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Research

Sexing pollen reveals female bias in a dioecious plant

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Summary

• Information on angiosperm sex ratios has largely been restricted to surveys of flowering individuals. These often deviate from equality, with male bias more commonly reported. Female-biased sex ratios are concentrated in a few taxa and have been linked to the possession of heteromorphic sex chromosomes and bias introduced during the gametophytic stage of the life cycle. It has been proposed that differences in gamete quantity and quality could give rise to female bias, although there is no direct evidence with which to evaluate this possibility.

• Here, we use flow cytometry to investigate microgametophytic 'sex ratios' in a flowering plant. We demonstrate that differences in DNA content between the sexes in *Rumex nivalis*, a species with heteromorphic sex chromosomes, make it possible to distinguish female- vs male-determining pollen nuclei.

• We found a small but significant female bias in microgametophytes produced by males (mean 0.515) with significant variation among family means (range 0.463–0.586), and 18 of 22 families averaging > 0.50.

• The observed female bias at the gametophytic stage of the life cycle is consistent with the direction of bias previously reported for seeds and vegetative and reproductive plants in wild populations of *R. nivalis*, but is insufficient to fully explain the degree of bias.

Key words: DNA content, flow cytometry, microgametophytic sex ratios, pollen, *Rumex*, sex chromosomes.

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Introduction

Sex ratio is a key factor in evolutionary biology, affecting population structure, mate competition and reproductive fitness. In species with separate sexes (dioecy), negative frequency-dependent selection should maintain unbiased sex ratios (Fisher, 1930; Lloyd, 1974a; Hardy, 2002). However, sex ratios in dioecious plants often diverge from equality, with male bias more commonly reported than female bias (Delph, 1999). Diverse mechanisms can influence sex ratios, and determining when and how biases are established during the life cycle is critical for understanding the evolution of sex ratios (Lloyd & Webb, 1977; Bierzychudek & Eckhart, 1988; Ågren *et al.*, 1999; Taylor, 1999; de Jong & Klinkhamer, 2002; Taylor & Ingvarsson, 2003). Investigation of plant sex ratios is complicated by the difficulty of distinguishing the sexes of individuals during the juvenile phases of the life history because of weakly developed secondary sex characteristics. As a result, gender determination has largely focused on reproductive individuals and has thus considered functional sex ratios only (Freeman *et al.*, 1976; Crawford & Balfour, 1990; Alström-Rapaport *et al.*, 1997; Carlsson-Graner *et al.*, 1998; Nicotra, 1998). A few studies of sex ratios in wild plant populations have included nonflowering individuals using sex-specific molecular markers (Eppley, 2001; Korpelainen, 2002; Hilfiker *et al.*, 2004; Stehlik & Barrett, 2005) or cytology in species with sex chromosomes (Żuk, 1963; Zarzycki & Rychlewski, 1972; Rychlewski & Zarzycki, 1975, 1986). Given the difficulty of assessing gender in early life stages, relatively little is known about the mechanisms responsible for biased sex ratios in plants.

Several dioecious species of *Rumex* (Polygonaceae) are characterized by predominantly female-biased sex ratios, and some effort has been made to understand the mechanism(s) responsible. Using experimental approaches and sex-specific molecular or chromosomal markers, differences in sex ratio among life-cycle stages have been revealed (Zarzycki & Rychlewski, 1972; Rychlewski & Zarzycki, 1975, 1986; Conn & Blum, 1981; Korpelainen, 1991, 2002; Stehlik & Barrett, 2005). For example, Stehlik & Barrett (2005) demonstrated male-biased mortality during the life history of sporophytes in *Rumex nivalis*, resulting in an increase in female frequencies from seed to vegetative and flowering cohorts. Similarly, flowering sex ratios in *Rumex acetosa* and *Rumex thyrsiflorus* are consistently more female-biased than seedling sex ratios (Żuk, 1963; Zarzycki & Rychlewski, 1972; Rychlewski & Zarzycki, 1975, 1986; Korpelainen, 1991). Seed sex ratios in *R. acetosa*, *R. thyrsiflorus* and *R. nivalis* are also influenced by pollen competition, with larger stigmatic pollen loads resulting in more female-biased sex ratios (Zarzycki & Rychlewski, 1972; Rychlewski & Zarzycki, 1975, 1986; Stehlik & Barrett, 2006). In *Silene latifolia*, another well-studied dioecious species with female-biased seed sex ratios, there is an association between biased progeny sex ratios and pollen abortion. This may reflect segregation distortion during microgametophyte development, although direct evidence for the mechanism is lacking (Taylor, 1994, 1999; Taylor & Ingvarsson, 2003). Segregation distortion refers to the situation when a gene or gene region enhances its own transmission to the detriment of other parts of the genome (selfish genetic element). This could result in an over-representation of X-chromosome over Y-chromosome bearing pollen. To what extent female-biased seed sex ratios in *Rumex* are influenced by the quantity and/or quality of pollen produced by males is not known.

The chromosomal sex determination system in *Rumex* subgenus *Acetosa* offers a unique opportunity to investigate the ratio of female- to male-determining pollen in dioecious plants. Species in this subgenus possess heteromorphic sex chromosomes, in which females ($2n = 14$) are homogametic with six autosomes and XX sex chromosomes, and males ($2n = 15$) are heterogametic with six autosomes and XY_1Y_2 sex chromosomes (Wagenitz, 1981; Navajas-Pérez *et al.*, 2005). This differs from most other species with heterogametic sex determination, in which males have only one Y chromosome (Ainsworth, 2000; Vyskot & Hobza, 2004). The gender-based difference in chromosome number, combined with a low autosome to sex chromosome ratio, produces conditions in which female- and male-determining pollen are potentially distinguishable using flow cytometry on the basis of nuclear DNA content.

Here, we used flow cytometry to measure the DNA content of nuclei from leaves and pollen of *R. nivalis*, an alpine plant with strongly female-biased sex ratios. Our study had three specific objectives: (i) to determine whether female and male sporophytes and female- and male-determining microgametophytes can be reliably distinguished on the basis of differences in DNA content; (ii) to investigate whether there is a numerical bias in the production of female- vs male-determining pollen in individual plants by counts of pollen nuclei in a bulk sample; (iii) to determine if there is a genetic component to the production of biased 'pollen sex ratios', as indicated by significant between-family variation in the ratios of female- and male-determining pollen. We also discuss the contribution of biased microgametophytic sex ratios to primary and secondary sex ratios in *R. nivalis* and evaluate the potential mechanisms involved.

Materials and Methods

The study system

Rumex nivalis Hegetschw. is a perennial wind-pollinated plant of European alpine snowfields (Wagenitz, 1981; Stehlik & Barrett, 2005). As males are the heterogametic sex, they produce two types of pollen: male-determining pollen with nuclei containing 6 autosomes (A) + Y_1Y_2 = 8 chromosomes and female-determining pollen with 6 A + X = 7 chromosomes.

Plant material and pollen collection

We selected half-sib seed families from 22 maternal parents from 16 of the 18 populations used in Stehlik & Barrett (2005). The 22 seed families resulted from two generations of open-pollination in the glasshouse at the University of Toronto. By using a genetic admixture, we avoided any potential population-specific influences on pollen sex ratios.

Before the onset of flowering, we transferred all 22 maternal half-sib families to a glasshouse at the University of Guelph. Females were discarded and pollen from males was collected and dried in folded wax weighing paper with desiccant in a refrigerator for a minimum of 3 d, after which the pollen was transferred to 1.5-ml microcentrifuge tubes. We combined pollen samples collected on different days from the same plant so that each tube held approx. 4 mg of pollen. These tubes were stored in a container with desiccant in a refrigerator for up to 32 wk until further analysis. For each maternal seed family, we analyzed the pollen from a mean of 8.45 males (range: 4–11), resulting in a total of 186 males.

Number of nuclei in pollen

To establish the expected DNA content of female- and male-determining pollen nuclei, which depends on the number and ploidy of nuclei within each pollen grain, we determined whether pollen grains were binucleate or trinucleate. We placed whole pollen grains in a 10 mM $MgSO_4$ buffer containing 50 μg ml^{-1} of DNA-specific fluorochrome propidium iodide (PI) stain and examined them with a fluorescence microscope (Leica DMRB microscope; green 546/12 nm excitation filter). Images were captured using OpenLab 3.5 (Improvision, Lexington, MA, USA).

Flow cytometry

We first estimated the absolute DNA content of nuclei in somatic tissue (leaves) from a subset of female and male plants using flow cytometry. We finely chopped fresh leaf tissue with a razor blade in 1.5 ml of ice-cold nucleus isolation buffer (NIB), containing 300 mM sucrose, 80 mM KCl, 20 mM NaCl, 15 mM HEPES, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM spermine, 0.25 mM polyvinyl pyrrolidone (PVP)-40, 0.2% Triton X-100, and 0.1% 2-mercaptoethanol, adjusted to pH 8.0 (Bino *et al.*, 1992). *Epilobium hirsutum* leaf tissue was chopped with *R. nivalis* as an internal DNA content standard. The inbred line of *E. hirsutum* had a DNA content of 0.84 pg/2C (DNA content of diploid nuclei in this species), as estimated relative to *Sorghum bicolor* Pioneer 8695 (1.67 pg/2C; Price *et al.*, 2005). We then ran each sample through a 30-μm filter, centrifuged the sample at 56 ***g*** for 12 min at 4°C, removed the supernatant, and added 0.4 ml of staining solution (9.6 mM $MgSO_4$, 47 mM KCl, 4.7 mM HEPES, 6.1 mM DL-dithiothreitol (DTT), 2.4% Triton X-100 and 50 μg ml^{-1} RNAse A, with 50 μg ml^{-1} PI) for 20 min to 2 h. Each sample was run for 3 min on a FACSCalibur flow cytometer (BD Biosciences, San Jose, MA, USA) at low pressure. We obtained total (integrated) fluorescence of nuclei, measured with a 585/42 nm photodetector, using CellQuest Pro 4.0.2 (BD Biosciences).

To analyze the pollen nuclei, we added 1 ml of NIB to each 1.5-ml microcentrifuge tube of pollen, vortexed for approx. 20 s and placed the tube on its side in a –20°C freezer overnight. The tube was taken from the freezer and the frozen block of buffer was chopped with a razor blade until it was completely thawed. We then filtered the resulting solution. For the first part of the study (113 samples), we filtered samples through 30-μm filters, but this allowed most intact pollen grains to pass through into the sample. In the latter part of the study (73 samples), we used a 10-μm filter, which excluded whole pollen grains. After filtering, we treated samples similarly to leaf samples (already described in the previous paragraph), except that they were run for 5–6 min on the flow cytometer. In a few samples, we co-chopped female and male leaf tissue with pollen to directly compare the fluorescence values of male and female somatic nuclei to those of putative female- and male-determining pollen nuclei.

Analysis of pollen fluorescence histograms

We examined the integrated fluorescence histograms to identify distinct peaks corresponding to female- and male-determining pollen nuclei, to quantify the number of nuclei within each peak, and to quantify the fluorescence (and thus DNA content) of each peak relative to other peaks and to somatic nuclei. For all samples, we used MODFIT LT (Mac 3.1 SP2; Verity Software, Topsham, ME, USA) to measure peak positions and the number of nuclei per peak, after gating out debris using a plot of fluorescence intensity measured with 585 nm vs 670 nm

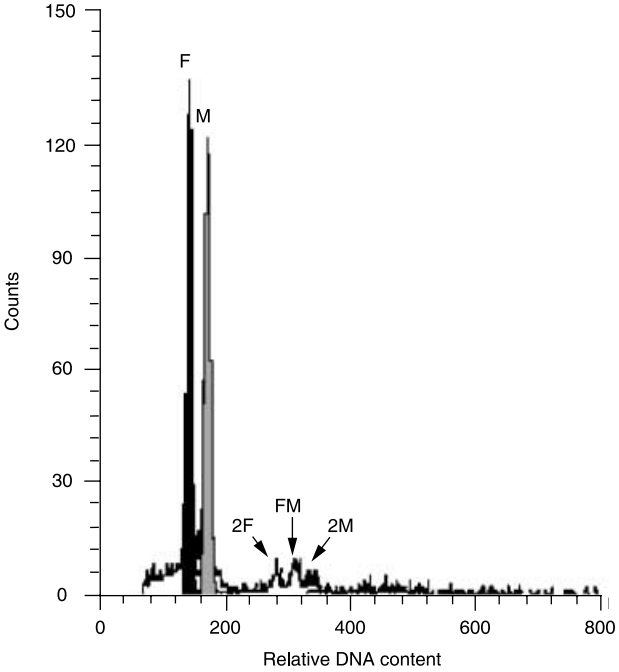

Fig. 1 A typical relative fluorescence histogram of *Rumex nivalis* pollen nuclei. Shown are main female-determining (F) and male-determining (M) peaks, as well as smaller secondary peaks at twice the fluorescence of the F and M peaks (2F and 2M, respectively), and a peak intermediate to the 2F and 2M peaks (FM).

photodetectors. We used a resolution of 256 channels to maximize events per channel and to improve the reliability of the curve-fitting algorithms. We excluded pollen samples from the final data set if they were too poor in quality to be fit by MODFIT LT, or if the total number of nuclei in the main female- and male-determining peaks was less than 1000. Our choice to use a cut-off based on total number of nuclei (rather than a per-peak criterion) was to avoid excluding strongly female- or male-biased samples, but all but one sample also passed the criterion of having at least 500 nuclei per peak.

In addition to the two main fluorescence peaks corresponding to female- and male-determining nuclei in pollen, small secondary peaks were typically detected at fluorescence levels corresponding to roughly twice the DNA content of the main peaks (Fig. 1). The majority of the events within these peaks were probably doublets (aggregates of two nuclei), based on the presence of three peaks corresponding to the position of the three possible doublet types and a reduction in the size of these peaks when the finer (10-μm) filter was used. For our main analysis, we assumed that the secondary peaks were entirely composed of aggregates and that female- and male-determining nuclei aggregate similarly. Thus, these secondary peaks were ignored because, under these assumptions, they would not affect our estimates of the proportion of female- to male-determining pollen. The same would be true if they consisted of contaminating somatic nuclei. However, we also considered the possibility that some events in these higher-fluorescence peaks were polyploid

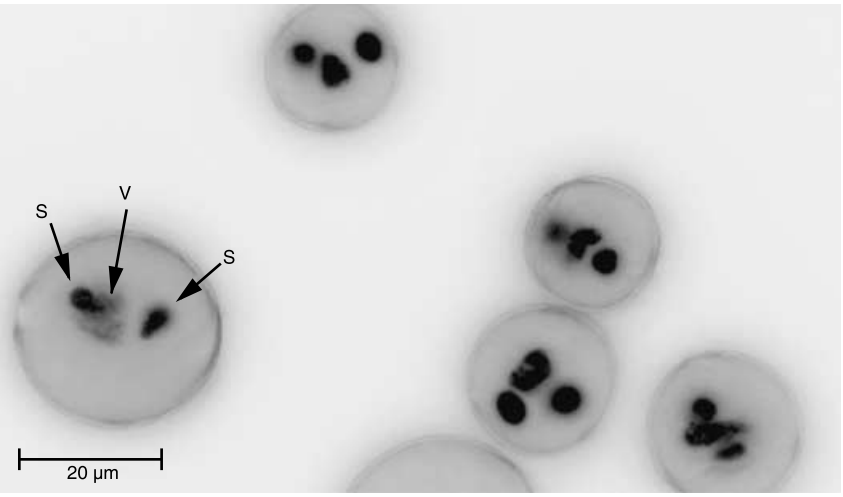

Fig. 2 Pollen grains of *Rumex nivalis*, each with one vegetative (V) and two sperm (S) nuclei. Pollen was stained with propidium iodide and examined under a fluorescence microscope (Leica DMRB microscope, green 546/12 nm excitation). The image is black/white inverted.

nuclei or generative pollen nuclei that had not yet divided into two sperm nuclei. We repeated the analyses under these assumptions, estimating debris, aggregates, and unexplained events in the secondary peaks using MODFIT LT, and modifying the total numbers of female- and male-determining nuclei appropriately. Under the 'generative nuclei' assumption, we doubled the number of putative generative nuclei before adding them to the totals of nuclei, because each generative nucleus corresponds to two sperm nuclei.

We first compared the mean proportion of female-determining pollen across all plants to an expected value of 0.50 (unbiased), using the nonparametric Wilcoxon signed-rank test because data were not normally distributed. We then examined the effect of maternal family on the proportion of female-determining pollen using analysis of variance (ANOVA), with filter pore size (small vs large) included as a covariate. Finally, we examined the distribution of family least square means (LSM) from this analysis, comparing the mean to 0.50 using a *t*-test.

Results

Number of nuclei in pollen

Mature pollen grains of *R. nivalis* were trinucleate, with two sperm and one vegetative nucleus (Fig. 2). Therefore, all three nuclei in pollen are expected to have the same DNA content and should generate a single fluorescence peak when examined using flow cytometry (Bino *et al.*, 1990).

Sex-specific DNA contents

Flow cytometry revealed that leaves of *R. nivalis* are endopolyploid, with nuclei in the 2C, 4C and 8C condition (Fig. 3a). The 8C and 4C nuclei combined were more numerous than 2C nuclei. Female plants had a nuclear DNA content of 6.74 pg/2C (± 0.03 standard error (SE)), which was significantly lower than the 7.41 pg/2C of male plants (± 0.02 SE; $t = -19.11$, $P < 0.0001$; $n = 7$ females, 8 males). In samples containing leaves from both female and male plants, there was a clear separation of female and male nuclei peaks (Fig. 3b). When pollen and leaves were run together, peaks identified as female- and male-determining nuclei were detected at the expected fluorescence intensities, i.e. the female-determining pollen peak was at half the fluorescence of female somatic tissue, and the separation between female- and male-determining pollen peaks was the same as the separation between female and male 2C somatic peaks (Fig. 3c). The difference in DNA content between male and female 2C nuclei was 9.5%, which corresponds to an 18.1% difference between male- and female-determining pollen nuclei.

Ratio of female- vs male-determining pollen

Among all 186 males of *R. nivalis*, the mean proportion of female-determining pollen was 0.515 (± 0.004 SE; range 0.274–0.758; Fig. 4). This female bias was significantly greater than 0.5 ($P < 0.001$; Wilcoxon signed-rank test). Mean female bias was higher for samples filtered with 10-µm filters (0.524; ± 0.006 SE) than for those sampled with 30-µm filters (0.509; ± 0.005 SE; $t = -1.966$; $P = 0.051$).

The female bias was stronger when samples were filtered with 10-µm filters. This is most likely attributable to improved histogram quality, rather than selective filtering of male-determining nuclei. Given the size of whole pollen grains (mean diameter ~20 µm; Fig. 2) there is little reason to believe that nuclei would be impeded by the 10-µm filter. It is even less probable that this filter size would form the cut-off between the sizes of female- and male-determining nuclei, as a potential difference in nucleus diameter arising from an 18.1% difference in (haploid) DNA content would be small relative to the filter size. Also, the female bias was present in samples filtered with 30-µm filters, which are large enough to allow whole pollen grains to easily pass through and do not impede nuclei at all.

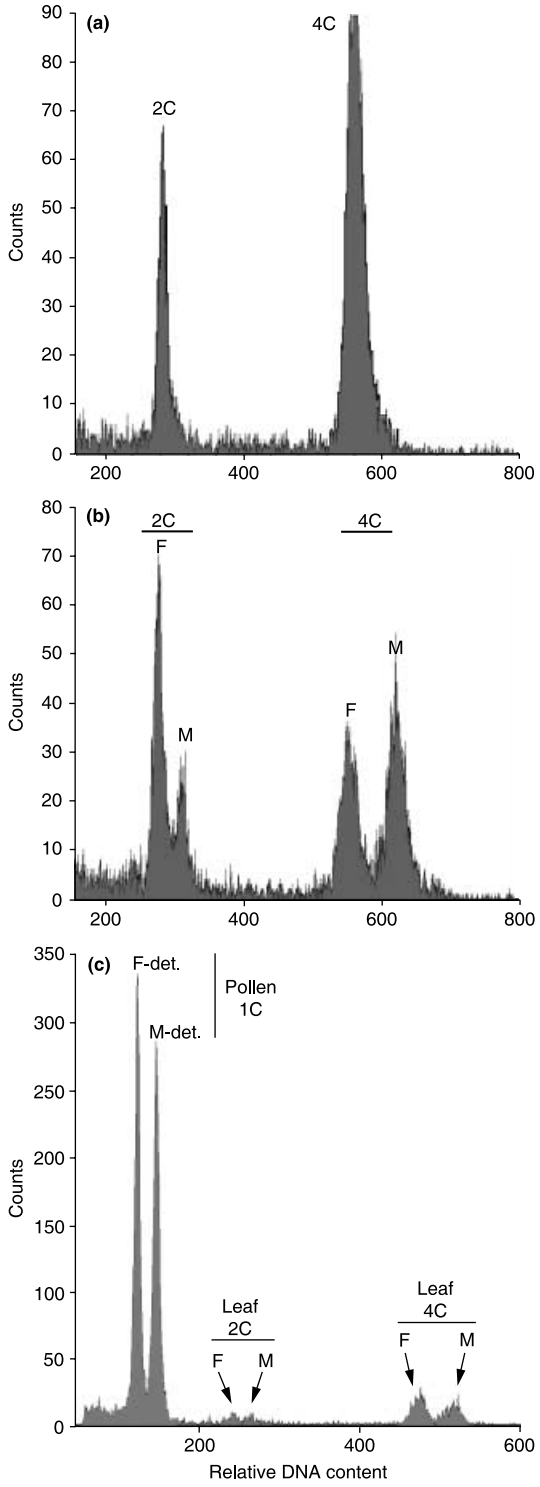

Fig. 3 (a) Relative fluorescence histogram of nuclei from female *Rumex nivalis* leaf tissue. 2C, 4C, and 8C nuclei are present in this endopolyploid species, although with the scale used in this study, the 8C peak position is not shown. (b) Integrated fluorescence histogram of nuclei from a bulk sample of female (F) and male (M) leaf tissue. (c) Integrated fluorescence histogram of nuclei from a bulk sample of female and male leaf tissue and of female- and male-determining pollen.

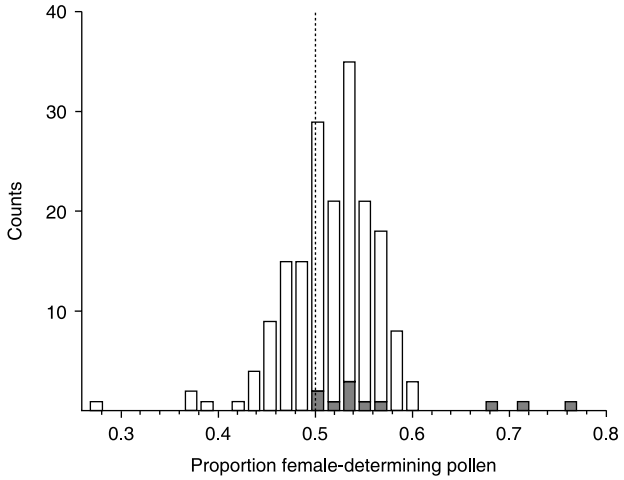

Fig. 4 The distribution of male plants of *Rumex nivalis* with different ratios of female- vs male-determining pollen. n = 186 plants from 22 families. Family 436 is highlighted (gray bars) because it was most strongly female-biased. The dashed line indicates an unbiased pollen ratio.

Table 1 Effect of maternal family on the ratio of female- vs male-determining pollen in *Rumex nivalis*

Source	SS	Mean square	d.f.	F	P
Family and random	0.1005	0.0048	21	2.119	0.0048
Filter	0.0274	0.0274	1	12.152	0.0006

See the Materials and Methods section for details.
d.f., degrees of freedom; SS, sums of squares.

Because samples were cleaner and produced higher quality histograms with the 10-μm filter, it is more likely that results using these filters are also more accurate. Nevertheless, we present additional results below using all data to be conservative. When the ratio of pollen types was calculated assuming that secondary peaks were polyploid or consisted of generative nuclei, the female bias decreased to 0.512 (± 0.004 SE) and 0.509 (± 0.004 SE), respectively, but in both cases the mean was still significantly different from 0.5 ($P < 0.001$ and $P = 0.002$, respectively; Wilcoxon signed-rank test).

There was significant variation among families in the proportion of female-determining pollen when the effect of filter size was taken into consideration ($P = 0.0048$; Table 1). Because the effect of filter size was also significant ($P = 0.0006$; Table 1), we used family LSMs in subsequent analyses, in order to take this effect into account. The mean of family means was 0.516 (± 0.0005 SE; range 0.463–0.586), which was significantly different from 0.50 ($t = 2.950$, $P = 0.0076$); 18 of 22 families had female-biased pollen sex ratios (i.e. > 0.50; Fig. 5). The family effect was largely driven by a single family (436), which included three males with strongly female-biased pollen (Figs 4, 5). The mean proportion of female-determining pollen in this family

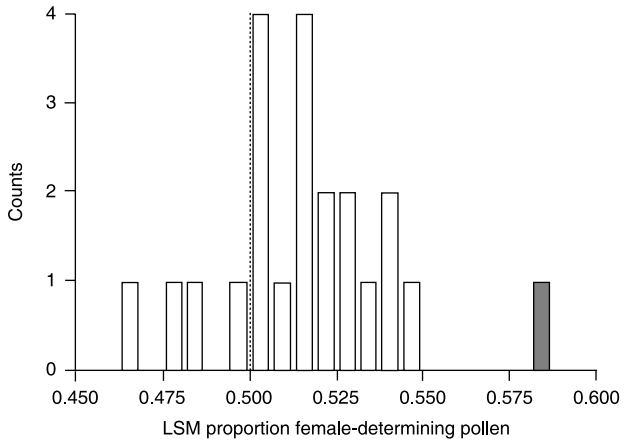

Fig. 5 The distribution of *Rumex nivalis* families containing male half-sib plants with different ratios of female- vs male-determining pollen. Values plotted are least-square means (LSMs) from analysis of variance (see Table 1). Family 436 is highlighted (filled bar) because it exhibited the most female-biased pollen sex ratio. The dashed line indicates unbiased pollen ratio.

was 0.586 ($n = 11$ plants; Fig. 5). When we repeated the analysis with this family excluded, the family effect was no longer significant ($P = 0.313$), although the overall mean across families (0.512) was still significantly higher than 0.5 ($t = 2.950$; $P = 0.008$). Tests for family effects assuming that secondary peaks were either polyploid or composed of generative nuclei also yielded significant family effects ($P < 0.008$ in each case). The mean of 0.513 (± 0.005 SE) using the assumption of polyploidy was significantly different from 0.50 ($t = 2.482$; $P = 0.022$), while the value for the assumption of generative nuclei of 0.510 (± 0.005 SE) was close to significant ($t = 1.998$; $P = 0.059$).

Discussion

Our investigation of the dioecious plant *R. nivalis* revealed that both female and male sporophytic tissue and female- and male-determining microgametophytes could be reliably distinguished using flow cytometry. This enabled us to determine whether bias was evident in the ratios of the two pollen types produced by male plants. Our earlier work on this species demonstrated female-biased sex ratios during various stages of the sporophytic life cycle (Stehlik & Barrett, 2005, 2006). We found that, on average, pollen production was significantly biased towards female-determining gametophytes, with males from 18 of 22 half-sib families producing more 'female' pollen. Additionally, we detected significant between-family variation in the ratios of female- and male-determining pollen, suggesting a genetic component to the production of biased pollen sex ratios.

Diagnosing sex using flow cytometry

Using flow cytometry, we were able to clearly distinguish female from male sporophytic nuclei in *R. nivalis* (Fig. 3b). This was undoubtedly because of the low ratio of autosomes to heterogametic sex chromosomes, the comparatively large size of the sex chromosomes, and the possession of two Y-chromosomes instead of one in males (Wagenitz, 1981; Navajas-Pérez *et al.*, 2005). These features of the genetic system of *R. nivalis* resulted in male nuclei containing 9.5% more DNA than female nuclei at the diploid level, with DNA contents of 6.74 pg/2C for females and 7.41 pg/2C for males. These values are comparable to flow cytometric estimates for the related *R. acetosa*, also in subgenus *Acetosa* (Błocka-Wandas *et al.*, in press), although the reported difference (7.0 pg/2C for females; 7.5 pg/2C for males) is smaller than for our species. The difference in DNA content between the sexes of *R. nivalis* is similar to an estimate from *Humulus japonicus* (9.8%; Grabowska-Joachimiak *et al.*, 2006), an unrelated dioecious species also with heteromorphic sex chromosomes. Doležel & Göhde (1995) also distinguished female and male nuclei peaks with differences of 3.9% and 4.5% in two *Melandrium* species. Therefore, gender assessment in species with sex chromosomes by flow cytometry can provide a reliable alternative to polymerase chain reaction (PCR)-based approaches using sex-specific sequence characterized amplified region (SCAR) markers (Stehlik & Blattner, 2004).

In addition to 2C nuclei, we also identified a high frequency of 4C and 8C nuclei in leaves of *R. nivalis*, indicating the occurrence of endopolyploid tissue in somatic cells (Fig. 3a; only 2C and 4C peaks shown). Cell polyploidization results from endoreduplication, which consists of one or more rounds of DNA synthesis in the absence of mitosis (Joubès & Chevalier, 2000). Endoreduplicated cells are commonly found in many tissues, especially those undergoing differentiation or expansion (Galbraith *et al.*, 1991), such as in our case with the leaves of *R. nivalis*. Thus, *R. nivalis* can be added to the growing list of plant species in which endoreduplication has been detected (Joubès & Chevalier, 2000). The occurrence of somatic endopolyploidization in *R. nivalis* posed no problem for distinguishing female and male plants (Fig. 3b), nor did it affect our ability to distinguish female from male microgametophytic nuclei.

In addition to diagnosing gender in sporophytes of *R. nivalis*, we also detected distinct fluorescence peaks for female- and male-determining microgametophytes (Fig. 1). In accord with our cytological observations of stained pollen grains, female- and male-determining pollen each produced one main fluorescence peak (Fig. 1). This was expected because all pollen grains we examined were trinucleate (Fig. 2) and all three nuclei are expected to be haploid and to have the same DNA content (Bino *et al.*, 1990). Using flow cytometry, Błocka-Wandas *et al.* (in press) were also able to distinguish female- from male-determining pollen in *R. acetosa*, indicating that this approach for 'sexing pollen grains' may have general applicability in *Rumex* species with the XX/XY_1Y_2 genetic system. However, the differences in DNA content in other angiosperm families with heteromorphic sex chromosomes may often be too small

for reliable diagnosis of sex differences between pollen grains (Parker, 1990; Ainsworth, 2000).

Flow cytometry studies of pollen nuclei, with few exceptions, usually identify small secondary peaks at fluorescence intensities corresponding to approximately twice the DNA content of the main peaks (reviewed in Suda *et al.*, 2007). Such secondary peaks are often interpreted, with varying degrees of support, as diploidized pollen nuclei resulting from a failure in reduction division of the chromosomes during meiosis (e.g. Bino *et al.*, 1990), or the postmeiotic failure of mitosis in pollen grains (Pan *et al.*, 2004; Błocka-Wandas *et al.*, in press). In our study, we detected three small secondary peaks (Fig. 1). Because the positions of these peaks correspond well to the predicted positions of the three possible doublet types, we interpret them as pollen aggregations of two female-determining, two male-determining, and one female-determining plus one male-determining pollen grain (2F, 2M, and FM, respectively; Fig. 1). This interpretation is further corroborated by the fact that when we used the finer 10-μm filter, which generally yielded stronger and clearer histograms, the smaller 2C peaks were substantially reduced in size. Finally, if substantial numbers of polyploid nuclei were present in addition to doublets, the first and third of these small peaks (2F and 2M in Fig. 1) should be larger than the central peak (FM, Fig. 1). However, we did not observe this pattern in our study. Clearly, caution is necessary when interpreting multiple peaks in histogram profiles of pollen using flow cytometry. Future work on 'pollen sex ratios' should pay particular attention to filter size because of the differences we observed between 10- and 30-μm filters. We emphasize, however, that interpreting these 2C peaks as either polyploid nuclei or undivided generative nuclei did not qualitatively affect our results.

Explanations for female-biased sex ratios

We detected a significant overall bias in the production of female- vs male-determining pollen in *R. nivalis* using flow cytometry. The degree of bias was sensitive to filter size and several assumptions that we made concerning the interpretation of peaks in our histograms. However, 18 of the 22 families that we examined produced pollen with a prevalence of female-determining microgametophytes (Fig. 5) and an ANOVA detecting between-family variation provided evidence of a genetic component to biased pollen sex ratios. We are therefore confident that our finding of female-biased pollen sex ratios in *R. nivalis*, although involving a relatively small numerical difference, represents a real biological phenomenon. Błocka-Wandas *et al.* (in press) also investigated pollen sex ratios using flow cytometry in an unspecified number of *R. acetosa* plants from a meadow in Poland. They also demonstrated female bias (0.55, a value slightly higher than we found in *R. nivalis*). These results are significant because of earlier reports of sex ratio bias in populations of *Rumex* species as these also involve female bias. These findings raise the question of what mechanism(s) might account for the pattern of sex ratio bias in male gametophytes.

During microgametophyte development in *R. nivalis*, diploid microspore mother cells located in anthers should contain the full complement of XY_1Y_2 sex chromosomes, as in all other somatic cells. At meiosis, it would be expected that microspore mother cells divide to form two classes of microspores containing either X or Y_1Y_2 sex chromosomes. As anther development proceeds, these microspores mature to become gametophytes and equal ratios of female- and male-determining pollen are generally expected, although this has never been verified directly.

Our results suggest that unknown mechanisms cause deviations from the expected 1 : 1 ratio of female- and male-determining pollen. The number of female-determining pollen could be inflated if meiotic disruptions generate microspores with single Y-chromosomes that are indistinguishable from those with X-chromosomes. However, cytological studies of *R. acetosa* provide no evidence for this phenomenon (Błocka-Wandas *et al.*, in press) and we consider this explanation unlikely. If such an effect were to occur, and if pollen with single Y-chromosomes were inviable, a smaller female bias would still be present, with meiotic disruption being one mechanism contributing to the loss of male pollen. Stehlik & Barrett (2005, 2006) review several additional hypotheses that might influence the quantity and quality of pollen in *Rumex* and affect sex ratio bias. These ideas, summarized in the following paragraph, are functionally associated with the particular type of chromosomal sex determination that occurs in *Rumex* section *Acetosa.*

Female-biased pollen sex ratios are most easily explained by the mortality of microspores or male-determining pollen during early gametophyte development. This could result from trivalent formation and nondisjunction of the sex chromosomes during meiosis as a result of the difference in chromosome numbers between the sexes. However, a study of *R. acetosa* provided no evidence of disturbance during meiosis (e.g. anaphase bridges or delayed chromosomes) and the presence of irregular nuclei or micronuclei was not observed (Błocka-Wandas *et al.*, in press). In their study, ~2% of pollen grains were considered inviable based on Alexander's test. *Rumex acetosa* and *R. nivalis* (both subgenus *Acetosa*) share the same XY_1Y_2 chromosomal system and similar female biases in sporophytic and gametophytic life stages (Zarzycki & Rychlewski, 1972; Rychlewski & Zarzycki, 1975, 1986; Błocka-Wandas *et al.*, in press). It is thus possible that similar mechanisms causing female bias operate in both species. Assuming that all inviable pollen is male-determining, the estimate of inviable pollen in *R. acetosa* is similar to our mean bias toward female-determining pollen. Future studies in *R. nivalis* are required to confirm that meiosis is regular and to establish levels of pollen viability. In particular, it would be important to determine if variation in pollen viability is correlated with the degree of female bias in families.

Another hypothesis that could explain sex ratio biases in *Rumex* species involves Y-chromosome degeneration and the accumulation of deleterious mutations (Smith, 1963; Lloyd,

1974b; Charlesworth, 2002; Stehlik & Barrett, 2005). Y-chromosomes in section *Acetosa* appear to be heterochromatic and there is some evidence for chromosome degeneration (Żuk, 1969; Negrutiu *et al.*, 2001; Vyskot & Hobza, 2004; Mosiolek *et al.*, 2005). These effects may be attributable to a lack of recombination and the accumulation of slightly deleterious mutations (Vyskot & Hobza, 2004). This degenerative process is likely to be particularly accelerated on Y-chromosomes because they are present in only one gender and, because of reduced effective population size, are more susceptible to random processes such as genetic drift, Muller's ratchet, the Hill–Robertson effect or genetic hitchhiking (Vyskot & Hobza, 2004). The detrimental effects of Y-chromosome degeneration should be most strongly expressed at the haploid stage and could lead to the abortion of male-determining microgametophytes and the observed bias towards female-determining pollen. In common with the previous explanation based on nondisjunction, evidence for irregular microspores or inviable pollen could provide supporting evidence for the Y-chromosome degeneration hypothesis. Whether the ~2% estimate of pollen inviability in *R. acetosa* (Błocka-Wandas *et al.*, in press) is associated with Y-chromosome degeneration is not known, but the value is not very different from the average degree of female bias that would occur with this amount of male-determining pollen death.

Another mechanism that could potentially cause biased ratios of female vs male-determining pollen involves a system of sex ratio distorters and restorers of the type reported in dioecious *Silene latifolia* (Taylor, 1994, 1999). This species shares several similarities with *Rumex*, including female-biased sex ratios and heteromorphic sex chromosomes, in which females are homogametic and males are heterogametic (Westergaard, 1958). However, the mechanisms responsible for sex ratio bias in these taxa appear to be quite different, particularly with respect to the relative roles of genetics and ecology. Therefore we doubt that the biases reported in *Rumex* species have a similar mechanistic basis.

In *S. latifolia*, female bias is caused by segregation distortion during microgametophyte development in one class of males termed 'driving males' (Taylor, 1994, 1999; Taylor & Ingvarsson, 2003). Pollen production in driving males diverges strongly from an expected unbiased female- to male-determining ratio as driving males produce little to no male-determining pollen. Sporophytic sex ratios of seeds and adults in *S. latifolia* depend solely on the bias established by the pollen of driving males involved at fertilization, with no indication of environmentally induced sex-biased mortality among adults (Taylor, 1994, 1999). By contrast, *R. nivalis* and several other species of *Rumex* section *Acetosa* exhibit different seed and adult sex ratios (*R. acetosa*, *Rumex hastatulus* and *R. thyrsiflorus*; Żuk, 1963; Putwain & Harper, 1972; Zarzycki & Rychlewski, 1972; Rychlewski & Zarzycki, 1975; Conn & Blum, 1981; Korpelainen, 1991). For example, in *R. nivalis*, the degree of bias increases progressively from the gametophytic to the sporophytic generation, and from seed to flowering as a result of gender-based differences in mortality (Fig. 6). Moreover, experimental studies clearly indicate that the amount of pollen captured by stigmas influences the degree of female bias in seeds (Stehlik & Barrett, 2006), indicating another way in which environmental factors play a role in determining sex ratios.

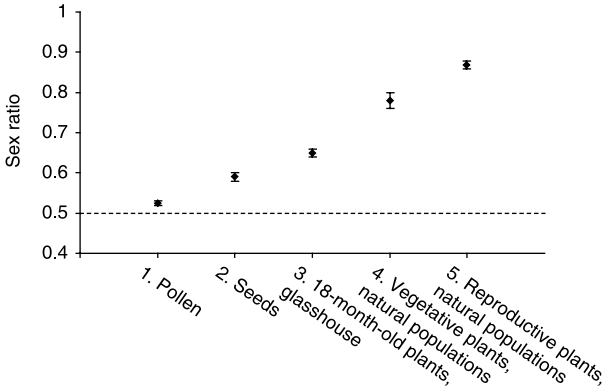

Fig. 6 Sex ratio dynamics during the life cycle of *Rumex nivalis*. Values plotted are the mean proportion of female-determining pollen and the sex ratio at different life-history stages (with standard errors). (1) pollen (this study); (2) open-pollinated seeds from the field; (3) 18-month-old plants in the glasshouse; (4) vegetative plants in the field; (5) flowering plants in the field. (2)–(5) are from Stehlik & Barrett (2005). Sex ratios (4) and (5) were assessed in 18 natural populations from Switzerland. Ratios (2) and (3) are from seed and from an adult glasshouse population grown from seed collected from the same populations. The pollen ratio (1) is from the second offspring generation (open-pollinated) of (3). The dashed line indicates an unbiased ratio.

Changes in sex ratio during the life cycle and the role of pollination intensity in determining seed sex ratios suggest a strong ecological component to sex ratio-variation in *Rumex* species of section *Acetosa*. This pattern would not be predicted if a system of genetic sex ratio distorters were involved. Different mechanisms involved in sex ratio bias in *Silene* and *Rumex* may reflect the independent origins and ages of sex chromosomes in these two groups. Sex chromosomes in *Rumex* section *Acetosa* are considered to be the most evolutionary advanced and also the most degenerate among the 11 flowering plant families known to possess sex chromosomes (Matsunaga & Kawano, 2001; Vyskot & Hobza, 2004). There are two distinct systems of sex determination in *Rumex* (XX/XY and XX/XY_1Y_2) and phylogenetic evidence clearly indicates that the *Acetosa* type is derived (Navajas-Pérez *et al.*, 2005). It would therefore be valuable to compare the patterns of sex ratio bias in sporophytes and gametophytes of *Rumex* species with different sex determination systems to determine if the degree of sex-chromosome differentiation is the key factor in causing female bias.

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