Sex ratio variation and sex determination in Urtica dioica

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Heritable variation in seed sex ratio of the stinging nettle (*Urtica dioica*)

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Male and female flowering plants of the dioecious *Urtica dioica* occur in approximately equal numbers in our study area at the coastal sand dune area of Meijendel. The seed sex ratio (SSR, fraction of sons) collected from female plants in the field varied between 0.05 and 0.76, and differed significantly between maternal parents. After one generation of selection for either high or low SSR, female plants produced seed batches with sex ratios as extreme as 0.08 and 0.73. Natural populations of *U. dioica* harbour considerable genetic variation in SSR.

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In well-mixed (panmictic), outcrossing populations, selection favours equal allocation of resources to sons and daughters (Fisher 1930, Charnov 1982, Uyenoyama and Bengtsson 1982) and, when sons and daughters are equally costly, they should be produced in equal numbers. In most common sex determination systems, meiosis is expected to produce equal numbers of males and females. It is often assumed that diploid organisms have unbiased sex ratios in their offspring, with very limited genetic variation (Charnov 1982, Hardy 2002). With respect to dioecious plants, which have separate male and female individuals, it is sometimes taken for granted that the seed sex ratio (SSR, fraction males in the seeds) is 0.5 (Delph 1999).

However, Fisher's (1930) idea only applies under a number of assumptions (Bull and Charnov 1988) that are not necessarily met in plant populations. Most plant populations are not panmictic but have distinct spatial genetic structure (Vekemans and Hardy 2004), result-

ing in mating between related offspring. Models with genetic structure show that, under fairly general conditions, selection favours a SSR different from 0.5 (PD Taylor 1994, de Jong et al. 2002). This SSR could be male or female biased, depending on the dispersal distances of pollen and seeds, which govern the relative importance of Local Mate Competition and Local Resource Competition (de Jong et al. 2002).

Also, Fisher's model assumed that nuclear genes control SSR, but control of sex ratio may be more complex. It is logical to hypothesize that cytoplasmic genes can influence sex ratio, since the cytoplasm is often only passed on through female gametes. In other words, when cytoplasmic genes are not transmitted through pollen, a cytoplasmic gene in a male plant is at a dead end and zero copies of this cytotype are passed on to the next generation, irrespective of whether the male lives or dies (Cosmides and Tooby 1981). Therefore cytoplasmic genes should favour the production of females. The conflict between cytoplasmic and nuclear genes in determining sex ratio is relatively unexplored in dioecious plants (but see Taylor 1994). However, intragenomic conflict over allocation to pollen versus seeds has been convincingly demonstrated in some well-studied gynodioecious species (females and cosexuals in the same species; van Damme 1991, Koelewijn and van Damme 1995, Frank and Barr 2001) and may also play a major role in dioecious plants.

Some dioecious plant species have been shown to produce a biased SSR (Alström-Rapaport et al. 1997, Taylor 1999) and it is therefore timely to revisit SSRs of dioecious plants. How often do biased sex ratios occur in the seeds of dioecious plants? How much genetic variation for SSR is maintained in natural populations? If this variation is small, as in other diploid organisms like humans and birds (Hardy 2002), is this due to constraints?

Few dioecious plants have been studied with respect to variation in SSR. Meagher (1981) found an average SSR of 0.5, and no genetic variation in SSR, in the lily *Chamaelirium luteum*. In *Silene latifolia* and *S. dioica*, SSR differed between genotypes from the same population and was most frequently female-biased (Correns 1928, Taylor 1999). These *Silene* species have distinct sex chromosomes with the male as the heterogametic (XY) sex. Meiotic drive could lead to an overrepresentation of X-bearing gametes in the pollen, and this

explanation has been invoked as the cause of the biased sex ratio in the seeds (Taylor et al. 1999). In interpopulation crosses with *Salix viminalis*, Alström-Rapaport et al. (1997) documented SSR variation. On average SSR, was female-biased, as was the case for flowering plants in natural populations of this willow species. With regard to the underlying mechanism, Alström-Rapaport et al. (1997) suggested that multiple genes are involved in sex determination, so that SSR is a quantitative trait. A multilocus sex determination system has been documented for *Mercurialis annua* (Louis 1989, Durand and Durand 1991), but such systems are considered to be rare (Chattopadhyan and Sharma 1991, Ainsworth et al. 1998).

At the moment it is obscure how common variation in SSR is in dioecious plants and what causes this variation. Furthermore most dioecious plants are long-lived trees and shrubs and these species pose serious practical problems for detailed genetic investigations of SSR: it takes many years before plants enter the reproductive stage and during this vegetative period mortality may differ between male and female plants. We therefore chose the stinging nettle, *Urtica dioica*, as a model species. Individuals flower within 2 months time and the germination rate and survival rate are both close to 100% (see later) under controlled conditions, so that errors caused by possible differences in germination and survival between the sexes are minimal.

In this paper, we first examine sex ratio of flowering individuals in the field. Such data reflect variation in SSR, but also possible differences in survival and age of first reproduction. These data provide a background for the work under controlled conditions. To obtain an exact picture of within-population variation in SSR, we collected seeds from many open-pollinated female plants in the field and grew these seeds to maturity under controlled conditions. We examine if neighbouring plants have similar SSRs. Finally, we investigate if variation in SSR is heritable.

MATERIALS AND METHODS

Species description

Urtica dioica is an allo-tetraploid species with a worldwide distribution (Greig-Smith 1948). It is dioecious, but monoecious individuals occur at low frequency (approx. 5%) in our study area at the Meijendel

dune reserve (near The Hague, The Netherlands) and throughout the geographical range (Kay and Stevens 1986). In Meijendel *U. dioica* grows along roads, near eutrophic lakes and in natural woodland, especially in association with *Populus nigra*. New rhizomes are produced in autumn. These rhizomes grow just beneath the soil surface and form new shoots in spring. Clones consisting of a single genotype may form patches of several square meters. Seeds have no special dispersal features and are dispersed by gravity. The species is wind pollinated, with limited gene flow according to Pollard and Briggs (1984).

Early reports on sex chromosomes in *U. dioica* have not been confirmed (Westergaard 1958). The old literature (listed in Greig-Smith 1948 and Westergaard 1958) stated that the male is the heterogametic sex and the female the homogametic sex, as is by far the most common situation in dioecious plants. We have tested extensively if the sex of male or female plants could be changed at different moments in the life cycle, by varying environmental conditions or by applying hormones (Glawe and de Jong 2005). Since, we have not been able to change the gender of a single male or female plant in our experiments, we consider sex determination in *U. dioica* to be entirely genetic. Contrary to this finding, monoecious plants produced 100% male flowers when grown under more favourable conditions (Glawe and de Jong 2005) and these monoecious individuals are considered as males in this paper (see Discussion) when computing sex ratios. The sex ratio of the flowers of monoecious plants is disregarded in this paper.

Experiment 1: Sex ratio of flowering plants in the field

To estimate variation in the ratio of the numbers of male and female individuals in nature, we counted in 1997 individuals of *Urtica dioica* in five areas at the sand dunes of Meijendel, which were chosen to reflect the largest possible range in height and vitality. Unisexual patches of *U. dioica* usually consisted of 1-100 flowering stems that apparently all belonged to a single genotype. When two patches overlapped, differences in gender, colour, leaf shape, and opening of the first flower usually allowed a distinction between the two genotypes. When in doubt, a patch was considered to consist of a single individual. We recorded for each plant that was regarded as an individual, the

number of flowering stems and the height of the longest stem, to the nearest centimetre. Such data show if any sexual dimorphism exists in plant growth. If a dimorphism exists it may affect the sex ratio of flowering plants in the field, for instance, when male plants are smaller and have a higher mortality rate as in *Rumex acetosella* (Korpelainen 1992).

Experiment 2: Variation in SSR

We estimated SSR from maternal plants from each of two populations (population 1 and 2), to test whether SSR differs among families and populations. In the autumn of 1997 seeds were collected from 8 female plants at Meijendel (population 1). Seeds from each sample from a mother plant (family) were therefore at least half sibs; all seeds from one family had the same mother but not necessarily the same father. The plants were all located within 100 m of each other. The sampling was repeated in a second population (population 2) in 1999, when seeds were sampled from 25 females along 800m of roadside. We only sampled maternal parents that were at least 2m apart, with no *Urtica dioica* individuals in between, so that we were certain that they did not belong to the same individual. In the second sampling, we made note of the position of plants. Seeds were kept separate according to maternal parent, and were germinated on moist filter paper in Petri dishes (over 90% germination). Different numbers of seedlings per maternal parent (60 for population 1 and 40 for population 2) were then placed separately in 1.3 litre pots with a 50/50 mixture of sand and potting soil, which were placed in a randomized design. Plants were grown in growth cabinets at 20°C during 16 h light and 15°C during an 8 h dark period. Humidity was 70% and light intensity was 180-200 μmol m^{-2} s^{-1} PPTD at plant level. Under these 'standard conditions', most plants flowered after about 2 months. Only 22 seedlings (4 from population 1 and 18 from population 2) died before flowering, so mortality was negligible under standard conditions. In total we determined the sex of 1644 flowering plants grown from seed.

Experiment 3: Selection on SSR

We tested whether SSR is heritable and responsive to selection. We used seeds from the same seed batches as in experiment 2 and select-

ed maternal families with a low (L, significantly less than 50% males) or high (H, significantly more than 50% males) sex ratio in experiment 2; these families are denoted as L1, L2, H1 and H2. At least 55 seedlings per family were grown under standard conditions (see experiment 2). After flowering we checked if the sex ratio in these families was as expected according the results of experiment 2. There was no seedling mortality in experiment 3. As soon as plants began to flower, a selection of five female plants from family L1 was placed together in a pollination chamber with five males from L2. For the reciprocal cross, five females from family L2 were placed in a chamber with five males from L1. Similarly, five females from H1 were crossed with five males from H2 and vice versa. In this setup crosses were made between families with a similar sex ratio, but we avoided crosses between sibs (i.e. female of L1 × male of L1). After setting of seeds, seeds were collected from all five maternal plants in each of the four crosses. At least 60 F_1 seeds per maternal parent were sown and grown until maturity under standard conditions and the gender of individuals was established. Only 9 seedlings died before flowering, so that mortality was again negligible.

RESULTS

Experiment 1: Sex ratio of flowering plants in the field

Among 207 plants counted in the field, 12 plants were monoecious (6.2%), 94 were male and 101 plants were female. The numbers of male (monoecious plants included) and female plants did not differ significantly from equality (binomial test, two-sided P=0.73). There were no significant differences in the sex ratio of flowering plants when comparing the five areas (χ^2=3.15, P>0.05). Males and females were very similar with respect to stem number (±SE): 20.7 ± 2.5 *vs.* 20.5 ± 2.3 and height 132.4 ± 4.9 cm *vs.* 132.6 ± 3.9 cm, for males and females, respectively.

Experiment 2: Variation in SSR

From the seeds collected from 8 mother plants in population 1, and raised to maturity, 238 plants were female, 234 plants were male and 4 plants were monoecious. SSR per family varied between 0.31 and 0.68 and was heterogeneous between families (χ^2=32.31, 7 d.f.,

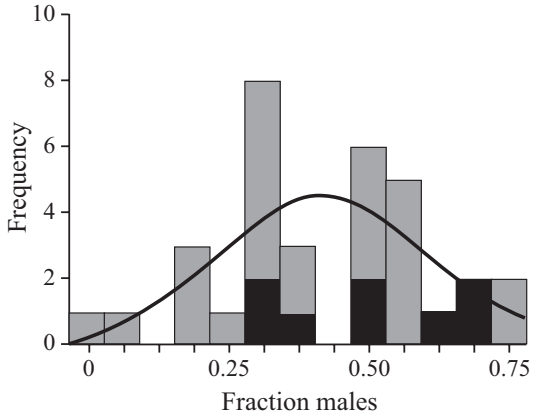

FIGURE 2.1 – Frequency distribution of sex ratio of seeds (SSR=fraction males and monoecious plants) collected from 33 maternal plants that received natural pollination in the field. Bars present the pooled data of the two populations, and are subdivided in population 1 (dark) and population 2 (shaded). For each observation 60 seedlings per mother plant were grown until maturity in population 1, and 40 seedlings per mother were grown in population 2.

$P<0.001$). Four families in population 1 produced sex ratios that were significantly biased (two-sided $P<0.05$ in a binomial test), in two cases SSR was significantly male biased and in two cases SSR was significantly female biased. Because mortality was negligible (less than 1%) such differences between families are not due to sex differential mortality; the differences originate in the seed stage.

From 982 seedlings that were raised to maturity for the second population; 568 plants were female, 358 were male and 56 were monoecious. The numbers of male (monoecious individuals included) and female plants differed significantly from equality (binomial test $P<<0.001$). SSR per family varied between 0.05 and 0.76 and, as in population 1, the data were heterogeneous ($\chi^2=124.53$, 24 d.f. $P<<0.001$). Ten families in population 2 produced sex ratios that were significantly biased (two-sided $P<0.05$ in a binomial test), in seven cases SSR was significantly female-biased and in three cases SSR was significantly male-biased. The frequency distribution of the SSR per family in the two populations is shown in Figure 2.1. The distribution of the pooled data from population 1 and 2 is approximately normal

TABLE 2.1 – Seed sex ratio (SSR=fraction male and monoecious plants) in A) seed samples (families) from four female parents that were open pollinated in the field (experiment 2), B) the same seed samples in a replicate estimate (experiment 3), and C) the offspring of five female F_1 plants when crossings were made between two families with low (L) and between two families with high (H) SSR.

A) Selected families from experiment 2					
Family	Male	Female	Monoecious	SSR	
L1	20	40	1	0.34	
L2	19	41	0	0.32	
H1	41	19	0	0.68	
H2	34	21	2	0.65	
B) Replicate (experiment 3)					
Family	Male	Female	Monoecious	SSR	
L1	16	43	1	0.28	
L2	18	34	5	0.40	
H1	48	7	2	0.88	
H2	46	17	2	0.74	
C) Cross	Male	Female	Monoecious	SSR	range SSR[2]
L1 × L2[1]	121	170	5	0.42	0.30-0.46
L2 × L1	31	248	16	0.16	0.08-0.14
H1 × H2	171	132	13	0.58	0.43-0.69
H2 × H1	211	118	6	0.65	0.58-0.73

[1]the maternal parent is listed first; [2]refers to range of SSR of the 5 female parents

(the *P*-value of the Kolmogorov Smirnov test on normality is 0.95) with an average SSR of 0.42 and standard deviation of 0.18.

In population 2 no spatial structure in SSR was found; in a multiple regression the variance in the SSR of a focal plant, explained by the SSR of the left and right neighbour on the road, was only 0.3% (P=0.96, n=23).

Experiment 3: Selection on SSR

When we took seeds from the same seed batches and grew plants to maturity to estimate the SSR, the results were very similar to that obtained in experiment 2 (compare Table 2.1B and 2.1A). After crossing plants from families L1 and L2, we grew the F_1 to maturity. It mattered greatly for the sex ratio in the offspring whether the father came from family L1 and the mother from L2 or the other way around (Table 2.1C). The crosses between females and males from H families resulted in high proportions males in the offspring (Table 2.1C). Here it made no difference whether the maternal parent came from family H1 or H2.

Results in Table 2.1C show that SSR is heritable under standard conditions in the lab. There are too few independent data points to quantify heritability in a reliable way. However, if we use the standard procedure of estimating narrow sense heritability (h^2) as the slope of the least-squares regression of offspring SSR on midparent value, we obtain an estimate of h^2=0.85 (r=0.84). Since the error in the midparent values (X-axis) is similar to the error in the offspring sex ratio (Y-axis), model II regression is probably more appropriate here. The slope of model II regression is equal to the slope of ordinary least-squares regression, divided by the correlation coefficient (Sokal and Rohlf, 1998). Model II regression thus yields a slope of 0.85/0.84=1.01.

DISCUSSION

Maintenance of variation

We found an unbiased sex ratio in the flowering plants of *U. dioica* in the field. Nevertheless, under the surface, there was considerable variation in SSR (Fig. 2.1). This variation was heritable (Table 2.1). These findings are inconsistent with the conventional view of limited variation due to balancing selection on SSR and/or constraints in meiosis. What explains the maintenance of large genetic variation in SSR within populations of *U. dioica?*

Firstly, selection on SSR may be weak so that, with autosomal genes in full control of sex ratio, mutation-selection balance can maintain some variation. While we cannot rule out this possibility completely, it is an unlikely explanation as to why some species harbour no variation in SSR (Meagher 1981), while other species like *Silene latifolia*, *Salix viminalis* and *U. dioica*, show such large variation in SSR.

Secondly, it is possible that SSR is not strictly under control of nuclear genes but is also influenced by mitochondrial genes. This could result in cyclical changes in gene frequency as has been suggested for gynodioecious plants (Gouyon et al. 1991, reviewed in Frank and Barr 2001 and Saur and Wade 2003) and maintenance of genetic variation in a metapopulation (McCauley and Taylor 1997, Couvet et al. 1998). We observed a maternal effect in the crosses between plants from families with a low sex ratio (Table 2.1C). We