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## **Sex ratio variation and sex determination in *Urtica dioica***

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## Summary and conclusions

Generally, it has been implicitly assumed that genetic factors lead to balanced sex ratios in seeds. However, in natural populations of dioecious plant species (with separate male and female individuals), both male- and female-biased sex ratios among flowering plants have been observed. Frequently, sex-based differences in life history traits (e.g. mortality, vegetative growth, longevity) have been invoked to explain such skewed sex ratios. Yet, investigation of primary (seed) sex ratios under controlled conditions revealed that there is also considerable variation among progeny sex ratios. These findings clearly show that, in order to fully interpret the sex ratio bias among flowering plants in the field, it is necessary to include the analysis of progeny sex ratios as well. Moreover, no complete understanding of the evolution of sex ratios can be claimed in the absence of a thorough understanding of the sex determination mechanism. Although the genetics of sex determination has been studied for more than a century – and continued rapidly with the advent of the molecular age – we are still far from a general picture of plant sex determination mechanisms and their evolution.

In my thesis, I studied the mechanism of sex ratio variation and different aspects of sex determination in the (sub-)dioecious plant species *Urtica dioica*. In the following, I will give a summary of the results obtained from these experiments and draw some final conclusions concerning the origin of sex ratio variation, the mechanism of sex determination and inheritance of the sex ratio trait in this particular plant species.

### SEX RATIO VARIATION

The motivation of this thesis is based on the finding that natural *U. dioica* populations at our field site in Meijendel harbour considerable variation in progeny sex ratios (fraction of males), although male and

female flowering plants occur at approximately equal numbers (CHAPTER 2). In particular, seeds collected individually from 33 female plants (=33 families) in the field and grown to flowering plants under laboratory conditions revealed progeny sex ratios that varied between 5% and 76% male offspring.

Beside variation in progeny sex ratios we also detected significant variation among male and female flowering shoots (between 7% and 82% male shoots) in other natural populations of *U. dioica*, inhabiting a range of environmental conditions (CHAPTER 3). The ratio of male to female reproductive shoots varied between and among habitats. In order to discriminate whether the sex ratio bias originated already in the seeds or resulted from sex-based differences in survival or vegetative reproduction of male and female flowering plants at some later point during lifetime, we studied several life history traits of both sexes. In a common garden experiment, male and female clones were monitored during the course of three growing seasons with respect to mortality and vegetative growth. Sex-differential mortality was not observed and mortality rate generally was low. Also, there was no difference in the number of shoots produced between female and male individuals. In a laboratory experiment, in which the plants were grown under varying environmental conditions for one season, female and male individuals developed approximately equal numbers of shoots and rhizomes. Plant biomass and plant height, measured both in the field and the common garden, did not differ significantly between the sexes.

Because we detected no sexual dimorphism in the life history traits measured, we believe that the sex ratio bias in flowering plants mirrors variation in the primary sex ratio. In Chapter 2 we clearly showed that there is considerable between-family variation in progeny sex ratios.

#### SEX DETERMINATION MECHANISM

As a first step towards understanding the causes and maintenance of variation in progeny sex ratios, we investigated the extent to which primary sex ratios and sex expression of *U. dioica* plants is influenced by varying environmental and physiological factors (CHAPTER 4). In particular we analyzed (1) whether nutrient availability of a parental plant affects the sex ratio of its progeny, (2) whether the primary sex

ratio can be affected by different environmental conditions before flowering, and (3) whether sex expression can be modified by various environmental conditions or by exogenous application of plant growth substances (phyto-hormones) after initiating flowering. The parents that were grown under different nutrient regimes during growth and pollination always produced approximately the same sex ratio in their progeny. Furthermore, the primary sex ratio did not change significantly when progenies were raised under benign, poor or garden conditions. Sex expression in flowering male and female plants did not change with varying conditions or hormone application, indicating that sex expression in both male and female plants is stable.

In contrast, sex expression in monoecious plants (with both male and female flowers) was found to be labile since the fraction of male flowers increased as plants were grown under benign conditions. Here, nearly half of the plants that at first showed a monoecious phenotype, exclusively produced male flowers when grown under favourable conditions. Such outcomes may suggest that monoecious individuals are inconstant males (i.e. they are genetic males that occasionally produce seeds) which alter the fraction of male flowers according to environmental conditions.

The findings presented in Chapter 4 provided strong evidence that the observed sex ratio bias in the progeny did not result from environmental sensitivity at different stages in the life cycle of *U. dioica*. Such biases mainly arise from genetic factors leading to skewed sex ratios in the offspring. To analyze the genetics of sex determination we performed in CHAPTER 5 a series of experimental crosses among male, female and monoecious individuals of *U. dioica*, addressing the following questions: (1) which is the homo-/heterogametic sex, and (2) are there more genes involved in sex determination beside the XX/XY scheme?

The heterogametic sex can be identified by self-pollination of male or female plants when, through manipulation, some flowers of the opposite sex can be induced. However, because sex expression of male and female individuals could not be altered experimentally (see Chapter 3) we had to revert to using naturally occurring plants of *U. dioica* bearing both male and female flowers. Self-pollination of such monoecious plants resulted in different types of offspring (female, male and monoecious), showing that monoecious plants were het-

erozygous at a sex determination locus. The monoecious types were then crossed to male and female plants to identify the homo- and heterogametic sex. In such crosses, the homogametic sex should result in less phenotypic variation in the offspring compared to the sex that is heterogametic. The outcomes of the crosses were largely consistent with females being the homogametic sex and males being the heterogametic sex: crosses between females and monoecious individuals yielded almost entirely female offspring, whereas crosses between males and monoecious individuals predominantly resulted in male and female offspring.

Another set of crosses, including males, females and monoecious plants that were all obtained after self-pollination of monoecious individuals, also suggested that a single locus has a major effect on sex determination. According to a simple scheme of sex inheritance, such as an XX/XY system, crosses between a single male and a single female individual are expected to produce male and female offspring in approximately equal numbers. Because bi-parental crosses between male and female individuals resulted in variable progeny sex ratios that neither could be explained by a major sex determination locus nor attributed to sex-based differences in germination or seedling mortality, we postulate that additional genes, on other loci, distort sex segregation. As an alternative, we discuss a quantitative genetic model involving numerous genes that determine gender. According to this model, the gender of the offspring will be determined by the sum of genetic effects of genes over different loci, thereby resulting in variable progeny sex ratios that are heritable.

To sum up, our findings in the genetic crosses described in Chapter 5 indicated the following results. (1) In *U. dioica*, males represent the heterogametic sex and females represent the homogametic sex. Monoecious individuals are regarded as inconstant males because they develop exclusively male flowers while growing under benign conditions and produce variable sex types when selfed (i.e. they are heterozygous at a sex determining locus). (2) Sex determination in *U. dioica* does not follow a clear-cut scheme. Apparently, also genes on different loci are involved in sex determination.

The aim of the following study (CHAPTER 6) was to investigate the outcomes in the genetic crosses by using cytological analysis and molecular markers. Karyological analysis of mitotic chromosomes

## SUMMARY AND CONCLUSIONS

showed no evidence for the presence of heteromorphic sex chromosomes. To identify sex-linked markers, we investigated male and female offspring that was produced in a bi-parental cross. None of the primers tested amplified markers that were 100% associated with sex, but seven markers showed significant sex-linkage. The sex markers were either segregating in the male parent or in both parents, but not in the female parent. Two sex-linked markers could be placed in a genetic map, but in different linkage groups. According to a discriminant analysis, sex expression in 72% of the offspring could be predicted correctly, using four out of seven sex markers. Although molecular studies need to be continued to complete the genetic map and a major sex determination locus still may be present, this locus was not detected yet. We conclude that sex determination in *U. dioica* apparently also involves an interaction of multiple genes on different loci. Such a scheme may explain the appearance of unexpected sexual phenotypes in the crossing experiment in Chapter 5.

### INHERITANCE OF THE SEX RATIO TRAIT

To understand the mechanism(s) that generate(s) the variation in progeny sex ratio in *U. dioica*, we examined inheritance of sex ratio by performing crosses among progeny of families with different levels of sex ratio bias (CHAPTER 7). For the families studied here, maternal plants strongly contribute to the variation in the primary sex ratio whereas paternal plants seem to have little effect. Also, sex ratios from reciprocal crosses were significantly different, demonstrating that the sex ratio of the progeny depends on which plant is used as the maternal and which as the paternal parent. Our results generally showed that sex ratios produced by the females resembled the sex ratios that were produced by their maternal parents. The genotype of the male parent had no effect on the sex ratio of its progeny. It should be noted though that only three male individuals were used in the crosses and more extensive sampling might reveal male genotypes that show some effect. The present study therefore demonstrates that the female parent incurs substantial influence on the sex ratio of its progeny. What are the mechanisms that could account for maternal control? We can rule out selective seed abortion as a cause for the sex ratio bias because seed-ovule ratios among maternal plants from different sex ratio families were all high and did not differ from each

other. But two other mechanisms are possible. Firstly, with a simple sex determination mechanism such as the XX/XY system the maternal parent may selectively promote germination or relative growth of X- vs. Y-bearing pollen tubes. Secondly, with a polygenic sex determination system which also involves a feminizing effect of the maternal cytoplasm pushing the sex ratio towards a female-bias, our findings can be explained. Cytoplasmic factors are typically transmitted through seeds but not through pollen. Consequently, these genes are selected to produce female-biased sex ratios, thereby increasing their transmission to future generations. Of course, an interaction of both mechanisms is also possible.

It has recently been argued for animals that genetic conflict plays a role in maintaining sex ratio variation among progenies and that such conflict also explains why sex determination mechanisms often turn out to be complex. In the plant kingdom, genetic conflict has been revealed to occur in gynodioecious plant species (female and cosexual individuals). Here, the interest of nuclear genes that are selected to produce a balanced ratio of pollen and ovules conflict with the interests of cytoplasmic genes that are typically only passed on through seeds, selecting for femaleness. In dioecious plant species, the locus of genetic conflict between the allocation of reproductive resources between pollen and ovules is transferred to the sex ratio of the progeny. Maternal inheritance of the sex ratio trait as it is documented in this thesis for *U. dioica* may suggest an influence of cytoplasmic factors. It is quite likely that the large variation in sex ratio in this species is maintained through natural selection affecting nuclear and cytoplasmic genes differentially. The mechanisms behind sex ratio variation and sex determination in *U. dioica* remain an interesting question for further in-depth analyses.