biased SSR's (Table 4). Only one family (M24) was found to produce a ratio that did not differ significantly from 1:1 female: male (binominal test, $P = 0.904$), while four families showed significant deviations from a 1:1 sex ratio [M31 ($P < 0.0001$), M18 ($P = 0.02$), M16 ($P = 0.046$), M14 ($P < 0.0001$)]. The sex ratio of the offspring in these biparental crosses is strikingly similar to the sex ratio of seed batches collected in the field from which the parents in Table 4 were randomly selected (Table 4).

Interestingly, these biparental crosses yielded monoecious progeny in considerable proportions, varying from 0 to 4.7% (Table 4). Self-pollination of the monoecious individuals from the F_1 always resulted in female, monoecious and male offspring, consistent with Table 2 (data not shown).

The monoecious trait and its transmission

Crosses among full sibs obtained after self-pollination of monoecious M31a

The cross between a monoecious individual (putative genotype $a^M_♀a^M_♂$) and female ($a^M_♀a^M_♀$) should yield 50% monoecious ($a^M_♀a^M_♂$) and 50% female progeny ($a^M_♀a^M_♂$). The approximate ratios are indeed observed in the progeny of M31a (Table 5a). Crosses between monoecious individuals ($a^M_♀a^M_♂$) and males ($a^M_♂a^M_♂$) should produce 50% monoecious and 50% male plants, and this is indeed close to the result for M31a in Table 5b. However, the few female progeny produced in these crosses (Table 5b) are unexpected.

Crosses between females ($a^M_♀a^M_♀$) and males ($a^M_♂a^M_♂$) should produce a single genotype $a^M_♀a^M_♂$, with a monoecious phenotype. Indeed for M31a we find predominantly (80%) monoecious offspring (Table 5c). If the phenotype of $a^M_♀a^M_♂$ is unstable and individuals are

Table 5 Crosses among female, monoecious and male offspring, all obtained after selfing monoecious M31a

Parents		*N*	Progeny % (sexual phenotype)		
Mother	Father		Female	Monoecious	Male
(a) M31a female × M31a monoecious					
M31-5	M31-4	44	50.0	45.5	4.5
(b) M31a monoecious × M31a male					
M31-2	M31-7	69	7.3	49.2	43.5
M31-2	M31-8	29	6.9	48.3	44.8
M31-1	M31-8	59	8.4	45.8	45.8
		G_{het}	df = 4	0.20	$P = 0.99$
		$G_{het\ (monoecious\ and\ males\ pooled)}$	df = 2	0.09	$P = 0.96$
(c) M31a female × M31a male					
M31-5	M31-7	63	9.5	82.6	7.9
M31-5	M31-8	45	8.9	77.8	13.3
M31-6	M31-7	98	6.1	81.7	12.2
M31-6	M31-8	57	8.8	75.4	15.8
		G_{het}	df = 6	2.56	$P = 0.86$
		$G_{het\ (monoecious\ and\ males\ pooled)}$	df = 3	0.75	$P = 0.86$

sometimes male, this may explain the male progeny. The few female progeny in Table 5c are however unexpected.

In conclusion, the monoecious trait was readily transferred to a large proportion of the offspring, also when crosses were performed between female and male offspring of the monoecious plant M31a. Except for a few unexpected females, results followed Mendelian inheritance according to the scheme in Table 1.

Crosses among plants from the dioecious and monoecious system

When M31a males ($a^M_{\male}a^M_{\male}$) are crossed with true females ($a^D_{\female}a^D_{\female}$), all genotypes in the progeny are $a^D_{\female}a^M_{\male}$ and if the allele from the dioecious system dominates, these plants are all female (Table 1). On average, many (83.7%) females were obtained (Table 6a). Results suggest that the $a^D_{\female}a^M_{\male}$ genotype does not always show a female phenotype but is monoecious in 10.0% and male in 6.3% of the cases. Dominance of the female allele from the dioecious system is incomplete and/or that other genes are involved in sex determination. The 10.0% of monoecious progeny (Table 6a), show that the factor associated with monoecy is passed on through the pollen of M31a super males. Similar results were obtained in crosses with true females from families M16 and M24.

When M31a females ($a^M_{\female}a^M_{\female}$) are crossed with true males ($a^D_{\female}a^D_{\male}$), 50% females ($a^M_{\female}a^D_{\female}$) and 50% males ($a^M_{\female}a^D_{\male}$) are expected in the progeny. We observed on average 34.2% female and 53.6% monoecious individuals, while the remaining 12.2% of the plants showed a male phenotype (Table 6b). Because the $a^D_{\female}a^M_{\female}$ genotype must be female, this result implies that the genotype $a^M_{\female}a^D_{\male}$ is much more likely to be monoecious than male, i.e. the male allele from the true male and the female allele from the monoecious mother are co-dominant. The results in Table 6b also clearly demonstrate that the monoecious trait in the M31a mother was passed on through the seeds.

Table 6 Crosses between (a) true females and males obtained after selfing M31a and between (b) true males and females obtained after selfing M31a

Parents		*N*	Progeny % (sexual phenotype)		
Mother	Father		Female	Monoecious	Male
(a) True female × M31a male					
M31c	M31-7	67	88.0	7.6	4.5
M31d	M31-8	71	81.9	12.0	4.1
M16b	M31-7	72	81.1	16.7	2.2
M24b	M31-8	62	82.3	3.2	14.5
		G_{het}	df = 6	14.69	$P = 0.02$
		$G_{het\ (monoecious\ and\ males\ pooled)}$	df = 3	1.58	$P = 0.66$
(b) M31a female × true male					
M31-5	M31e	62	29.0	59.7	11.3
M31-6	M31f	61	39.4	47.5	13.1
		G_{het}	df = 2	1.85	$P = 0.40$
		$G_{het\ (monoecious\ and\ males\ pooled)}$	df = 1	1.44	$P = 0.23$

Discussion

The heterogametic sex

After self-pollination, the offspring of monoecious plants segregated in female, monoecious, and male offspring, mostly in a ratio of 1 female on 3 male plus monoecious individuals. Monoecious plants are therefore heterogametic. Monoecious plants produce 100% male flowers under favourable conditions, showing their similarity to constant males. Nevertheless the genotypes must be different. When the monoecious plant is the paternal parent in a cross with a true female, this results in different sex ratios in the progeny than when a true male is used in the same cross (compare Table 3a and Table 4). We suggest that true males are also heterogametic, as in Correns' (1928) study of *Bryonia*. Also, in the great majority of plant species investigated the male is considered as heterogametic and the female as homogametic (Westergaard 1958).

Nevertheless the picture that emerges is more complex than the simple dichotomy between homo- and heterogametic sex suggests and this is probably due to the dominance relations (Table 1). From Table 2 we know that monoecious plants are heterogametic, yet when these individuals are crossed to true females, 87.5% of the offspring is female. This implies that some of these female offspring are heterozygous ($a^D_{\varphi} a^M_{\sigma}$) at the sex-determination locus with the female allele from the maternal parent from the dioecious system dominating the male allele from the monoecious paternal parent.

Using 68 polymorphic markers Glawe (2006) found 7 markers that were significantly associated with sex. Two markers could be placed in linkage groups that inherited independently of each other. Together the markers helped to predict gender accurately for a rather low proportion (72%) of the plants. Thus we have not detected a major sex determination gene. With $2n = 52$ chromosomes, the 68 markers are insufficient to cover the genetic map of *U. dioica*. Nevertheless these results show that several genes are involved in sex determination in this species. Plants may be heterozygous at a major locus but the strength of the male and female allele at this locus may differ. Further additional genes

influence gender, so that the phenotype is not always as would be expected from the genotype at the major locus.

The results of Shannon and Holsinger (2007) on *U. dioica* spp. *dioica* in the US and Canada are consistent with some of our findings. They also report SSR variation in crosses between male and female plants, the occurrence of monoecious individuals in the offspring of such crosses and monoecious plants behaving much more similar in crosses to true males than to true females. However, certain aspects of sex determination in these North American populations are strikingly different from ours. Upon selfing two monoecious plants, Shannon and Holsinger (2007) obtained only c.8% female offspring, which is much lower than the many cases in which found 25% females and the two exceptional cases in which we found 40.8–41.0% females. Therefore the genetic basis of monoecy differs between these studies. Our result of 25% female offspring after selfing monoecious plants was the basis for proposing a simple model of sex determination, but clearly this model cannot be applied to the North American data. The crosses that Shannon and Holsinger (2007) made between monoecious plants and true male or true female plants.

In their crosses monoecious plants produced only 3% females in their offspring when pollinated by true males (as compared to the 31.2% we found). True females pollinated by a monoecious parent, produced 45% female offspring (as compared to our result of 87.5%). We found for Dutch *U. dioica* a more balanced distribution over the three sex types in crosses between monoecious individuals and true males as compared to crosses between monoecious individuals and true females (Table 3). The US study presents the opposite result. Shannon and Holsinger (2007) also made crosses with *U. dioica* spp. *gracilis* from another area. The subspecies *gracilis* is monoecious and supposedly homozygous at the sex-determination locus. Crosses between females of *U. dioica* spp. *dioica* and monoecious spp. *gracilis* yielded about 57% females and 43% male plus monoecious plants. Crosses between male *U. dioica* spp. *dioica* and monoecious spp. *gracilis* yielded 80% females and 20% male monoecious. This suggests that the female produces two types of gametes in equal proportions while the male produces mostly one type of gamete. Shannon and Holsinger (2007) concluded that many genes are involved in sex determination, including cytoplasmic genes. While this general conclusion fits our data on Dutch *U. dioica* well, some results differ markedly from ours and even suggest that in North America the female, rather than the male, is the heterogametic sex.

When viewed in a population context sex determination is even more complex. Many of our crosses involve genotypes from family M31, but Table 2 already suggests that different monoecious plants have different genotypes. Therefore our one-locus-four-alleles model should not be taken to suggest that sex determination in *U. dioica* is simple and within populations abundant genetic variation occurs to produce male, female and monoecious plants in various proportions.

Four-allele model with dominance

In crosses between true males and true females monoecious progeny was generated in rather high frequency (up to 5%) and the monoecious phenotype persisted in further crosses. We could well imagine that an epigenetic mutation, involving perhaps methylation of the major sex determination gene and reduction of gene activity, makes plants monoecious. Epigenetic mutations are readily passed on to future generations in plants (Tadeka and Paszkowski 2006; Bond and Finnegan 2007). If the differences between a_{δ}^{M} and a_{δ}^{D} and between a_{φ}^{M} and a_{φ}^{D} are indeed of this nature, the monoecious plants have the same genes as males but are epigenetically different.

The predictions of the one-locus-four-alleles model and assumed dominance relations were summarized in Table 1. The predictions of the model are fair for the crosses within the monoecious system. The model cannot explain the biased sex ratios in crosses between males and females in the dioecious system. For predicting the results of crosses between plants of the two systems there are two problems. First, the $a_{♀}^{D}a_{♂}^{M}$ genotype does not have the female phenotype according to Table 1. Nevertheless Tables 3a, b and 6a suggest that this genotype can also have sometimes a monoecious or male phenotype. Second, genotype $a_{♀}^{M}a_{♂}^{D}$ is supposedly male according to Table 1. This is confirmed by Table 3b but Table 6b suggests that this genotype most often has a monoecious phenotype. Probably in these cases additional genes or different strength of alleles at the major locus also mattered for the phenotype of the offspring.

For the female allele of the true female parent, Westergaard (1958) reported a variable picture with respect to dominance. Allele $a_{♀}^{D}$ can be either dominant over $a_{♂}^{M}$ (*B. dioica*; Correns 1928) or recessive (*Ecballium elaterium*, Mather 1949; Galán 1951, or *Spinacia oleracea*, Janick and Stevenson 1955). According to Westergaard (1958) the $a_{♂}^{D}$ allele always dominates. Our *Urtica* data are apparently an exception in which $a_{♂}^{D}$ and $a_{♀}^{M}$ are co-dominant (Table 6b).

Alternative genetic models

Apart from the one-locus-four-alleles model that we developed, several alternative models can be proposed that all seem less appropriate for explaining our *U. dioica* data.

First, Mather (1949) and Galán (1951) proposed a one-locus model with three alleles for *Ecballium elaterium*. The dioecious type and the monoecious type of *E. elaterium* occur in different populations and the monoecious type is homozygous for the a^{M} allele. As our results clearly indicated that monoecious plants of *U. dioica* were not homozygous but their offspring segregated in female, male and monoecious individuals, this model does not apply to *U. dioica*.

Second, Charlesworth and Guttman (1999) modelled the evolution of dioecy via gynodioecy, assuming two linked loci for male and female fertility. Dorken and Barrett (2004) successfully applied the model to sub-dioecious *Sagittaria latifolia*, a species with monoecious and sub-dioecious populations. In their model, monoecious plants produce after selfing either 100% monoecious offspring or 75% monoecious and 25% female offspring. Since we always find males, females and monoecious plants in the progeny of selfed monoecious plants, the model of Charlesworth and Guttman (1999) misses some of the characteristic features of the *U. dioica* system in Europe and the US (see also Shannon and Holsinger 2007).

Third, monoecious plants may have a feminising cytoplasm. Feminising cytotypes are well known from gynodioecious species (Saumitou-Laprade et al. 1994). In dioecious species a neutral cytoplasm in a male would pass on zero copies of itself to the next generation and therefore a feminising cytoplasm that restores female function would be strongly selected for. In most plant species cytoplasmic DNA is inherited through the seed and not through the pollen (Corriveau and Coleman 1989). *U. dioica* was not included in this survey, but additional work by Zhang et al. (2003) on other *Urticaceae* suggested that inheritance of cytoplasmic DNA is strictly maternal. Our results showed that the monoecious trait was transmitted through both seed and pollen. Hence, this model is not considered for further analysis.

Fourth, we can assume an additional minor feminising locus on another chromosome so that genotype *FF* or *Ff* makes male ($a_{♂}a_{♀}$) plants monoecious, while it does not affect the

phenotype of females ($a_{\mathcal{F}}a_{\mathcal{F}}$) and super males ($a_{\mathcal{J}}a_{\mathcal{J}}$). Writing down the equations for the two-locus model becomes rather cumbersome and the interested reader may wish to check these in the online thesis by Glawe (2006). Many predictions of the two-locus model are similar to the one-locus-four-alleles model we analysed here.

Fifth, one could develop a quantitative genetic model of sex determination in which there is an underlying continuous scale of maleness with two thresholds that separate discrete female, monoecious and male phenotypes (Lynch and Walsh 1998, pp 736–744). Such a model would allow a calculation of heritability of SSR variation (Bull et al. 1982) and is an attractive way to explain SSR variation in the dioecious system. However, the results in Table 3 are homogeneous and this is not what one would expect when using males or females from widely different SSR families in crossings with monoecious plants. Therefore some of our results, especially when plants from the dioecious and monoecious system are crossed, remain difficult to explain.

Sex determination genes versus sex ratio genes

In *U. dioica* SSR differs between seed samples taken from different female plants in the field (de Jong et al. 2005) and biparental crosses between true males and true females (Table 4). The question remains whether the sex determination system (Table 1) gives a satisfactory explanation for variation in SSR in *U. dioica*, as suggested for *M. annua* by Louis (1989). Or do other factors like meiotic drive, pollen competition and selection by the maternal parent, play an additional role (see also Werren and Beukeboom 1998)?

Urtica dioica differs in several respects from *M. annua*. Monoecy does not occur in diploid *M. annua*, while in *U. dioica* this is a significant part of the system. Gender is stable in *U. dioica* and male plants could not be feminized. Additional sex ratio genes appear likely in *U. dioica*. (i) Crosses between true males and true females yielded strongly biased sex ratios (Table 4) and in these crosses no monoecious plants were involved. (ii) Glawe and de Jong (2007) showed that SSR was inherited only through the maternal parent, which could suggest female choice or some other epigenetic mechanism in the maternal parent to affect the gender of its offspring. (iii) Monoecious plants are quite rare in the field and are therefore unlikely to cause the large bias in SSR (between 0.05 and 0.75, de Jong et al. 2005) that was observed.

Although sex determination genes and sex ratio genes are conceptually different they will be difficult to separate from each other in *U. dioica*.

The maintenance of monoecious plants in natural populations

Monoecious individuals were frequently produced in crosses between true males and true females and this character was passed on to the offspring (also reported for *U. dioica* by Shannon and Holsinger 2007). To our knowledge, only one study reported on monoecious progeny to appear in biparental crosses among true males and true females (*Actinidia deliciosa*, Testolin et al. 1995). In *A. deliciosa* however, monoecious individuals did not persist after self-pollination. The presence of monoecious individuals in kiwifruit is viewed as a threshold character that only is expressed when the genetic and/or environmental conditions create a hormonal equilibrium (Seal and McNeilage 1989).

In natural populations of *U. dioica* in the Netherlands, monoecious plants occur at frequencies between 0 and 7% (Glawe 2006). While we observed populations consisting only of male and female individuals, we never found monoecious plants to dominate populations or to occur on their own. We have shown that, for example, self-pollination of monoecious

plants can result in high numbers of monoecious offspring. Our data also suggest that the transition occurs in one direction, from dioecy to monoecy, and not in the opposite direction. So, what keeps monoecious plants from increasing? Apparently, monoecy is selected against. Monoecious plants may have high selfing rates in the field accompanied by high inbreeding depression (e.g., Rottenberg 2000). We did not observe an obvious depression of germination rate or growth in the selfed offspring of monoecious plants under the non-competitive lab conditions that we used. It is certainly worthwhile to further detail inbreeding depression, both in the lab and under more stressful field conditions.

Conclusion

The investigation of the mechanism of sex determination revealed a complex pattern in *U. dioica*. In dioecious and sub-dioecious species it is generally assumed that one sex is heterogametic, whereas the other is homogametic. While this is generally true for *U. dioica*, to explain our findings we postulated homogametic 'super-males' and heterogametic female individuals to occur as well. The occurrence of such sex types in natural populations would affect SSR. However, the large variation of SSR in crosses between true male and female individuals suggests that there are other ways to modify SSR.

The general ideas about sex determination in dioecious plants are to a large extent determined by studies on *Silene* and *Rumex* species, i.e. species with heteromorphic sex chromosomes. Sex chromosomes that differ in size and morphology occur only in a handful of species. Most dioecious species have distinct male and female genotypes but one or a few genes determine these differences. Westergaard's (1958) seminal review of dioecy provides many examples in which crosses yielded some unexpected phenotypes in frequencies, which are as high as observed in this paper. Therefore the complex sex determination in *U. dioica* may be more representative for dioecious plants in general than the oversimplified picture of heterogametic males and homogametic females that always produce a 50% sex ratio in the seeds.

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