**A sex-chromosome mutation in *Silene latifolia***

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Abstract  *Silene latifolia* is dioecious, yet rare hermaphrodites have been found, and such natural mutants can provide valuable insight into genetic mechanisms. Here, we describe a hermaphrodite-inducing mutation that is almost certainly localized to the gynoecium-suppression region of the Y chromosome in *S. latifolia*. The mutant Y chromosome was passed through the megaspore, and the presence of two X chromosomes was not necessary for seed development in the parent. This result supports a lack of degeneration of the Y chromosome in *S. latifolia*, consistent with the relatively recent formation of the sex chromosomes in this species. When crossed to wild-type plants, hermaphrodites performed poorly as females, producing low seed numbers. When hermaphrodites were pollen donors, the sex ratio of offspring they produced through crosses was biased towards females. This suggests that hermaphroditic *S. latifolia* would fail to thrive and potentially explains the rarity of hermaphrodites in natural populations of *S. latifolia*. These results indicate that the Y chromosome in *Silene latifolia* remains very similar to the X, perhaps mostly differing in the primary sex determination regions.

Keywords  Chromosomes • Dioecious • Hermaphroditic • *Melandrium* • Sex chromosomes • *Silene latifolia*

Introduction

Most extant angiosperms have hermaphroditic flowers, and this is almost certainly the ancestral state for flowering plants (Ainsworth et al. 1998; Tanurdzic and Banks 2004). Dioecy, production of unisexual flowers on separate plants, is uncommon, though it has apparently evolved repeatedly. Renner and Ricklefs (1995) surveyed 240,000 species and found that ~6% were dioecious; Cronquist (1988) found that 43% of angiosperm families include at least one dioecious species. Evolutionary models suggest that X and Y sex chromosomes evolve from homologous autosomes (Charlesworth 1978, 1991). Only a small subset of dioecious lineages has identifiable sex chromosomes (Grant et al. 1994; Ainsworth et al. 1998). Among them are species in the genus *Silene*, section Elisanthe (Caryophyllaceae), whose well-developed sex chromosomes have been studied throughout the 20th century (for example, Winge 1931; Warmke and Blakeslee 1939; Westergaard 1940). *Silene latifolia* Port (=*Melandrium album* (Miller) Garcke = *Lychnis alba* Miller also *Silene alba*) is diploid with 2N = 24 chromosomes; XY males and XX females. Based on morphological and genetic data, phylogenies show that sex chromosomes in *Silene* evolved relatively recently (Desfeux and Lejeune 1996; Zhang et al. 1998), with molecular clock estimates at ~20 MYA (Charlesworth 2002). The Y chromosome plays a key role in sex determination in *S. latifolia*, and three sex-determining regions have been identified on the Y: the female suppressor region (Westergaard 1946; Lardon et al. 1999), an early stamen development region (Farbos et al. 1999), and a late stamen development region (Westergaard 1946).

Treatment with 5-azacytidine (Janousek et al. 1998) induced androhermaphroditism in *S. latifolia* through heritable hypomethylation (Janousek et al. 1996), and these
plants were crossed with x-ray-induced hermaphrodites to show that their Y chromosome was not transmitted through the female line (Janousek et al. 1998). This was interpreted as the result of either absence of an X chromosome resulting in failure of embryo sac formation or an imbalance of X/Y chromosomes in developing endosperm (Janousek et al. 1998). Similarly, Lardon et al. (1999) created bisexual mutants in S. latifolia via $^{60}$Co pollen irradiation and identified two regions with female suppression function, one located on the Y chromosome and one on an autosome.

Chemical treatment or irradiation mutagenesis, despite its great value in genetic studies, is likely to produce widespread changes in plant genomes (e.g. Batista et al. 2008) and can complicate interpretation of results, making studies of natural variation important (Alonso-Blancoa and Koornneef 2000). Natural mutations causing reversion to hermaphroditism in S. latifolia are rare, but can occur (van Nigtevecht 1966; Desfeux and Lejeune 1996). Here, we investigate the genetic basis of rare hermaphroditic individuals with perfect flowers found in natural populations. We described the effects of the trait on flower morphology and addressed the following questions: (1) what is the genetic nature of the mutation causing hermaphroditism and (2) can the mutated Y chromosome pass through the megaspore? We addressed these questions using a series of crosses with the hermaphroditic individuals as either the female (seed plant) or the male (pollen donor) parent. Y-specific molecular markers were used to test the hypothesis that the Y chromosome was present in hermaphrodites. We used sex ratio data from the crosses to test whether the reversion to hermaphroditism was caused by a mutation in the gynoecium-suppression region on (a) an autosome or (b) the Y chromosome (Lardon et al. 1999). To test the hypothesis that the Y chromosome passed through the megaspore, we used sex ratio data combined with molecular markers to rule out the possibility of self-fertilization.

Materials and methods

A mutant individual, designated 17W, that produced hermaphroditic flowers with functional male and female parts was discovered in seed grown from a wild accession of S. latifolia collected near the University of Massachusetts, Boston campus. These seeds produced offspring that were hermaphrodites with all perfect flowers and androhermaphrodites possessing both perfect and male flowers. The majority of flowers on the original plant and its subsequent progeny were hermaphroditic with functional male and female parts. This population was designated H300 and used to generate additional selfed and sib-mated lines, as well as F1 populations with plants from typical dioecious populations of S. latifolia, originating from Massachusetts, Ohio, and Italy. Additional crosses were made using the AH plants as females (seed plants) and wild-type male S. latifolia as pollen donors. Plants produced from these crosses were given population numbers H500 to H511. In all crosses, flowers were covered before opening to prevent any pollen contamination and kept covered until the seed had set and begun to mature.

All plants were inspected over at least two flowering seasons. Almost all flowers were inspected over a minimum of 2 months during each flowering season. Stamens, if any, were counted on each flower.

DNA was extracted from young plant leaves following a procedure adapted from Bernatzky and Tanksley (1986). Y-chromosome screening was performed using seven Y-chromosome-specific sequence-characterized amplified region (SCAR) primers (Zhang et al. 1998). These primers amplify bands from unique sequences on the Y chromosomes of S. latifolia and S. dioica. All SCAR marker primers and protocols were previously described in Zhang et al. (1998). A comparison of the X-chromosome SLX-1 gene sequence (Filatov et al. 2000) was used to differentiate between the parental X chromosomes and test whether outcrossing had occurred in hermaphrodite offspring. SLX-primers and protocols were used as indicated in Filatov et al. (2000). The TempliPhi method (Nelson et al. 2002), by which cloned DNA in circular vectors undergoes “rolling circle amplification” using $\Phi 29$ DNA polymerase, was used to obtain template DNA for sequencing the SLX-1 gene from the parents and several offspring of the H507 population. A high annealing-temperature technique for performing random amplification of polymorphic DNA (RAPD) (Atienzar et al. 2000, 2002) was used to test for outcrossing in the H500 populations.

Results

Characterization of hermaphrodite and androhermaphrodite plants

Germination of self-pollinated seed from the original androhermaphrodite, 17W, was low but produced 15 hermaphrodites (H), 5 androhermaphrodites (AH), and 17 females (F). This population was designated H300, and all H and AH plants carried the Y chromosome (designated Y$^{m}$), as shown by the presence of all 7 SCAR markers. The 17 females from this population did not possess any of the SCAR markers.

Over two flowering seasons, hermaphrodites continued to produce only perfect flowers (one H plant died after one flowering season), and AH plants continued to produce
some staminate flowers. Number of styles on perfect flowers of H300 varied from 1 to 5, and plants that had some staminate flowers had lower mean style numbers than hermaphrodites (Fig. 1, Table 1), but statistical evidence for this difference was equivocal (ANOVA, \( P = 0.08 \)). Style number on perfect flowers was more than twice as variable in AH plants (range = 1–5 styles, coefficient of variation \( CV = 70.3\% \)), compared with hermaphrodites (range = 1–5, \( CV = 33.3\% \)).

Hermaphrodites had low seed production in self-crosses and outcrosses. The average number of seeds produced per flower in H self or HxH sib matings was 49.97 (SE = 4.13, \( n = 22 \)). In H crosses to wild-type males the average was higher, 85.75 (SE = 11.17, \( n = 15 \)). In crosses with H as pollen donor crossed to wild-type female flowers, however, mean seed production was 384.3 (SE = 18.6, \( n = 15 \)).

Transmission of the hermaphroditic trait

Plants were grown from 6 self-crosses and one sibling cross, carried out with H and AH plants (Table 2). One male plant is indicated in the total; however, this plant was unhealthy, produced few flowers before dying, and was excluded from the segregation analyses. We attempted to emasculate the perfect flowers (>70 flowers) before pollination, but this triggered flower dehiscence without seed set. One percent auxin and lanolin cream applied to the flower stem after emasculation did not prevent dehiscence (van Doorn and Stead 1997). These crosses were then completed using buds on which the pollen had not matured. All crosses produced seed that did not germinate well, and offspring data were pooled for the sex ratio analysis (Table 2). If the \( Y^m \) chromosome was transmitted through the ovary in the \( XY^m \times XY^m \) cross and \( Y^mY^m \) individuals were viable and male/hermaphrodite, a hermaphrodite to female ratio of 3:1 would be expected. If \( Y^mY^m \) individuals were not viable, a 2:1 ratio would be expected. The offspring produced did not fit either of these ratios \( (P < 0.001, \text{Table 3}) \). The hermaphroditic (H and AH phenotypes combined) and female morphs segregated in a 1:1 ratio \( (\chi^2 = 0.093, P = 0.76) \), which is the expected ratio if the Y was not transmitted through the ovule. The expected 2:1 and 3:1 ratios for transmission of the Y chromosome through both the female and the male germ lines, however, assume that individuals bearing the mutant Y are as viable as those that do not. If they are less viable, the ratios may approach the 1:1 ratio of the alternative hypothesis in which the Y is not transmitted through the female germ line. This alternative was explored through further testing described below.

**Table 1** Style counts for AH population

| Individual plant # | # Perfect | # Male | Total styles | Average |
|---------------------|-----------|--------|--------------|---------|
| H300 #3             | 33        | 3      | 148          | 4.11    |
| H300 #6             | 9         | 0      | 45           | 5       |
| H300 #4             | 18        | 0      | 90           | 5       |
| H300 #10            | 10        | 1      | 26           | 2.36    |
| H300 #11            | 11        | 1      | 49           | 4.08    |
| H301 #6             | 6         | 4      | 16           | 1.6     |
| H301 #9             | 10        | 1      | 21           | 1.9     |
| H301 #11            | 14        | 7      | 70           | 3.3     |
| H301 #12            | 21        | 0      | 35           | 1.67    |
| H301 #13            | 6         | 4      | 9            | 0.9     |
| H301 #14            | 14        | 0      | 45           | 3.2     |

**Table 2** Plants grown from original seed and inbred crosses

| Population # | H | AH | F | M | Parent(s) |
|--------------|---|----|---|---|-----------|
| W-H300       | 15| 5  | 17| 0 | 17W       |
| X-H301       | 6 | 2  | 8 | 0 | H300 x AH300 |
| O-H302       | 6 | 1  | 8 | 0 | H300      |
| O-H303       | 0 | 2  | 3 | 1 | AH301     |
| O-H304       | 1 | 3  | 4 | 0 | H300      |
| O-H305       | 2 | 0  | 1 | 0 | H302      |
| O-H306       | 4 | 0  | 3 | 0 | H300      |
| O-H307       | 3 | 0  | 3 | 0 | H300      |
| Total        | 37| 13 | 47| 1 | H + AH = 50 |

Sex of plants is abbreviated as H Hermaphrodite, AH Androhermaphrodite, F Female, M Male

* Seed source W Original from wild collected plant, X Cross of two original H plants, O Self of H plant

Fig. 1 *Silene latifolia* mutant hermaphrodite male flower with part of the calyx and petals removed to reveal the gynoecium with 2 styles and the stamen
Crosses between wild-type female (XX) and H300 AH (XY<sup>hm</sup>) plants as the male parent produced androhermaphrodite and female offspring (12AH and 22 females). Two of the F1 AH plants produced were subsequently crossed to wild-type females again. Fifty seeds from these crosses were planted and produced 10AH, 4 males, and 15 females. Crosses between hermaphrodites (XY<sup>hm</sup>) as the female parent and wild-type males (XY) produced populations designated H500 to H514, which segregated for hermaphrodites, females, and males (Table 4). H and AH offspring were produced from these crosses, and the pooled sex ratio was 24:39:24 AH?H:F:M. This sex ratio was consistent with the hypothesis that the H or AH parent passed the mutant Y<sup>hm</sup> through the ovule ($\chi^2 = 5.172$, $P = 0.08$).

To confirm Y<sup>hm</sup> transmission through the ovule, and rule out the possibility that H/AH plants from the above cross were actually self-fertilized, selected H500 plants were screened using RAPD markers. Comparisons of band patterns were made between them and the wild-type male parent to attempt to confirm outcrossing. In two populations whose band patterns supported outcrossing, further testing was performed using sequence data comparing the X-chromosome SLX-1 gene sequence (Filatov et al. 2000). In the H507 population, the H and the wild-type male used as parents for this cross revealed several indels and nucleotide differences that could be used to differentiate between the chromosomes and confirm outcrossing had occurred in the hermaphrodite offspring. The majority of the differences were found at the 3' and 5' ends of the ~2,300 bp sequence, so these regions (~600 bp each) were used for comparison. Four of the possible eight F1 progeny were selected for testing. Three of these had the wild-type male parent SLX-1 sequence, indicating they were produced through outcrossing (Genbank accession numbers: HM183079, HM183080, HM183081, HM183082, HM183083).

Discussion

The Y chromosome was present in the H and AH plants, as confirmed by Y-linked SCARs, and is the most likely site for the mutation that has caused sex reversion. The mutant Y chromosome can be transmitted through the pollen and the ovule of H and AH plants. When self-pollinated, or used as a pollen donor in crosses with wild-type female <i>S. latifolia</i>, only AH and female offspring were produced. There is at least one autosomal mutation in <i>S. latifolia</i> (Janousek et al. 1996, 1998), and in <i>S. dioica</i> (<i>Melandrium dioicum</i>, van Nigtevecht 1966), a close relative of <i>S. latifolia</i>, which could cause reversion to hermaphroditism (see also Lardon et al. 1999). A mutation caused by an autosomal dominant would be expected to segregate independently from the Y chromosome (Janousek et al. 1998): a self-fertilized hermaphrodite plant heterozygous for this autosomal mutation should segregate 50% hermaphrodites, 33% females, and 17% males assuming the Y chromosome is passed through the ovule and YY individuals do not survive. If the Y chromosome did not pass through the ovule, the offspring would be 37.5% hermaphrodite, 50% female, and 12.5% male. This was not the case in our mutant lineage, where self-pollination did not produce male offspring (Table 1). An autosomal recessive would only produce wild-type offspring in an outcross, whether it

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**Table 3** Sex ratio test of H300 population plants from original seed and inbred crosses

|       | Observed | Expected (1:3)<sup>a</sup> | Expected (1:2)<sup>b</sup> | Expected (1:1)<sup>c</sup> |
|-------|----------|-----------------------------|-----------------------------|-----------------------------|
| Female| 47       | 24.25                       | 32.33                       | 48.5                        |
| Hermaphrodite| 50    | 72.75                       | 64.66                       | 48.5                        |

Chi-square $P = 9.6 \times 10^{-8}$, $P = 0.0016$, $P = 0.761$

<sup>a</sup> Test for Y<sup>hm</sup> passage through ovule and Y<sup>hm</sup>Y viable

<sup>b</sup> Test for Y<sup>hm</sup> passage through ovule and Y<sup>hm</sup>Y lethal

<sup>c</sup> Test for normal sex ratio

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**Table 4** Results of outcrosses between androhermaphrodites (AH), as seed plants, and wild-type male <i>S. latifolia</i>

| AH parent | Male parent | Population # | H | AH | F | M |
|-----------|-------------|--------------|----|----|---|---|
| H300H #1  | B31         | H500         | 1  | 0  | 0 | 0 |
| H300H #1  | B72         | H501         | 0  | 0  | 1 | 1 |
| H300AH #1 | B31         | H502         | 1  | 0  | 0 | 0 |
| H300H #6  | B31         | H503         | 6  | 0  | 6 | 1 |
| H300H #6  | O26         | H504         | 1  | 0  | 2 | 0 |
| H300H #6  | O56         | H505         | 3  | 0  | 4 | 1 |
| H302H #2  | B31         | H506         | 1  | 0  | 3 | 1 |
| H302H #2  | Amy5        | H507         | 8  | 0  | 15| 14|
| H300H #6  | Amy5        | H508         | 0  | 2  | 3 | 0 |
| H302H #2  | B23         | H509         | 0  | 0  | 2 | 2 |
| H300H #7  | Amy5        | H511         | 1  | 0  | 3 | 4 |
| Total     |             |              | 22 | 2  | 39| 24|

$H + AH = 24$

Sex of offspring produced is abbreviated as AH Androhermaphrodite, H Hermaphrodite, F Female, M Male offspring
was used as the seed plant or pollen donor. This also did not occur in our cross results (Table 4), further supporting linkage of the mutation to the Y chromosome.

Westergaard (1940, 1946, 1958) suggested that there were three regions on the Y chromosome that were important for sex determination in *S. latifolia*: gynoecium suppression, stamen initiation, and stamen maturation, and recent research has continued to support this general view (e.g. Farbos et al. 1999; Lardon et al. 1999; Lebel-Hardenack et al. 2002). A mutation in the gynoecium-suppression region is the most parsimonious explanation for the hermaphroditism seen here. The mutant Y chromosome in the H300 population, in which some plants had up to 50% staminate flowers, apparently had a semifunctional female suppression region (Table 1). There was also variation in the expression of the mutation, as the hermaphroditic flowers had from one to five styles instead of the consistent 5 styles seen in typical females. There was no pattern to the placement of the staminate flowers versus hermaphroditic flowers on the inflorescence, as was noted by Janousek et al. (1996) who found that bisexual flowers increased in the later branches that developed on the inflorescence. Lardon et al. (1999) found that decreases in carpel number were correlated with the frequency of staminate flowers in hermaphrodites. Our data only marginally support this finding (ANOVA, $P = 0.08$), but a higher coefficient of variation (70.3%) for style number was associated with plants with high frequencies of staminate flowers.

Janousek et al. (1998) found that in mutant lineages created with 5-azacytidine, the Y chromosome was not transmitted through the AH when used as a seed plant. Lardon et al. (1999) stated that their radiation-mutated Y chromosomes did pass through the seed plant, but this finding was not confirmed with genetic markers, and self-pollination could occur prior to flower opening in perfect flowers of *Silene* (Davis and Delph 2005). Janousek et al. (1998) speculated that lack of Y transmission through the ovule could be due to absence of an X chromosome resulting in failure of embryo sac formation, or an imbalance of X/Y chromosomes in developing endosperm. In our natural AH population, SLX-1 sequence data confirmed the finding that a mutant Y chromosome can pass through the seed parent. This suggests that if genes on the X are responsible for embryo sac formation, they are sufficiently conserved on the Y to remain functional. Furthermore, X/Y imbalance apparently does not play a role in endosperm formation. It may be that the chemical mutagenesis used by Janousek et al. (1998) caused changes in the X chromosome that precluded normal seed formation.

In the H507 population (Table 4), at least three of the eight H offspring had the X chromosome of the wild-type parent. If the mutant Y passed through the ovule, it should have altered the sex ratio in self-crosses (Table 3) and outcrosses to wild-type males (Table 4). We did not find this to be true, nor did Lardon et al. (1999). There is often a sex ratio bias to female in *S. latifolia*, which may have affected this result (van Nigtevecht 1966; Mulcahy 1967; Lyons et al. 1994, 1995; Taylor 1994a, b, 1996; Taylor et al. 1999). In addition, if seeds or plants produced by Ym passing through the ovule are less viable, the ratios would not reflect this mode of transmission.

The seed set per flower for the H and AH plants after self-pollination averaged only 57.4, which may have been the result of inbreeding depression. Outcrossing has been suggested as one of the positive selective forces leading to evolution of dioecy, as self-pollination may lead to homozygosity and exposure of recessive lethal mutations (Lloyd and Gregg 1975; Charlesworth 1978; Maynard Smith 1978; Charnov 1982). Hermaphrodites also had sharply reduced seed number when outcrossed to wild-type *S. latifolia* males, averaging only 85.75 seeds per flower, indicating that inbreeding is not the sole reason for low seed set in these individuals. When H and AH plants were used as pollen donors with wild-type female *S. latifolia*, however, the females produced >3× more seed per flower (mean = 384.25). Westergaard (1946) found that average seed production in crosses of wild-type *S. latifolia* was 451 seeds/flower, and Purrington (1993) got an average as high as 368 seeds/flower in studies with varying nutrient regimens. Our H and AH plants fall within this range for seed production when used as pollen donors, suggesting that they functioned normally as male plants, and further indicating that the Y-chromosome mutation was isolated to the gynoecium-suppression region. The F1 offspring from the crosses with wild-type females, however, had a strong sex ratio bias with 22 female and 12 H and AH offspring. Sex allocation theory suggests that trade-offs occur between the sexual functions when a plant is hermaphrodite, as resources for reproduction are limited (Charnov 1982; Campbell 2000). Such a trade-off may apply to our hermaphrodites, as they function much better as male plants, and may be allocating more energy to pollen production. As they are the result of a mutation, however, it is probable that the female function is being affected by more than resource allocation. These hermaphrodites would likely be unsuccessful in competition with wild-type *S. latifolia* males, as they do produce more female offspring than expected whether they act as pollen donors or seed plants. Other rare bisexual mutants have also been found to be less successful in either one or both of the functional gender roles (Rottenberg 2000). These results may explain why *S. latifolia* hermaphroditic mutants are only rarely detected in natural populations (van Nigtevecht 1966; Desfeux and Lejeune 1996), as they would be unlikely to invade and displace a dioecious population.
Our results support the notion of recent derivation of sex chromosomes in *Silene latifolia* and consequently little degeneration of the Y chromosome in this species. This suggests that the mutated Y chromosome was relatively intact, and not, for example, missing large regions, as was the case with mutated Y chromosomes that arose in polyploids studied by Westergaard (1946, 1958). If that were the case, some of the SCAR markers would likely have been absent, and important genes missing, preventing pollen that bore the mutation from functioning. Although some incipient degeneracy is apparent on the Y chromosome of *S. latifolia* (reviewed by Bernasconi et al. 2009), comparisons of sequences of several pairs of homologous loci found on the X and Y chromosomes in *S. latifolia* have found differing levels of divergence, and overall relatively little differentiation, with retention of functionality (Filatov et al. 2000; Atanassov et al. 2001; Marais et al. 2008). Taken together, these results support the view that degeneration of the Y chromosome in *S. latifolia* is in the early stages and that this species is fertile ground for further research on sex-chromosome evolution.

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