# Sex ratio variation and sex determination in Urtica dioica

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# The genetic basis of sex determination in sub-dioecious *Urtica dioica*

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To interpret sex ratio variation in the sub-dioecious species *Urtica dioica*, a series of experimental crosses was conducted to analyse the genetic basis of sex determination. At our study site, populations of this wind-pollinated perennial consist of low proportions of monoecious individuals (inconstant males) beside male and female plants. In *U. dioica*, a single locus appears to have a major effect on sex determination. Our data suggest that, in dioecious plants, males represent the heterogametic sex, with maleness dominant over femaleness. Monoecious plants were found as being heterogametic, however in this sex type, maleness appears to be co-dominant to femaleness. Self-pollination of monoecious plants generally resulted in 1:3 female:male plus monoecious offspring. When crossed with monoecious plants, females produced mostly one sex type (females) whereas crosses between monoecious plants and males mostly yielded two sex types (females and males). Full-sib crosses among progeny obtained after self-pollination showed that the bisexual trait generally was inherited following Mendelian rules. The bisexual trait was transmitted via both pollen and seeds. However, crosses among dioecious plants and individuals that were obtained after self-pollination of a monoecious plant led to unexpected alterations in sex determination in the new arisen genotypes. Here, multiple alleles seem to interact in several different ways, resulting in variations in the type of dominance and different phenotypic effects (e.g., heterozygous females). We discuss the importance of the genetic sex determination mechanism to explain the sex ratio variation observed in crosses between female and male plants.

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The occurrence of biased sex ratios in natural populations of dioecious (separate sexes) plant species is often considered as a consequence of sex-specific life histories (e.g., sex-differential mortality, sex-differential reproductive investment) thereby implicitly assuming that the two sexes are produced in approximately equal numbers (e.g., Delph 1999). For many dioecious species, especially long-lived trees or shrubs, details on the seed sex ratio (SSR, fraction of males) are, however, lacking. Interestingly, several studies on dioecious species revealed that sex ratios already can be biased in the seeds (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 natural populations from which the seeds were sampled. We are just beginning to understand the evolution of sex ratios and therefore it is timely to revisit the subject of sex determination with an emphasis on the factors that cause variation in sex ratios.

*Urtica dioica* flowers within two months from germination under laboratory conditions, making it particularly suitable to study the evolution of sex ratio. Both SSR (de Jong et al. 2005) and sex ratio of flowering plants in natural populations (fraction of male ramets; Glawe et al., Chapter 3) have been found to vary considerably. The cause of this variation still needs to be established. A series of experiments with *U. dioica* indicated that neither varying conditions for the parent (pre-zygotic stage) nor for the seed or seedling (post-zygotic stage) affected SSR (Glawe and de Jong 2005). We concluded that the enormous variation in SSR in this species may be entirely genetically based. The genetic sex determination mechanism has only been poorly characterised in *U. dioica*. The older literature (Strasburger 1910) stated male heterogamy and female homogamy. Heteromorphic sex chromosomes have, however, not been convincingly demonstrated (Meurman 1925).

Inasmuch as Strasburger's (1910) findings were not documented by data, it appeared to us of importance to study once more in more detail the sex inheritance in sub-dioecious *U. dioica*. Typically in sexually dimorphic plants, one of the sexes is heterogametic, producing two types of gametes, and the other is homogametic, producing one type of gamete. Westergaard (1958) already reviewed in detail the different ways by which the heterogametic sex can be identified.

The most obvious method is to reveal the existence of heteromorphic sex chromosomes by cytological investigation. However, only in a small number of plant species sex chromosomes have been convincingly demonstrated to 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 naturally occurring bisexual individuals from sub-dioecious species. In sub-dioecious species, male and/or female plants occasionally produce flowers of the opposite sex (Westergaard 1958). Unlike bisexual animals that are sterile intersexes, such bisexual plants are almost always fertile and can be used to study inheritance of sex. For example, in natural populations of *Asparagus officinale*, male plants with a few perfect flowers (male plants with occasionally female or hermaphrodite flowers are called sub-androecious) are found every now and then (Rick and Hanna 1943). Self-pollination of these sub-androecious individuals yielded female (XX) and male (XY, YY) offspring in the proportion 1:3. Such an 1:3 ratio of females to males suggests that the male sex is heterogametic and homozygous 'super-males' (YY) are viable. A third method to identify the heterogametic sex is the method that Correns (1928) applied in his classical *Bryonia* studies. In the two *Bryonia* species he used the dioecious trait was dominant over the bisexual one. When *B. dioica* females were pollinated by monoecious (separate male and female flowers on one plant) *B. alba* almost all-female offspring were produced. When monoecious individuals from *B. alba* were cross-pollinated with *B. dioica* males predominantly female and male offspring were obtained.

Low frequencies of bisexual (monoecious) plants have been found to occur beside unisexual individuals from *U. dioica* in the Meijendel population (Heemskerk et al. 1998, de Jong et al. 2005) but also throughout Europe (Greig-Smith 1948, Kay and Stevens 1986). While sex expression in male and female plants was stable, gender in monoecious individuals was found to be labile (Glawe and de Jong 2005). Because the fraction of male flowers increased towards 100% under benign conditions, monoecious plants from the Meijendel population can be regarded as inconstant males. While a large body of literature documents that sexual lability in sub-dioecious species is confined to certain genotypes (e.g., McArthur et al. 1992, Barrett et al. 1999, Ueno and Kadono 2001, Dorken and Barrett 2004), little is known

about the genetic mechanism of sex determination of these genotypes (but see Mather 1949, Janick and Stevenson 1955, Dorken and Barrett 2004). The study of the genetic background of the different sex types may give valuable insight into the evolution of (sub-)dioecy.

In this study, a series of experimental selfings and crosses was conducted to determine the genetic basis of sex determination in sub-dioecious *U. dioica*, addressing 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 bisexual trait transmitted? In addition to providing basic information regarding the complex mechanism of sex determination in a sub-dioecious species, knowledge of its mechanism sheds some light on how sex ratios can be influenced in the presence of bisexual individuals.

## MATERIAL AND METHODS

### *Study organism*

*Urtica dioica* is a sub-dioecious perennial. In The Netherlands, monoecious individuals have been found to coexist with males and females at frequencies which vary between zero and about 7% (Glawe, unpublished data). In our study site at Meijendel (near The Hague), 6.2% of the flowering plants was monoecious (de Jong et al. 2005). Thirty-five females that received open pollination at the Meijendel field site produced between 0 and 18% monoecious offspring (de Jong, unpublished data). *U. 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 Koselka 2002; Mutikainen, personnel communication). Tetrasomic inheritance with a single dominant male factor (males are AAAB and females are AAAA) gives 50% male progeny. Tetrasomic inheritance with more than one male factor is unstable and reduces quickly to a system with a single male factor. For these reasons, 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). Different female (M31-5,-6), male (M31-7,-8) and monoecious (M31-1,-2,-4) individuals were selected from the $F_1$ progeny that was obtained after self-pollination of monoecious M31a. All cross combinations included pollination among individuals of family M31 to minimise genetic variability that might exist between the families.

For individuals that were used repeatedly in different crosses, cuttings were obtained from the plants and cultured in vitro (MS 0 medium). Prior to cloning, gender expression of true males and true females (both are assumed to originate from crosses between dioecious individuals) of the different families was observed for 2 flowering seasons, and was found to be constant. Sex expression in females and males that were obtained after self-pollination of monoecious M31a was checked according to a method described by Glawe and de Jong (experiment 3a, 2005) and was also found to be stable. Seed germination and plant growth were carried out at standard conditions (Glawe and de Jong 2005). Because seed set in monoecious individuals that produced only a low number of female flowers was limited, different numbers of seed per self- or cross-pollination were planted. Gender was determined approximately four weeks after plants began flowering. Germination and survival rate of the progeny of each single cross exceeded 82% and 88%, respectively.

### *The heterogametic sex*

Self-pollination of monoecious individuals

Twenty monoecious plants which all came from open-pollinated female plants at Meijendel were selfed (M14, M18, M24, M31; family background of the other individuals is not known). Flower sex ratios (FSR, fraction of male flowers) of monoecious individuals were found to vary considerably between clones from the same plant between treatments but also within treatments (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 their FSRs.

Cross-pollination 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 (both are assumed to originate from crosses between dioecious individuals). In crosses in which monoecious individuals represented 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*

To estimate SSRs for the five families (M14, M16, M18, M24, M31) used in the crossing program, crosses were performed between a true female and a true male from the same family. Also, since these male and female plants were half-sibs (or even full-sibs) to monoecious plants we were interested if the bisexual trait was unique to monoecious offspring or if it also extended to other sex types in the progeny. The SSRs followed by the percentages of monoecious offspring produced by the selected open-pollinated females 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%]. For each cross, SSR was calculated as the proportion males and monoecious individuals to total progeny.

*The bisexual trait and its transmission*

Another set of crosses was carried out to analyse the mode of sex inheritance in female, male and monoecious individuals that were obtained after self-pollination of a monoecious plant, and to follow the transmission of the bisexual trait (i.e. whether the bisexual trait was passed on through pollen and/or seeds). For that purpose we (1) performed full-sib crosses among progeny that were obtained after self-pollination of the monoecious plant M31a, and (2) carried out crosses among dioecious plants and individuals that were obtained after self-pollination.

*Sex types*

In our crossing program we distinguish between the following sex types: (1) 'True male' plants with male flowers only, originating from crosses among dioecious individuals. (2) 'True female' plants with

female flowers only, originating from crosses among dioecious individuals. (3) 'Monoecious' plants are plants with varying proportions of male and female flowers. The monoecious plants used in the crosses were obtained after self-pollination of monoecious M31a. Glawe and de Jong (2005) regarded monoecious individuals from the Meijendel population as 'inconstant males' because FSR dramatically increased toward 100% maleness under benign conditions. This category of plants has also been designated as 'sub-androecious' or 'fruiting male' by various authors. (4) 'M31a male' plants or 'super-males' (YY) with male flowers only were obtained after self-pollination of monoecious M31a. (5) 'M31a female' plants with female flowers only were obtained after self-pollination of monoecious M31a.

### *Data analysis*

We compared the observed sex ratios from each cross type to the expected sex ratios from one-locus and two-locus genetic models (see Table 5.1) using $\chi^2$-tests (Table 5.7). Most of the cross types include multiple crosses, with maternal and paternal parents from different families. Therefore prior to the comparison, the segregation of sex phenotypes in the progenies of each cross type was tested against heterogeneity (G-test for heterogeneity). Different cross types are designated as 2a (self-pollination of monoecious individuals from the P generation), 2b (self-pollination of monoecious individuals obtained in the $F_1$ generation of monoecious M31a), 3a (cross between true female and monoecious plant), et cetera. For comparison, we either used models that already were established for other sub-dioecious species (Model 1 and 4) or developed new models. Based on progeny sex ratios, we estimated the genotype of each parental plant for the new generated models, allowing us to determine if the genotypes were consistent among different crosses in which we used the same parental plant. According to our results regarding the heterogametic sex, true males and monoecious plants are assumed as being heterogametic, while true females are assumed as being homogametic.

<u>One-locus three-allele model (*Ecballium elaterium*, Mather 1949 and Galán 1951)</u>

In *E. elaterium*, the dioecious type and the monoecious type occur apart from each other. In dioecious types, males are heterozygous for $a^D$ and $a^d$ alleles and females are homozygous for $a^d$ alleles. There is

only one monoecious type which is homozygous for a different allele $a^+$. $a^D$ is dominant to $a^+$, while $a^d$ is recessive to $a^+$. As our results clearly indicated that monoecious plants of *U. dioica* were not homozygous but segregated in female, monoecious, and male offspring, this model was not considered for further analysis.

One-locus four-allele model, with dioecy dominant over monoecy

This model is based on the outcomes that Correns (1928) obtained from his *Bryonia* studies (dioecious trait was dominant to monoecious one). Although Correns performed crosses between two different species, monoecious *B. alba* and dioecious *B. dioica*, the same might be true for monoecious and dioecious plants of *U. dioica* if we assume the monoecious condition to be more primitive than the dioecious, as is generally believed in flowering plants (Lewis 1942). Based on this hypothesis, in the dioecious (D) type true males are denoted as being heterozygous for $A^DB^D$ alleles, with maleness (B) being dominant, and true females are homozygous for $A^D$ alleles. The monoecious (M) type is denoted as being heterozygous for $A^MB^M$ alleles, with maleness being co-dominant to femaleness [a monoecious plant segregates, when selfed, in female ($A^MA^M$), monoecious ($A^MB^M$), and male ($B^MB^M$, 'super-male') offspring]. According to the model we would expect alleles from the dioecious type to be dominant over alleles from the monoecious type, i.e. $A^DB^M$ is female and $A^MB^D$ is male (see Tables 5.3 and 5.6).

Two-locus model with minor feminizing factor

The data indicate that true males and monoecious plants are both heterozygous at a major sex determination locus. In this model we hypothesize that a minor modifying gene(s), F, at another locus is responsible for the feminization of males, turning them into bisexual (monoecious) individuals (ABF_, i.e. ABFF or ABFf). Since the bisexual trait was found to be genetically based (monoecious plants recurred at high frequencies after selfing), we assume the feminizing factor to be transmitted from the monoecious parent to its entire seed progeny, so that female and male offspring that were obtained after self-pollination both posses the feminizing factor (female and male offspring are AAF_ and BBF_, respectively). The feminizing factor is considered to only affect male progeny heterozygous at the major sex

determination locus (ABF_), while progeny with the putative genotype BBF_ possess two major male factors and are phenotypic males ('super-males'), regardless of the presence of the minor feminizing factor. As compared to plants carrying the feminizing factor (AAF_, ABF_, BBF_), true male and female individuals are designated AAff and ABff.

Two linked loci for female and male fertility (Charlesworth and Guttman 1999)

According to the model of Charlesworth and Guttman (1999) for the evolution of dioecy via gynodioecy, two linked loci for male and female fertility determine sex expression in male, female and monoecious plants. Dorken and Barrett (2004) successfully applied the model to sub-dioecious *Sagittaria latifolia*, a species with monoecious and dioecious populations (inconstant males were found also in the latter). Based on the model, a bisexual population (MfMf) is invaded by a recessive male-sterility mutation (m), leading to the establishment of gynodioecy (females are mfmf; bisexuals which are inconstant males are Mfmf). Subsequently, a dominant suppressor of female fertility (F) among bisexuals is operating, leading to the establishment of dioecy (males are MFmf).

Cytoplasmic male sterility

As our data show, male plants were found which were able to produce female flowers (here designated as monoecious plants or inconstant males). In analogy to the feminising cytotypes that are well known from gyno-dioecious species (Saumitou-Laprade et al. 1994), one would expect that a feminising cytoplasm that transforms a male into a monoecious individual would be 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 the survey of Corriveau & Coleman (1989) but additional work by Zhang et al. (2003) on other *Urticaceae* suggested that inheritance of cytoplasmic DNA is strictly maternal. Our results show however that the bisexual trait was transmitted through seed and pollen. Therefore, this model is not considered for further analysis.

TABLE 5.1 – Genetic models of sex determination and seed sex ratios predicted by each model.

| Model | Putative genotypes | | | Seed sex ratios (female: monoecious: male) expected from cross types in | | | |
|---|---|---|---|---|---|---|---|
| | True female | Monoecious | True male | Table 5.2 | Table 5.3 | Table 5.5 | Table 5.6 |
| One-locus four-allele model (D dominant over M) | $A^DA^D$ | $A^MB^M$ | $A^DB^D$ | a) 1: 2: 1<br>b) 1: 2: 1 | a) 1: 0: 0<br>b) 1: 0: 1 | a) 1: 1: 0<br>b) 0: 1: 1<br>c) 0: 1: 0 | a) 1: 0: 0<br>b) 1: 0: 1 |
| Two-locus model with minor feminizing factor | AAff | ABFF | ABff | a) 1: 2: 1<br>b) 1: 2: 1 | a) 1: 1: 0<br>b) 1: 2: 1 | a) 1: 1: 0<br>b) 0: 1: 1<br>c) 0: 1: 0 | a) 0: 1: 0<br>b) 1: 1: 0 |
| | AAff | ABFf | ABff | a) 2: 3: 3<br>b) *p*=2/3 2: 3: 3<br>*p*=1/3 1: 2: 1 | a) *p*=2/3 2: 1: 1<br>*p*=1/3 1: 1: 0<br>b) *p*=2/3 1: 1: 2<br>*p*=1/3 1: 2: 1 | a) *p*=1/2 1: 1: 0<br>*p*=1/3 4: 3: 1<br>*p*=1/6 2: 1: 1<br>b) *p*=1/3 0: 1: 1<br>*p*=2/9 1: 1: 2<br>*p*=2/9 0: 3: 5<br>*p*=1/9 1: 2: 1<br>*p*=1/9 0: 1: 3<br>c) *p*=7/24 0: 1: 0<br>*p*=1/6 0: 1: 1<br>*p*=1/6 2: 1: 1<br>*p*=1/6 0: 3: 1<br>*p*=1/12 1: 1: 0<br>*p*=1/12 1: 0: 1<br>*p*=1/24 0: 0: 1 | a) *p*=2/6 0: 1: 1<br>*p*=2/6 1: 0: 1<br>*p*=1/6 0: 0: 1<br>*p*=1/6 0: 1: 0<br>b) *p*=1/2 2: 1: 1<br>*p*=1/4 1: 1: 0<br>*p*=1/4 1: 0: 1 |
| Two linked loci for female and male fertility (adapted from Charlesworth and Guttman 1999) | mf/mf | Mf/mf | MF/mf | a) 1: 3: 0<br>b) *p*=2/3 1: 3: 0<br>*p*=1/3 0: 1: 0 | a) 1: 1: 0<br>b) 1: 1: 2 | a) *p*=2/3 1: 1: 0<br>*p*=1/3 0: 1: 0<br>b) NA<br>c) NA | a) NA<br>b) 1: 0: 1 |

The monoecious genotypes refer to monoecious M31a ($A^MB^M$) that was selfed in Table 5.2a. Depending on the genotype of M31a, the genotype of monoecious plants in the $F_1$ generation can differ (see results for genotype $A^MB^D$ in the text). Note that cross types include multiple crosses (see Tables 5.2, 5.3, 5.5 and 5.6).
NA, not applicable; the male genotype does not exist.
*p*, probability of sex segregation.

## RESULTS

### *The heterogametic sex*

Self-pollination of monoecious individuals

Regardless of their FSR, all 20 monoecious individuals segregated in female, monoecious, and male offspring (Table 5.2a), demonstrating that all monoecious plants examined were heterozygous at the sex determination locus. In Table 5.2a, 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 four of the 20 selfings, the progeny conformed to a 1:2:1 female:monoecious:male distribution (among them two from family M31, data on the other two are not given in detail but are included in: other 15 plants; Table 5.2a). Progeny resulting from self-pollination of the 16 other individuals were significantly different from a 1:2:1 female:monoecious:male ratio. When both male and monoecious offspring were pooled and regarded as males, the distribution of sex phenotypes of 14 of the 16 selfings conformed to a 1:3 female:male distribution (Table 5.2a). In the progeny of the other two selfings (M18a, M24a; Table 5.2a) too many females and too few monoecious individuals were recovered, contrasting with the 1:3 female:male distribution. Altogether, excluding the $F_1$ progenies of M18a and M24a, all other $F_1$ progenies conformed to a 1:3 female:male distribution (G-test for heterogeneity, $G_{34}$=41.66, $P$=0.17). All $F_2$ progenies of selected plants from family M31a segregated again in three sex phenotypes and therefore corroborated the heterozygous state of monoecious plants in the $F_1$ progeny of M31a (Table 5.2b). For three selfings (M31-1, M31-2, and M31-4), the progeny conformed to a 1:2:1 female:monoecious:male distribution, while for one selfing (M31-3) the distribution of sex phenotypes significantly differed from both 1:2:1 female:monoecious:male distribution and 1:3 female:male distribution (Table 5.2b). Here, too many females and too few male offspring were obtained. Since monoecious plants recurred in high frequencies in the offspring after self-pollination of monoecious individuals, the bisexual state is clearly genetically based.

TABLE 5.2 – Sex distribution of a) $F_1$ and b) $F_2$ progenies of monoecious plants of *U. dioica*.

| Family and FSR | Number of progeny | Progeny in % (Sexual phenotype) Females | Monoecious | Males | $\chi^2$ for 1:2:1 ratio | $\chi^2$ for 1:3 ratio |
|---|---|---|---|---|---|---|
| *a) Generation 1* | | | | | | |
| M31a / FSR=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 (M18a, M24a excl.) | 968 | 27.3 | 42.0 | 30.7 | 26.75*** | 2.67 ns |
| *b) Generation 2 (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* |

The monoecious plants that were selfed to generate $F_1$ progeny were from seed batches (family: M14, M18, M24, M31; families of the other plants not known) collected from open-pollinated females in the field. To confirm the heterozygous state of monoecious plants from the $F_1$ progeny, four individuals were selected that were obtained after self-pollination of M31a to generate $F_2$ progeny. To increase the possibility to detect different sex determination genotypes that might exist, plants producing different flower sex ratios (FSR, fraction of male flowers) were chosen. *, **, ***, ns: $P<0.05$, $P<0.01$, $P<0.001$, not significant, respectively.

## Cross-pollination between true females/true males and monoecious plants

In crosses between true females and monoecious plants, one female (M18b) gave rise to female and monoecious progeny, the other two (M14b, M31c) segregated in female, monoecious and male progeny (Table 5.3a). On average 88% of the offspring consisted of plants showing a female phenotype. This high proportion of phenotypic females was observed in all three crosses. Crosses between true males and monoecious individuals always resulted in three sex phenotypes: besides females and males, fairly low frequencies of monoecious plants occurred in this type of cross (Table 5.3b). In other words, crosses between true females and monoecious individuals yielded almost entirely female offspring, whereas crosses between true males and monoecious individuals predominantly resulted in female and

TABLE 5.3 – Segregation of female, monoecious and male plants of *Urtica dioica* in the $F_1$ progeny of 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 x 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 x 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* |

TABLE 5.4 – Sex segregation ratio in five families of *Urtica dioica*, in which biparental crosses were performed between a true female and a true male plant from the same family.

| Parents | | N | Progeny % (Sexual phenotype) | | | | |
|---|---|---|---|---|---|---|---|
| Mother | Father | | Female | Monoecious | | Male | |
| *True female x True male* | | | | | | | |
| M31c | M31e | 61 | 24.6 | 1.6 | | 73.8 | |
| M24b | M24c | 67 | 50.7 | - | | 49.3 | |
| M18b | M18c | 63 | 34.9 | - | | 65.1 | |
| M16b | M16c | 64 | 62.5 | 4.7 | | 32.8 | |
| M14b | M14c | 64 | 84.4 | 3.1 | | 12.5 | |
| | | | $G_{het}$ | | *df=8* | *66.38* | P<*0.0001* |
| | | | $G_{het\ (monoecious\ and\ males\ pooled)}$ | | *df=4* | *58.71* | P<*0.0001* |

male offspring. Our findings are very similar to those obtained in crosses between dioecious *B. dioica* and monoecious *B. alba* (Correns 1928) and suggest male heterogamy in *U. dioica*.

### *Seed sex ratios in crosses between true females and true males*

Crosses between true female and true male individuals from the same family resulted in the production of different SSRs (Table 5.4). Only one family was found to produce a ratio that did not differ significantly from 1:1 female:male [binomial test, M24 ($P$=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)]. Here, two crosses produced offspring with a male bias [M31 (SSR=0.75), M18 (SSR=0.65)], and two crosses resulted in female-biased progeny [M14 (SSR=0.16), M16 (SSR= 0.38)]. Monoecious plants were found in proportions varying from 0-4.7% (Table 5.4). Self-pollination of several monoecious individuals from the $F_1$ all resulted in female, monoecious and male offspring consistent with Table 5.2 (Glawe, data not shown).

### *The bisexual trait and its transmission*

Full-sib crosses among progeny obtained after self-pollination of monoecious M31a

Crosses between monoecious M31a individuals and M31a females or M31a males yielded the same sexual phenotypes as their parents at a 1:1 ratio (Table 5.5a, b), and cross-pollination between M31a females and M31a males resulted in predominantly monoecious offspring (on average 80% monoecious, Table 5.5c). Overall, the bisexual trait was not only transmitted to the next generation when monoecious plants were used as maternal or paternal parent, but also when crosses were performed between M31a female and M31a male.

Crosses among dioecious plants and individuals obtained after self-pollination of monoecious M31a

When M31a males were used as pollen donors in crosses with true females, on average 83% of the plants showing a female phenotype were obtained (Table 5.6a). At the same time, on average 10% monoecious offspring were obtained which is five fold more compared to the average of monoecious offspring occurring in crosses between true males and true females (Table 5.4), indicating that some factor associated with monoecy must be passed on through the pollen of M31a males. When M31a females were crossed with true males, on average 34% female and 54% monoecious individuals were obtained, the remaining plants showed a male phenotype (Table 5.6b). The results clearly demonstrate that the bisexual trait was inherited through the seeds of M31a females. Taken together, the bisexual trait is transmitted to the next generation via both pollen and seeds.

TABLE 5.5 – Sex distribution of $F_2$ progenies of *Urtica dioica* from full-sib crosses among different sex phenotypes (female, male and monoecious) from the $F_1$ progeny of the monoecious M31a.

| Parents | | N | Progeny % (Sexual phenotype) | | | | |
|---|---|---|---|---|---|---|---|
| Mother | Father | | Female | Monoecious | | Male | |
| *a) M31a female x M31a monoecious* | | | | | | | |
| M31-5 | M31-4 | 44 | 50.0 | 45.5 | | 4.5 | |
| *b) M31a monoecious x 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 x 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* |

TABLE 5.6 – Segregation of female, monoecious and male plants of *Urtica dioica* in the $F_1$ progeny of crosses between a) true females and M31a males, and b) true males and M31a females. Both M31a females and M31a males were obtained after self-pollination of the monoecious M31a.

| Parents | | N | Progeny % (Sexual phenotype) | | | | |
|---|---|---|---|---|---|---|---|
| Mother | Father | | Female | Monoecious | | Male | |
| *a) True female x 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 x 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* |

## *Genetic models*

One-locus four-allele model, with dioecy dominant over monoecy

Two out of six crosses (only crosses for which a $\chi^2$-test was applicable were considered here, the same holds for all other models) were significantly different from the expected ratios (Table 5.7). Although the ratio of female to male offspring in cross type 3b was in contrast to 1:1, female and male individuals were obtained at fairly high frequencies as compared to monoecious offspring. This is in line with our expectation. However, progeny sex ratios obtained in cross type 6b cannot be accounted for by assuming the dioecious trait to be dominant to the monoecious one: according to the results obtained in Table 5.3b we would expect pure males rather than monoecious individuals to occur in the progeny. In three further crosses (cross type 3a, 5c, 6a; Table 5.7) for which only one sexual phenotype was predicted to arise, relatively low frequencies of unexpected sex types (13-20%) also were recovered. Overall, if we ignore the results obtained in cross type 6b, all other findings can be predicted according to this model.

To further analyse the results it is useful to denote exactly from which parental genotype the A allele descends from. For the new arisen genotypes, this is outlined in Table 5.8.

A new genotype $A^MB^D$, showing a monoecious phenotype, is observed to result from crosses between M31a females and true males (Tables 5.6b and 5.8). Up to now we considered monoecious plants used in the crosses to be exclusively $A^MB^M$ (see Table 5.1). Alternatively, monoecious plants may also have the genotype $A^MB^D$. However, under this assumption the outcomes for this model fit less. For the purpose of clarity, the results are given here and not in Table 5.7: 2a/b [1:2:1; $\chi^2$=0.38, ns], 3a [1:0:1; $\chi^2$=129.40], 3b [1:2:1; $\chi^2$=32.46 or 1:0:3; $\chi^2$=6.74], 5a [1:1:0; =0.10, ns], 5b [0:1:1; $\chi^2$=0.17, ns], 5c [0:1:0; 20%], 6a [0:0:1; 93,75%], and 6b [1:1:0; $\chi^2$=12.79]. Therefore, we regard monoecious plants (monoecious M31a and monoecious offspring obtained after self-pollination) to have the genotype $A^MB^M$.

Two-locus model with minor feminizing factor

Assuming that there are two loci, a major sex determination locus and another minor modifying (feminizing) locus, we found that two out of seven crosses were significantly different from the expected ratios

TABLE 5.7 – The number of female (F), monoecious (Mon), and male (M) progeny resulting from nine different cross types and the goodness of fit ($\chi^2$) to the genetic models of sex determination (see Table 5.1).

| Cross type | Number of progeny | | | One-locus four-allele model | | | Two-locus model with minor feminizing factor | | | | | | Two linked loci for female and male fertility (Charlesworth & Guttman 1999) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | Mon | M | Predicted ratio | $\chi^2$ | S | Predicted ratio for ABFF[1] | $\chi^2$ | S | Predicted ratio for ABFf[2] | $\chi^2$ | S | Predicted ratio | $\chi^2$ | S |
| 2a | 17 | 34 | 20 | 1: 2: 1 | 0.38 | | 1: 2: 1 | 0.38 | | 2: 3: 3 | 3.73 | | 1: 3: 0 | 6.99* | |
| 2b | 65 | 90 | 52 | 1: 2: 1 | 5.15 | | 1: 2: 1 | 5.15 | | 2: 3: 3 | 13.82*** | | 1: 3: 0 | 30.82*** | |
| | | | | | | | | | | 1: 2: 1 | 5.15 | | 0: 1: 0 | NA | 56.5% |
| 3a | 157 | 13 | 10 | 1: 0: 0 | NA | 12.8% | 1: 1: 0 | 121.98*** | | 2: 1: 1 | 99.86*** | | 1: 1: 0 | 121.98*** | |
| | | | | | | | | | | 1: 1: 0 | 121.98*** | | | | |
| 3b | 63 | 13 | 127 | 1: 0: 1 | 105.78*** | | 1: 0: 1 | 105.78*** | | 1: 1: 2 | 71.85*** | | 1: 1: 2 | 71.85*** | |
| | | | | | | | | | | 1: 2: 1 | 82.73*** | | | | |
| 5a | 22 | 20 | 2 | 1: 1: 0 | 0.10 | | 1: 1: 0 | 0.10 | | 1: 1: 0 | 0.10 | | 1: 1: 0 | 0.10 | |
| | | | | | | | | | | 4: 3: 1 | 2.97 | | 0: 1: 0 | NA | 56.5% |
| | | | | | | | | | | 2: 1: 1 | 14.73*** | | | | |
| 5b | 12 | 75 | 70 | 0: 1: 1 | 0.17 | | 0: 1: 1 | 0.17 | | 0: 1: 1 | 0.17 | | | | |
| | | | | | | | | | | 1: 1: 2 | 49.86*** | | | | |
| | | | | | | | | | | 0: 3: 5 | 12.48*** | | | | |
| | | | | | | | | | | 1: 2: 1 | 41.18*** | | | | |
| | | | | | | | | | | 0: 1: 3 | 51.93*** | | | | |
| 5c | 21 | 212 | 32 | 0: 1: 0 | NA | 20.0% | 0: 1: 0 | NA | 20.0% | 0: 1: 0 | NA | 20.0% | | | |
| | | | | | | | | | | 0: 1: 1 | 132.79*** | | | | |
| | | | | | | | | | | 2: 1: 1 | 432.18*** | | | | |
| | | | | | | | | | | 0: 3: 1 | 18.59*** | | | | |
| | | | | | | | | | | 1: 1: 0 | 156.57*** | | | | |
| | | | | | | | | | | 1: 0: 1 | 2.28 | | | | |
| | | | | | | | | | | 0: 0: 1 | NA | 87.9% | | | |

TABLE 5.7 – Continued

| Cross type | Number of progeny | | | One-locus four-allele model | | | Two-locus model with minor feminizing factor | | | | | | Two linked loci for female and male fertility (Charlesworth & Guttman 1999) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F | Mon | M | Predicted ratio | $\chi^2$ | S | Predicted ratio for ABFF[1] | $\chi^2$ | S | Predicted ratio for ABFf [2] | $\chi^2$ | S | Predicted ratio | $\chi^2$ | S |
| 6a | 227 | 28 | 17 | 1: 0: 0 | NA | 16.5% | 0: 1: 0 | NA | 89.7% | 0: 1: 1 | 20.84*** | | | | |
| | | | | | | | | | | 1: 0: 1 | 180.74*** | | | | |
| | | | | | | | | | | 0: 0: 1 | NA | 93.8% | | | |
| | | | | | | | | | | 0: 1: 0 | NA | 89.7% | | | |
| 6b | 42 | 66 | 15 | 1: 0: 1 | 12.79*** | | 1: 1: 0 | 5.33 | | 2: 1: 1 | 54.66*** | | 1: 0: 1 | 12.79*** | |
| | | | | | | | | | | 1: 1: 0 | 5.33* | | | | |
| | | | | | | | | | | 1: 0: 1 | 12.79*** | | | | |

The progeny sex ratios from the following cross types (maternal parent x paternal parent) were analyzed: 2a = self-pollination of monoecious M31a, 2b = self-pollination of monoecious individuals obtained in the $F_1$ generation of monoecious M31a, 3a = true female x monoecious, 3b = true male x monoecious, 5a = M31a female x monoecious, 5b = monoecious x M31a male, 5c = M31a female x M31a male, 6a = true female x M31a male, 6b = M31a female x true male. Note that cross types include multiple crosses (see Tables 5.2-5.5). NA, not applicable; the value cannot be determined from the data.
S, percentage of sexual phenotypes in each cross type that deviated from expected.
[1]predicted ratio when monoecious M31a is assumed to be homozygous (ABFF) for the feminizing factor at the minor sex determination locus;
[2]predicted ratio when monoecious M31a is assumed to be heterozygous (ABFf) for the feminizing factor at the minor sex determination locus;
* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

when monoecious plants are regarded as being homozygous (monoecious M31a is ABFF, Table 5.7) at the minor feminizing locus. Again, although the data from cross type 3b have no fit to a 1:1 female:male ratio, male and female phenotypes were produced at fairly high frequencies as compared to the monoecious one. However, estimates of the maternal genotype from progeny sex ratios (females are assumed to be homozygous at the major sex determination locus) were not consistent across generations. This becomes obvious in cross type 6a when true females (putative genotype AAff) were pollinated by 'super-males' (putative genotype BBF_) that were obtained after self-pollination. The outcome was highly unexpected since all progeny would be heterozygous at the major sex determination locus and therefore none of the many observed phenotypic females could have the correct genotype. In two further crosses (cross type 5c, 6a; Table 5.7) for which only one sexual phenotype was expected to occur, other sex types were produced at low but also at high frequencies (20% and 90% for cross type 5c and 6a, respectively).

When monoecious plants are assumed to be heterozygous at the minor modifying locus (monoecious M31a is ABFf), however, we expect to find heterogeneous results for almost all cross types. This is simply because of segregation of the feminizing factor (e.g. in cross type 5a-c, and 6a, b). Nineteen out of 25 crosses had frequencies that were significantly different from the expected ratio (Table 5.7). Furthermore, in crosses in which only one sex type was expected to occur, other sexual phenotypes at fairly high frequencies (up to 94%) could be observed (Table 5.7, for example 6a and 6b). Altogether, the fit of the model appears to be poor.

Two linked loci for female and male fertility (Charlesworth and Guttman 1999)

Five out of seven crosses were significantly different from expected and two crosses for which the model predicted to result in one sexual type only, yielded 57% progeny that did not show the expected phenotype (Table 5.7). Under this model, self-pollination of the monoecious type (inconstant male) is expected to give a 1: 3 ratio of females to monoecious plants, but selfing of inconstant *U. dioica* males always resulted in the production of pure male offspring (i.e. 100% male flowers) as well. Full-sib crosses among offspring recovered from such a selfing demonstrated that inconstant males and pure males

were of two different genotypes in *U. dioica*. Furthermore, females from dioecious populations and females that were obtained after self-pollination are assumed to have the same genotype. However, crosses between true females and true males (Table 5.4) and crosses between female offspring from a monoecious type and true males (Table 5.6b) clearly showed that there are two types of females present in *U. dioica*. The Charlesworth and Guttman (1999) model is not supported by the data.

## DISCUSSION

The main focus of this article is the analysis of the genetic basis of sex determination of different sex types (unisexual, bisexual) in the sub-dioecious *U. dioica* to interpret the observed sex ratio variation (de Jong et al. 2005). Our study provides insight into a complex sex determination mechanism in a sub-dioecious plant species that appears to consist of many different sex genotypes.

### *The heterogametic sex*

After self- pollination, the monoecious plants (inconstant males) investigated here segregated in female, monoecious, and male offspring. For some selfings, the progeny conformed to a 1:2:1 female: monoecious:male distribution (among them M31a), but for most of them the progeny conformed to a 1:3 female:monoecious/male distribution. The monoecious plants and the males that were recovered from monoecious M31a are of two different types, as could be shown when they are crossed to the females which were obtained after self-pollination: the monoecious types gave females and monoecious individuals in equal proportion, while the males gave almost only monoecious offspring. The results in the selfings and crosses can be explained by assuming that the monoecious sex type is heterogametic and that both females and males are homogametic at the major sex determination locus.

The heterogametic sex in dioecious plants of *U. dioica* (designated as true female and true male) could be established according to Correns' *Bryonia* method (1928). Crosses between females and monoecious individuals yielded almost all-female offspring, whereas crosses between males and monoecious individuals predominantly gave female and male offspring. Even though heteromorphic sex

chromosomes could not be distinguished from mitotic chromosomes (Chapter 6), our results suggest that males from dioecious *U. dioica* are heterozygous at the sex determination locus.

### *Sex determination*

None of the genetic models was found to perfectly fit our data. Our results however suggest that a single locus has the major effect on sex determination in sub-dioecious *U. dioica*. The alleles at the major sex determination locus seem to differentiate between the reproductive types. In dioecious plants, females appear to be homozygous for $A^D$ and $A^D$ alleles and males appear to be heterozygous for $A^D$ and $B^D$ alleles, with maleness being dominant (see later). Monoecious plants appear to be heterozygous for $A^M$ and $B^M$ alleles, with $A^M$ co-dominant to $B^M$. Monoecious plants homozygous at the major sex determination locus have not been detected. Self-pollinations of monoecious individuals and full-sib crosses among progeny obtained after self-pollination indicate that the bisexual trait is generally inherited according to Mendelian inheritance. The low frequencies of unexpected males (Table 5.5a,c) may be ascribed to the unstable nature of the monoecious phenotype: whether the plant showed a male or monoecious sex expression depended on nutrients (even within the same environment sex expression was found to be labile; Glawe and de Jong 2005). Likewise, the low number of female offspring (Table 5.5b,c) might occur as a genotype-environment interaction. This seems likely because some of the plants that appeared as phenotypic females in the first flowering season were observed to carry both female and male flowers in the second. Given the data presented in Table 5.2 (e.g., M18a, M24a), we cannot exclude the possibility that there are different monoecious genotypes present in the Meijendel population.

Crosses among dioecious plants and individuals that were obtained after self-pollination however indicate a change of the dominance relationships between alleles in the new arisen genotypes. This may have led to the unexpected alterations in sex determination. For example, the occurrence of phenotypic females in cross type 6a which were found to be heterozygous at the sex determination locus suggest that the female trait was dominant to the monoecious one (see also Table 5.3a). Thus, when $A^D$ from a true female combines with $B^M$ from a monoecious plant or a 'super-male' $B^MB^M$, the gene combina-

TABLE 5.8 – Genetic model of sex determination in the sub-dioecious species *Urtica dioica*. The alleles at the major sex determination locus differentiate between the reproductive types. In dioecious plants, males are heterozygous with maleness dominant over femaleness. Monoecious plants are also heterozygous, but maleness is co-dominant to femaleness. Both female and male plants that were obtained after self-pollination are homozygous. In the new arisen genotypes, alleles interact in several different ways, resulting in variations in the type of dominance and different phenotypic effects. D (dioecious) and M (monoecious) designate the parental origin of the sex determination genes.

| Reproductive types | Putative genotype | Sex phenotype | Reference |
|---|---|---|---|
| Dioecious | $A^DA^D$ | female | Table 5.4 |
| | $A^DB^D$ | male | Table 5.4 |
| Monoecious | $A^MA^M$ | female | Tables 5.2 and 5.5b, c |
| | $A^MB^M$ | monoecious | Tables 5.2 and 5.5a, b |
| | $B^MB^M$ | male | Table 5.2 |
| New genotypes | $A^DA^M$ | female | Tables 5.3a, b and 5.6b |
| | $A^DB^M$ | female[1] | Tables 5.3a and 5.6a |
| | $A^MB^D$ | monoecious[2] | Table 5.6b |
| | $A^MB^D$ | male[3] | Table 5.6b |
| | $B^DB^M$ | male | Table 5.6b |
| Dominance relationships: | $B^D > A^D > A^M$ (monoecious plant) | | |
| | $A^D > B^M$ (monoecious plant / male plant) | | |
| | $B^D = A^M$ (female plant) | | |
| | $A^M$ (female plant) = $B^M$ (male plant) | | |

[1] $A^DB^M$ plants were produced when true females were cross-pollinated with monoecious or male plants both obtained after self-pollination; $A^D$ is assumed to be dominant to $B^M$. [2] $A^MB^D$ plants were produced when females that were obtained after self-pollination were mated with true males; $A^M$ is assumed to be co-dominant to $B^D$. [3] $A^MB^D$ plants were produced when monoecious individuals that were obtained after self-pollination were mated with true males; the $A^M$ is assumed to be recessive to $B^D$.

tion predominantly results in a female phenotype ($A^DB^M$), suggesting that $A^D$ is dominant to $B^M$. The results obtained in Table 5.6b indicate on the other hand that the monoecious trait is dominant to the male: when $A^M$ derives from a female that was obtained from a monoecious plant and combines with $B^D$ from a true male, the sexual phenotype is predominantly monoecious ($A^MB^D$, $A^M$ is co-dominant to $B^D$). This is a good example of what is called 'relative sexuality' by Hartmann (1956). However, the outcome in Table 5.3 rather suggests both female and male trait being dominant to the monoecious one since the

crosses between true males or true females and monoecious individuals gave predominantly male and female offspring in ratios which are in conformity with male heterogamy. Our results are very similar to those of Correns' *Bryonia* studies (1928), where dioecy was dominant to monoecy. Possibly in *U. dioica*, the findings in the different cross combinations (compare Table 5.3b and 5.6b) may be due to manifold interactions of the sex determination genes that strongly depend on the female genotype that is crossed with a true male. While Correns' data are based on crosses between two different species (monoecious *B. alba* and dioecious *B. dioica*), Mather (1949) and Galán (1951), Janick and Stevenson (1955) and Glawe and de Jong (this paper) performed crosses between monoecious individuals and true males or true females of the same species. Contrary to our results for *U. dioica*, in *Ecballium elaterium* (Mather 1949, Galán 1951) and *Spinacia oleracea* (Janick and Stevenson 1955) the female trait was recessive to the monoecious one.

Altogether, if we assume a one-locus four-allele model to determine sex in *U. dioica*, it is possible to suggest a mechanism of interaction of the alleles from their dominance relationships (Table 5.8). At least four additional genotypes arise in crosses among dioecious plants and individuals that were obtained after self-pollination from a monoecious plant, yielding a total of at least nine genotypes governing the occurrence of three sexual phenotypes (Table 5.8). In the new arisen genotypes, feminisation not only seems to depend on the origin of the allele (dioecious or monoecious), but also on the maternal plant (female or monoecious) that was crossed with a true male (Table 5.8).

However, the conclusion about the validity of this scheme must be tempered for different reasons. For example, seeds of the parental plants used in self- and cross-pollination were collected from open-pollinated females in the field. Because monoecious individuals were also found to occur beside female and male plants at our field site, cross-pollination between the different sex types can result in different genotypes that express the same sexual phenotype. So, the genotypes estimated based on the progeny sex ratios may not be tantamount to the sexual phenotypes (e.g., a true female, true male; both were assumed to originate from crosses between dioecious plants) we refer to in the text. Moreover, given the data from the selfings it

seems quite likely that more monoecious genotypes exist. Also, by chance we may have failed to detect monoecious individuals that are homozygous at the major sex determination locus.

### *Alternative model*

In the above model we tried to explain the results of our crosses by assuming that a single locus has the major effect on sex determination. However, we cannot rule out the possibility that multiple loci affect sex determination and in such a case a quantitative genetic model may be more appropriate. Bull et al. (1982) suggested that although individuals are observed to be either male or female, their gender may be determined by an underlying character X, which is continuous. If X is above a certain threshold T individuals are phenotypically male, while below this threshold they are phenotypically female. In *U. dioica* we have three sexual phenotypes: female, monoecious and male. A model with two thresholds $T_1$ and $T_2$ then seems appropriate. Individuals with the lowest value of X ($X<T_1$) are female, individuals with intermediate values ($T_1<X<T_2$) are monoecious and individuals with high values ($X>T_2$) are male (Lynch and Walsh 1998). Bull et al. (1982) further assumed in their model that when two individuals mate, the offspring have on average the value of X of their parents, but with some variance. The Bull et al. (1982) model could explain a number of features observed in crosses for *U. dioica*. Firstly, it predicts that every cross between a true female and a true male produces, by chance, some monoecious offspring for which X lies between $T_1$ and $T_2$. Monoecious plants were indeed observed in most of the crosses we did (Table 5.4). Secondly, it predicts that a female with a very low value of X ($X<<T_1$) would produce more female offspring than a female with an X value just below threshold $T_1$ ($X<T_1$). Likewise, a male with an extremely high value of X ($X>>T_2$) would produce more male offspring than a male with an X value just above $T_2$ ($X>T_2$). This is a novel, attractive explanation for the heritable variation in seed sex ratio we observed in crosses between true females and true males (Table 5.4 and de Jong et al. 2005). Thirdly, if we were to cross a monoecious plant with a true male, most of the offspring should have an X-value greater than $T_2$, i.e. they should be male. Similarly, a cross between a monoecious plant

and a true female should predominantly result in plants with X values below $T_1$, i.e. they should be females. This is consistent with the results shown in Table 5.3. However, the Bull et al. (1982) model assumed that inheritance of character X is independent of parental origin. In this case results should be symmetric when a monoecious plant is crossed with either a male or a female. This is clearly not the case and our data suggest that, in *U. dioica*, the male is heterogametic, i.e. produces two types of gametes, each with a different value for X. The model can be extended by taking this into account and can be fitted to our data using different assumptions about the variance in X in female and male gametes. This is, however, beyond the scope of the present paper. Other *Urtica* species are monoecious and in such species the development of flowers as either male or female must depend on some internal hormone threshold. We thus believe that a quantitative view of sex determination with an internal threshold is well worth considering.

### *Biased sex ratios in cross between males and females from the dioecious system*

The skewed seed sex ratios may be explained by a multi-locus sex determination mechanism or, if sex in *U. dioica* is determined by a major sex determination locus, modifying genes must be present to explain our results. Sex determination based on the first mechanism is considered to be rare and has so far only been reported for a single (sub-) dioecious species (*Mercurialis annua*). In *M. annua*, three independently segregating genes control sexuality and as a result seed sex ratios are enormously variable (Louis 1989). On the other hand, in species with sex chromosomes, pollen competition (Correns 1928), certation (Conn and Blum 1981), meiotic drive (Taylor and Ingvarsson 2003), nuclear sex ratio distorters or cytoplasmic factors (reviewed by Werren and Beukeboom 1998) have been invoked to explain biased seed sex ratios. For example in the *S. latifolia*, a dioecious plant species with an X/Y sex determination mechanism, sex-linked modifiers have been proposed to influence the seed sex ratio (Taylor 1994).

In *U. dioica*, two lines of evidence suggest a major-sex-determination-locus model. Firstly, in our study, none of the true female or male plants used in the cross combinations with different female,

monoecious and male individuals that were obtained after self-pollination produced progeny arrays with significantly different seed sex ratios (Tables 5.3 and 5.6). This is not what one would expect if the variation in seed sex ratio among crosses between true females and true males is due to a sex determination mechanism based on a multi-locus system. Secondly, current investigations of crosses among true males from a low seed sex ratio family and true females from a high seed sex ratio family, and vice versa, indicated that the sex ratio was inherited through the female (Glawe and de Jong, Chapter 7). In other words, the sex ratio produced by the females generally resembled the sex ratios produced by their maternal parents.

*Monoecious plants and their maintenance in natural populations*

Monoecious individuals (inconstant males) were rare and produced in a non-Mendelian fashion in crosses between true males and true females. The bisexual trait was found to occur at regular intervals as the phenomenon was observed in three out of five crosses. When selfed, monoecious plants recurred besides males and females and the distribution of the different sex phenotypes was similar to the monoecious individuals used in this study. To our knowledge, there has been one study reporting on inconstant males to appear in biparental crosses among true males and true females (*Actinidia deliciosa*, Testolin et al. 1995). In *A. deliciosa* however, inconstant males have not been obtained after self-pollination. While the presence of inconstant males 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), feminisation of males has been shown to be a heritable trait in *U. dioica* (i.e. monoecious plants recur after selfing).

At the moment it is unclear which mechanism may be responsible for the heritable modification (feminisation) of male individuals. Generally, the presence of bisexual progeny in female x male crosses is believed to be a consequence of recombination between different sex determination loci. In *U. dioica*, bisexual individuals were produced at regular intervals rather suggesting a multi-locus than a major-locus sex determination mechanism. While we cannot exclude the existence of other sex determination loci besides the major locus in plants of the dioecious system and therefore recombination to

occur, we also could imagine that sex expression in sub-dioecious *U. dioica* may be epigenetically controlled. Thus, the sexual phenotype of an individual depends on whether certain DNA fragments are active (demethylated) or inactive (methylated). Males may have turned into bisexual (monoecious) individuals as a result of a heritable epimutation (non-programmed epigenetic event). Recent studies have emphasised the role of imprinting in the early evolution of sex chromosomes (Jablonka 2004).

In natural populations, monoecious plants have been found to occur at low numbers (between 0 and 7%; Glawe, unpublished data) together with male and female individuals. While we observed populations consisting only of male and female individuals, we never have observed 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. So, we indeed may expect higher numbers of such sex types to occur in the field. Apparently, there is selection against monoecy. For example inbreeding depression may play a role, maintaining the frequency of monoecious individuals at low levels (e.g., Rottenberg 2000).

The occurrence of low proportions of monoecious types of *U. dioica* beside male and female plants may mean that the species is still in a transitional stage from monoecy to dioecy and at this moment, it remains an open question if the maintenance of bisexual individuals incurs an evolutionary significance in terms of population fitness.

## *Conclusion*

The investigation of the mechanism of sex determination revealed a complex sex pattern in *U. dioica*. In dioecious and sub-dioecious species it is generally assumed that one sex is heterogametic, whereas the other is homogametic. 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 thus would affect seed sex ratios. However, the considerable variation of SSRs in crosses among true male and true female individuals rather seems to be a consequence of SSR modification.

Possibly, our scheme is not the last word on sex determination in this species. The greater complexity of genotypes in the sub-dioecious breeding system, and the variety of sexual phenotypes

expressed by a single genotype, may give just some reasons why it has remained less well documented and understood than gyno-dioecious or andro-dioecious breeding systems. With this report, we hope to provide basic information regarding the genetic control of sex expression in a breeding system which might be not so rare as generally is believed.

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