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ORIGINAL ARTICLE

A new plant sex-linked gene with high sequence diversity and possible introgression of the X copy

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We describe patterns of DNA sequence diversity in a newly identified sex-linked gene, *SlX9/SlY9*, in *Silene latifolia* (Caryophyllaceae). The copies on both sex chromosomes seem to be functional, and each maps close to the respective X- and Y-linked copy of another sex-linked gene pair, *SlCypX/SlCypY*. The Y-linked copy has low diversity, similar to what has been found for several other Y-linked genes in *S. latifolia*, and consistent with the theoretical expectations of hitch-hiking processes occurring on a non-recombining chromosome. However, *SlX9* has higher diversity than other genes on the *S. latifolia* X chromosome. We evaluate the hypothesis of introgression from the closely related species *S. dioica* as an explanation for the high sequence diversity observed.
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Keywords: *Silene latifolia*; sex chromosomes; introgression; *SlX9*

Introduction

Measurements of neutral nucleotide diversity have been used to infer that Y chromosomes in some species, including neo-Y chromosomes are undergoing hitch-hiking processes that are expected to cause genetic degeneration in these non-recombining genome regions. In contrast to the situation for autosomal and X-linked loci, the non-recombining region of a Y chromosome is expected to experience selective interference effects among linked loci, such as weak selection Hill–Robertson interference (Hill and Robertson, 1966; McVean and Charlesworth, 2000; Comeron, 2008), genetic hitch-hiking because of positive selection (Maynard Smith and Haigh, 1974; Kaplan *et al*., 1989) or the elimination of strongly deleterious variants (background selection and Muller's ratchet (Muller, 1964; Charlesworth *et al*., 1993; Gordo *et al*., 2002)). These effects will reduce diversity values compared with those of genes on the X or autosomes, in addition to the expected reduction in diversity because of the smaller Y effective population size caused by the smaller number of Y chromosomes in the population.

Silene latifolia is a dioecious plant used to study the evolution of young sex chromosomes. Synonymous site diversity values of six X-linked genes studied (*SlX1*, *SlX4*, *DD44-X*, *SlssX*, *SlCyt* and *SlCyp*) vary between 0.07 and 5.1% (Atanassov *et al*., 2001; Laporte *et al*., 2005; Bergero *et al*., 2008; Kaiser *et al*., 2009); the lowest value (for *SlssX*) may be due to a recent selective sweep in the genomic region, which did not affect diversity at the nearby *DD44-X* (Filatov, 2008). In contrast, four Y-linked genes studied (*SlY1*, *SlY4*, *DD44-Y* and *SLAP3Y*) have silent diversity values between 0 and 0.28% (Atanassov *et al*., 2001; Matsunaga *et al*., 2003; Laporte *et al*., 2005), consistent with the predicted reduced Y chromosome N_e.

Interpretation of within-species polymorphism in *S. latifolia*, and comparison of X and Y diversity values, are, however, complicated by the possibility of introgression from its closely related sister species *Silene dioica*, which could increase diversity for some loci. *S. latifolia* forms natural hybrids with *S. dioica* (Desfeux *et al*., 1996; Minder *et al*., 2007; Minder and Widmer, 2008), although the species differ ecologically (Baker, 1947, 1948), with *S. latifolia* having white flowers, a generally wider distribution and growing in dry, open habitats, whereas *S. dioica* has red flowers and grows at the margins of woodlands. The pollinators also differ, with *S. latifolia* being mainly visited by the dusk-flying moth *Hadena bicruris*, and *S. dioica* visited during the day by bumblebees and butterflies (Bopp and Gottsberger, 2004; Minder *et al*., 2007). There are also some geographic distribution differences, with *S. dioica* being found mainly in Northern Europe (Baker, 1947, 1948; Karrenberg and Favre, 2008). However, hybrids with pink flowers are common in the wild, and data from three sex-linked genes, *DD44*, *SlX1* and *SlX4* (Ironside and Filatov, 2005; Laporte *et al*., 2005), as well as AFLP markers (Minder *et al*., 2007; Karrenberg and Favre, 2008; Minder and Widmer, 2008), suggest that introgression of *S. dioica* sequences into *S. latifolia* is common in nature.

To understand the early stages of sex chromosome evolution and specifically to test for Y chromosome degeneration and the effects of different chromosomal environments on nucleotide diversity, we have isolated genes from the *S. latifolia* X and Y chromosomes. In this study, we describe a newly identified sex-linked gene pair in *S. latifolia*, *SlX9/SlY9*, and analyze its diversity levels, which suggest Y chromosome degeneration and X chromosome introgression.

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Materials and methods

Plant materials

Sex-linkage of *SlX9* in *S. latifolia* was established using the mapping family H2005-1 (Bergero *et al*., 2007), which is a full-sib cross between F1 offspring whose parents came from different European populations (male E2004-17-1, from the Netherlands, and female E2004-11-1, from Canche, Northern France). Ninety-two plants from this family were used to map its location on the X chromosome. The mother of the mapping family is a heterozygote for two X-linked alleles that produced PCR products of different lengths (bands of approximately 450 and 600 bp, see Supplementary Figure 1), which were used for genetic mapping, as described below. The panel of 38 deletion mutants used to find the location of *SlY9* on the Y chromosome is described in Bergero *et al*. (2008).

To study sequence diversity, we used a sample of 46 *S. latifolia* males from 24 European populations, covering most of the range of the species. Nineteen *S. dioica* individuals were also sampled, from Scotland, France and Finland, including 13 males (Supplementary Tables 1 and 2).

PCR amplifications and primers

SlX9 was identified from a *S. latifolia* complementary DNA (cDNA) library derived from male leaf primordia, and shown to be a sex-linked gene (Kaiser *et al*., 2009). The complete cDNA sequence was obtained, and primers were designed based on this sequence. As described below, it proved very difficult to sequence this gene in its entirety from all our sampled individuals, and only partial genomic sequences were obtained, with different regions sequenced from different species, and from the X and Y copies. To obtain sequences, new primers were designed from the sequences yielded by the initial primers; these are listed in Supplementary Table 3. Figure 1 (below) diagrams the regions amplified for the diversity study, which differ for the X- and Y-linked alleles as follows. For *SlX9*, the sequences include part of intron 1, exon 2, intron 2, exon3, and part of intron 3 (totalling 255 coding and 178 non-coding sites). For *SlY9*, they include part of intron 2, exon 3, intron 3 and part of intron 4 (270 coding and 222 non-coding sites).

PCR amplification was generally carried out using Taq JumpStart (Sigma-Aldrich, Poole, UK), and the following conditions: initial denaturation at 95 °C for 5 min, 10 cycles of denaturation at 95 °C (30 s), annealing at 55–58 °C (30 s), extension at 72 °C (1–1.5 min), final extension at 72 °C for 15 min. PCR amplicons were cleaned using ExoSAP-IT (GE Healthcare, Little Chalfont, UK) and sequenced on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA, USA) and sequences edited using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA).

The two X-linked alleles in the mapping family (see above) were cloned from PCR products and sequenced. Primers used to amplify across introns 1, 2 and 3 are listed in Supplementary Table 3. The gene structure of *SlX9* was then inferred by comparing the *SlX9* genomic sequence with its cDNA sequence, as well as by comparisons with *Arabidopsis thaliana* and *Silene vulgaris* gene structures. A BLASTN search was performed at www.ncbi.nlm.nih.gov to identify homologous genes in other organisms and their functions.

Obtaining and mapping the Y-linked homologue

As described in Kaiser *et al*. (2009), using primers RB18_F and RB18_R (Supplementary Table 3) to amplify DNA from male and female plants from family H2005-1, yielded a male-specific PCR product of approximately 1.2 kb. To sequence the Y-linked homologue of *SlX9*, the longer, male-specific allele (see Supplementary Figure 1) was cut from the agarose gel, cloned and sequenced. To obtain the 5′ coding sequence of *SlY9*, which was not present in the region initially sequenced, the cloned PCR products from family H2005-1 were also used to design new, Y-specific primers from this sequence (Supplementary Table 3). These primers were also used for the *SlY9* diversity study (see below).

To test whether the Y-linked gene was expressed, we did nested PCR using cDNA derived from male flower tissue. The first round of PCR amplification used the primers TsShort and RB18_R (Supplementary Table 1), in which TsShort matched the sequence to which the cDNA was ligated. The second round used TsShort and the Y-specific primer RB18_Y_E3-R (Supplementary Table 3).

For deletion mapping *SlY9* on the Y chromosome, PCR amplifications were scored in the deletion mutants

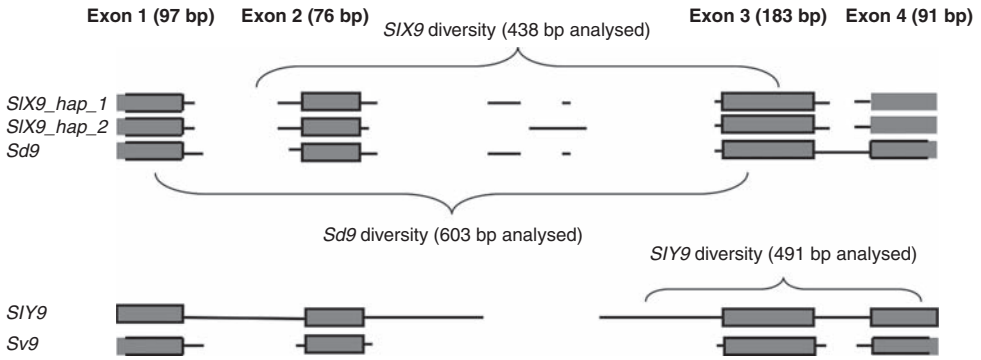

Figure 1 Schematic view of the alignment of *SlX9*, *SdX9*, *SlY9* and *Sv9*. Sizes of introns and exons are not drawn to scale. Both X-linked intronic variants are shown (*SlX9*_hap_1 and *SlX9*_hap_2). Thick lines around exons: sequences available for at least one individual. (The original *S. latifolia* cDNA clone from which the gene was identified contained the whole open reading frame; sizes of exons for which we do not have complete sequences are drawn based on the assumption that exon sizes are the same as for the cDNA.) Note: sequences used in the diversity studies of *SlX9*, *SdX9* and *SlY9* only cover parts of the gene sequences, as indicated.

(see above) using the primers RB18_F and RB18R (Supplementary Table 3). The location of *SlY9* was inferred by comparing the presence–absence of the Y-linked, larger, fragment to the presence–absence of other Y-linked genes (Zluvova *et al*., 2005, 2007; Bergero *et al*., 2008).

Diversity of *SlX9* and *SlY9* and linkage disequilibrium analysis

To study sequence diversity of *SlX9* and *SlY9*, parts of the genes (listed in Supplementary Table 3) were amplified in 46 *S. latifolia* male individuals (Supplementary Table 1). The X-linked copies were amplified using the primers RB18_exon_1_F and RB18_exon_4_R, and the Y-linked copies with either RB18-Intron2-male-F-2 and RB18-3′UTR, or RB18-Intron2-male-F-3 and RB18-3′UTR, respectively (Supplementary Table 3). The sequence fragments were assembled and aligned manually using the programme Se-Al v.2.0a11 (Se-Al: sequence alignment editor, http://evolve.zoo.ox.ac.uk/). The sequences have been deposited in GenBank under Accession numbers HM141608–HM141717.

Sequence diversity was analyzed using DnaSP 4.0 software (Rozas and Rozas, 1999), excluding indel polymorphisms. To estimate between-population differentiation, the *SlX9* and *SlY9* sequences were divided into four broad geographic groups based on their location of origin ('Northern Europe', 'North-Eastern Europe', 'Mediterranean group', and 'Spain and Portugal', see Supplementary Table 1), K_{ST} statistics were computed in DnaSP 4.0. An NJ tree of the X-linked sequences was constructed in MEGA 3.1 (Kumar *et al*., 2004).

To test for introgression from the sister species *S. dioica*, we used the principle that introgression will cause variants from one species to be found in the same haplotype more often than expected based on their frequencies in the hybrid population, that is, there will be positive linkage disequilibrium among segregating sites, specifically in regions containing multiple successive variants from *S. dioica*. The associations are expected to last until they are broken up by recombination events, so that the physical distance over which we observe positive LD can be used as a measure of the time when hybridization occurred and/or the strength of selection against hybrid individuals. We used DnaSP 4.0 to calculate D', a measure of linkage disequilibrium among segregating sites, standardized relative to its maximum possible value.

Tests of neutrality and recombination estimates

Using population samples of *SlX9* and *SlY9*, several neutrality test were performed in DnaSP 4.0, including Tajima's D, Fu and Li's D^* and F^*, Fu's F and Fay and Wu's H statistics (Tajima, 1989; Fu and Li, 1993; Fu, 1997; Fay and Wu, 2000). Levels of statistical significance were estimated using coalescent simulations in DnaSP 4.0, conservatively assuming no recombination (Tajima, 1989; Wall, 1999). We also used DnaSP 4.0 to calculate an estimate of the recombination parameter, $R = 3N_e r$ (for *SlX9*); the minimum number of recombination events, and Strobeck's S statistic, which gives the probability of sampling the same or smaller number of haplotypes as observed in the population sample, given an estimate of θ (Strobeck, 1987).

Comparisons with outgroup species

Using the primer pair RB18_exon_1_F and RB18_exon_4_R (Supplementary Table 3), the homologue of *SlX9*/*SlY9* was amplified in *S. vulgaris*, a gynodioecious species that lacks sex chromosome and forms an outgroup to the *S. latifolia*/*S. dioica* clade. PCR amplification of the homologue of *SlX9* in *S. dioica* was carried out using different combinations of primers, listed in Supplementary Table 3 (RB18-male-exon1-F and RB18_male_exon3-R; RB18_exon_1_F and RB18-E3-R-beg; RB18-E2-F-beg and RB18_exon_4_R; RB18-male-exon1-F and RB18-exon2-R-Male; RB18-male-exon1-F and RB18_exon_4_R).

We call these homologues *Sv9* (for *S. vulgaris*) and *SdX9* (for *S. dioica*); we infer that *SdX9* is X-linked, but we did not obtain the Y-linked homologue for this species (see Results). The HKA test (Hudson *et al*., 1987), as implemented in DnaSP 4.0, was used to compare *SlX9* and *SlY9* diversity, using *Sv9* as the outgroup sequence.

To test whether *SlY9* has an accelerated rate of mutation, all fourfold degenerate sites were extracted from the *SlX9*/*Y9* coding sequence using DnaSP 4.0. The baseml programme of PAML was used to compare the rates of evolution along the three branches of the phylogenetic tree, using the *Sv9* outgroup sequence. A model that assumes a single rate of evolution for all branches ('clock = 1′ in Table 2, below) was compared with a model that assumes a different rate for the Y-linked branch compared with the X-linked genes ('clock = 2′).

The codeml programme of PAML was used to estimate K_A/K_S ratios on the branches leading to *SlX9* and *SlY9*, respectively, again using *S. vulgaris* as an outgroup, and allowing each branch of the tree to have its own rate of evolution. The likelihood of obtaining the data under a model in which all three branches of the tree have the same K_A/K_S ('model = 0′ in Table 2, below) was compared with a model ('model = 2′) in which there was one K_A/K_S ratio in the *SlY9* branch, and one for the branches leading to *SlX9* and *Sv9*.

Divergence between the X- and Y-linked copies of *SlX9-Y9* was estimated using DnaSP 4.0. The exonic sequence of male E2004-15-1 (from Serre de Nogere, Portugal) was compared with the set of X-linked sequences amplified for the diversity study (255 coding sites).

Results

Discovery of the new gene

The segregation results for intron size variants in the mapping family (see Materials and methods) are shown in Supplementary Figure 1, and clearly indicate sex-linkage (Kaiser *et al*., 2009). The new sex-linked gene was named *SlXY9*. The original cDNA sequence contains a continuous open reading frame of 444-bp coding sites (148 amino acids), and its sequence identifies it as an X-linked allele, similar to *SlX9* in the mapping family. It is probably a housekeeping gene, as BLAST searches showed similarity to the photosystem I subunit of *A. thaliana* (At1g08380) and to undefined membrane proteins of tobacco, wheat and rice.

To test whether the Y-linked gene was expressed, we used nested PCR with cDNA derived from male flower tissue (see Materials and methods). We retrieved a cDNA

sequence that overlaps, and is identical to, part of the genomic *SlY9* sequence (whereas there were six differences from all X-linked sequences, including the original cDNA and the X-linked sequences in our diversity study, see below). Therefore, both Y and X copies produce transcripts in male flower buds.

Comparisons between the original cDNA sequence and genomic sequences of *SlX9* and *SlY9* (including those in our diversity study) show that there are four exons in *S. latifolia*, and three introns, one more than in the *A. thaliana* putative homologue (Figure 1). Intron 2 of the Y-linked copy in the mapping family has extra sequence (yielding a distinctive large band in males, in addition to length differences between the maternal and paternal X copies, see Supplementary Figure 1). The complete intron 2 genomic sequence was obtained only for this male, and *SlY9* in Figure 1 and the sizes in Supplementary Figure 1 are based on this plant (for the other male plants, in the diversity study, the forward primer was within intron 2, and at most 86 bp of intron 2 sequence was obtained, so the full length of intron 2 in these plants is unknown).

No BlastN or BlastX matches were found for the insertion in *SlY9*, and no repetitive sequences were detected using the RepeatMasker programme (www.repeatmasker.org). However, the insertion may be a TE of a new type, or changed too much to be recognizable. The other two *SlY9* introns are also longer than those of *SlX9* or the *S. vulgaris* homologue (*Sv9* in Figure 1). This suggests that the intron sizes have expanded in the Y-linked copy, consistent with previous findings of non-coding sequence accumulation on the *S. latifolia* Y chromosome (Hobza *et al*., 2006; Cermak *et al*., 2008), and longer introns for the Y-linked genes *DD44Y* and *SlX3* (Marais *et al*., 2008), and the observed expansion of the *Drosophila miranda* neo-Y and the non-recombining Y-like region of papaya (Liu *et al*., 2004; Bachtrog *et al*., 2008).

X and Y haplotypes

To study sequence diversity, we used 46 males from different European locations (Supplementary Table 1), and obtained 46 *SlY9* and 40 *SlX9* sequences (probably underestimating X diversity, because *SlX9* failed to amplify from a few plants, suggesting that sequence differences are present in their X alleles). Our PCR amplifications with X- or Y-specific primers always yielded just one amplicon, identifiable as either *SlX9* or *SlY9* by the intron length variant that distinguishes Y-linked alleles (see above). The gene is therefore single-copy in the genome, and is sex-linked throughout the species' range. No frame shift mutations or premature stop-codons were found in the coding regions in any of the *SlY9* (or *SlX9*) sequences.

The X-linked sequences are of two distinct sequence types. In 13 *SlX9* sequences, intron 2 was ~485-bp long (yielding a band of 585 bp, including the amplified portions of the flanking exons, which accounts for the ~600-bp X-linked band observed in the mapping family), whereas in the others it was only ~380 bp (corresponding to the 480-bp X band amplified in the mapping family). The intron sequences of the two types were highly diverged, and were aligned manually (see Materials and methods and Supplementary Figure 2).

Location of the *SlX/Y9* gene on the X and Y chromosomes, and X–Y divergence

SlX9 is closely linked to a previously described X-linked gene, *SlCypX* (Bergero *et al*., 2007; Kaiser *et al*., 2009). Deletion mapping (see Materials and methods) showed that *SlY9* is always co-deleted with *SlCypY*, suggesting that these genes have been physically close because recombination stopped in this sex chromosome region. Both genes should therefore have similar X–Y sequence divergence. This region probably stopped recombining before the *S. latifolia* and *S. dioica* split, because synonymous divergence between *SlCypX* and *SlCypY* is 6.1%, and *SlCypY* carries a MITE insertion in intron 2 of both species, which is absent from *SlCypX* (Bergero *et al*., 2007). Divergence between *SlX9* and *SlY9* can be estimated only roughly, because only parts of the sequences could be obtained. As discussed below, *SlX9* has high sequence diversity (and all non-synonymous differences between *SlX9* and *SlY9* were polymorphisms in the *SlX9* sequences), inflating the raw X–Y divergence estimates in Table 1. We therefore also computed net divergence, which estimates fixed X–Y differences. The results (Table 1) consistently support divergence in line with that of *SlCypXY*, whether we include all X sequences, or only the longer or shorter type.

A caveat is that, if *SlX9* and *SlY9* stopped recombining independently in *S. latifolia* and *S. dioica* after their split, the X-linked copy of each species should be more similar to its Y-linked copy than to the X of the other species, and introgression could then inflate the X–Y divergence. Different degrees of introgression of different regions of the X could then obscure the true times when recombination stopped. However, this is unlikely to affect our conclusions, because *SlX9–SlY9* divergence is much higher than divergence between the two species. Silent site divergence between the *S. dioica* sequence and that of *SlX9* is 3.0% (based on 227 sites), similar to estimates from other X-linked genes in these species (the highest previous synonymous divergence estimate is 4.4% for *SlCyp*-X, see Bergero and Charlesworth, 2009).

Divergence from outgroup species

Several sex-linked genes have been found to have higher Y than X mutation rates (Filatov and Charlesworth, 2002; Filatov, 2005), but *SlX9* and *SlY9* have similar divergence from the *S. vulgaris* homologue (Jukes–Cantor corrected divergence estimates for synonymous sites were 21% for both *SlX9*, based on 255 coding sites, and *SlY9*, using 261 coding sites). PAML analysis confirms that *SlY9* has not evolved significantly faster than the X-linked copy, using fourfold degenerate sites (72 sites) or all 444 alignable coding sites (Table 2).

The d_N/d_S ratio on the branch leading to *SlY9* is 0.077, and does not differ significantly from that on the other branches (Table 2). Together with the lack of frame shift mutations or premature stop-codons in *SlY9*, as well as its expression as mRNA (see above), these results suggest that the *SlY9* gene is still functional.

Sequence diversity within *S. latifolia*

We sequenced a portion of *SlX9* including parts of exons 2 and 3 and introns 1–3. Within *S. latifolia*, the estimated synonymous diversity (π_S) of the X-linked copy is much higher than for other X-linked genes (see above), but this is based on few codons; silent site diversity is also high (Table 1). As described further below, the high diversity results partly from the presence of two *SlX9* sequence types (the two X haplotypes with different intron sizes described above, see Figure 1). However, most diversity is within the types, not between them: silent site diversity with JC correction within either set of X-linked sequences was 3.2%, and the net silent site divergence is <1%.

The Y-linked copy, *SlY9*, has substantially lower diversity (Table 1), and there were only two single-nucleotide polymorphisms, both in introns. There was also one indel polymorphism in intron 3 of *SlY9*: the sequences from two males from a population in Greece had an insertion of the triplet TCA. Although the numbers of sites analyzed in both sets of sequences are small, an HKA test using all site types finds a significant difference in X–Y diversity estimates, taking into account the different ploidy level ($\chi^2 = 5.5$, $P < 0.05$). The X/Y diversity ratio is 24.5, using the species-wide silent site estimates in Table 1. *SlX9* showed significant differentiation between populations (K_{ST} estimate 0.109, $P < 0.001$). The estimate for *SlY9* was only slightly higher (0.122, $P < 0.05$), but is based on only two segregating sites.

Table 1 Sequence divergence estimates between *SlX9* and *SlY9* (*K* values), and diversity estimates (π values) within *SlX9* and in the *Silene dioica* homologue

Region compared (footnotes indicate the sequences that were used)	K_S *or* π_S *(synonymous or silent sites, see the left-hand column)*[a]	K_A *or* π_A *(non-synonymous sites)*
SlX9 versus SlY9: raw divergence values		
392 coding sites[b]	0.153	0.00069
255 nucleotides in exons 2 and 3 (85 codons)[c]	0.144	0.00026
SlX9 versus SlY9: net divergence (excluding SlX9 polymorphisms)[d]		
90 silent sites, *SlX9* versus *SlY9*	0.063	—
255 coding sequence sites (as above)	0.053	0 (small negative value)
Long X-linked copies only, versus *SlY9*, 255 coding sites	0.042	0
Short X-linked copies only, versus *SlY9*, 255 coding sites	0.062	0
Diversity within S. latifolia[e]		
X-linked copy, synonymous sites (85 codons in exons 2 and 3)	0.092	0.0005
X-linked copy, 240 silent sites	0.040	—
Long X-linked copies, 559 silent sites	0.032	—
Short X-linked copies, 412 silent sites	0.033	—
Y-linked copy, 283 silent sites[f]	0.0016	0
X-linked gene diversity within S. dioica		
514 silent sites[g]	0.018	—
117 coding sequence sites	0.066	0.0035

[a]With Jukes–Cantor correction for saturation.
[b]Comparing the longer (Y) sequence from the male parent of the mapping family with the original complementary DNA clone.
[c]*SlX9* from the diversity study and *SlY9* from the male parent of the mapping family.
[d]Estimated by subtracting the average of the diversity values.
[e]On the basis of single-nucleotide polymorphisms only.
[f]It is to be noted that the X and Y estimates of π are based on different gene regions (see Figure 1).
[g]From our sample of 18 X-linked sequences; as explained in the text, no Y-linked sequences were amplified from this species.

Tests for introgression from *S. dioica*

Given the genetic evidence mentioned above that there are no duplicate copies of these genes, we tested whether the two different *SlX9* sequence types in *S. latifolia* described in the preceding section, and the high *SlX9* diversity, reflect introgression from *S. dioica*. Very recent introgression seems unlikely, because the short length type was found in populations from the Mediterranean region, in which *S. dioica* is absent (Prentice *et al*., 2008). Both intron sizes were found within *S. latifolia* populations from Sweden and Poland (in which *S. dioica* is present), and in Greece (where *S. dioica* is absent), and the short *SlX9* sequence was also found in Italy and Spain.

To test this further, we sequenced portions of the gene (Figure 1) from *S. dioica* sampled from several geographic regions of Europe. All 13 *S. dioica* males yielded only single sequences, whereas 1 of the 6 females (A2009_2_female_1) contained several heterozygous single-nucleotide polymorphisms, and another female (FS14) was heterozygous for a 22-bp length variant in intron 2 (it is to be noted that this female yielded a short sequence, which was excluded from further analyses). It thus seems that only the X-linked copy amplified from *S. dioica*. The Y copy may have been deleted in *S. dioica*. Alternatively, given the high X–Y divergence in *S. latifolia* (Table 1), the *S. dioica* Y-linked sequence may be too diverged for the primers to work.

All 19 *S. dioica* X-linked sequences had the short haplotype (Figure 1). Diversity is slightly lower than for *S. latifolia* X-linked sequences (Table 1); for a region of

Table 2 PAML-based tests of rate differences between *SlX9* and *SlY9*, using *Sv9* as an outgroup sequence

Program	*Comparison*	*Number of sites*	*Model*	χ^2	*df*	P
Baseml	Fourfold degenerate sites	72	Clock = 1 Clock = 2	2.67	1	0.102
Baseml	All coding sites	444	Clock = 1 Clock = 2	0.32	1	0.57
Codeml	K_A/K_S	438	Model = 0 Model = 2	1.51	1	0.22

165 bp sequenced from both species, $\pi = 2.3\%$, versus 3.1% in *S. latifolia*. The shorter *S. latifolia SlX9* haplotypes have sequences similar to the *S. dioica* sequences (silent site divergence 2.7%, with Jukes–Cantor correction, based on 314 sites), whereas the long ones differ slightly more (5.7%, based on 277 silent sites). In the NJ tree (Figure 2), several *S. latifolia* sequences cluster closely with *S. dioica*, and all these have the short intron 2. Several individuals from Northern France and England are very similar in sequence, or identical (plant 38), to *Sd9* sequences, but the sequence from individual 6 is also similar to a group of *SdX9* sequences (Figure 2), although it comes from Southern France, in which *S. dioica* is not found.

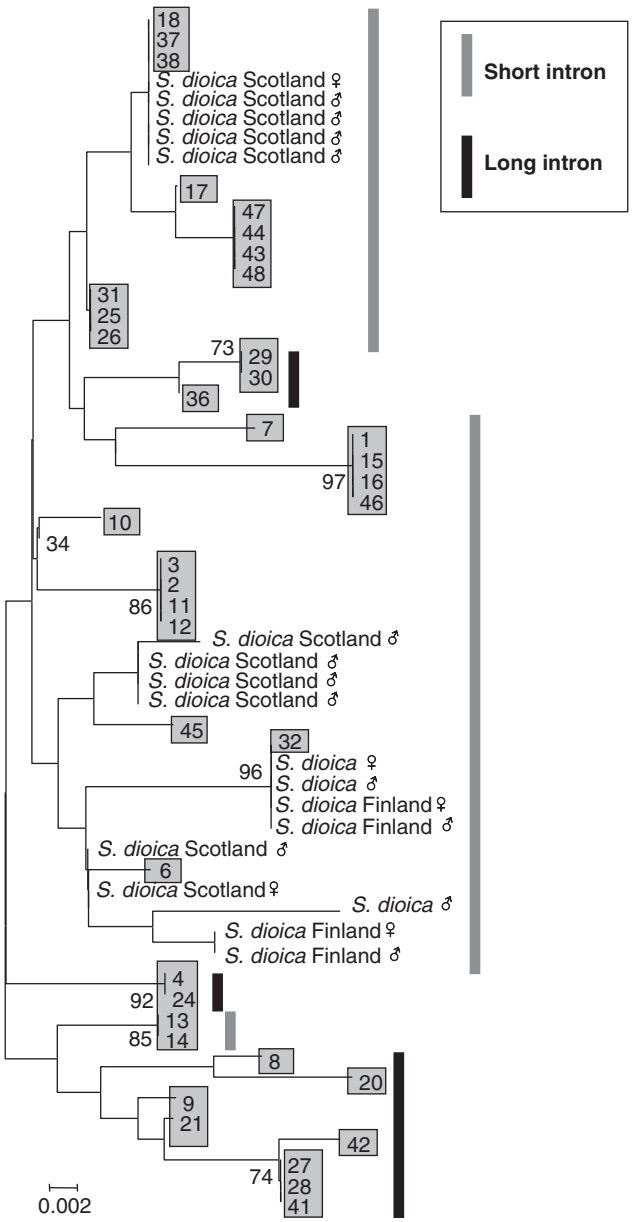

Figure 2 Neighbour-joining tree of *SlX9* and *SdX9*. The tree was constructed in MEGA, based on all sites, with complete deletion. Bootstrap support values >70% are shown at the branches. The *S. latifolia* male individuals (blue shading) are identified by their numbers in Table 1, and the *S. dioica* plants are identified by their populations of origin when known.

The part of the gene in which we have at least 2 X-linked sequences from each species contains 138 variable sites (including both species), of which 33 are in the intron 2 region that is absent from the short sequences. Our results from these sites suggest introgression. *SlX9* and *SdX9* share 20 polymorphisms, 12 at sites in regions present in both *S. latifolia* haplotypes and 8 in the intron 2 region that is present only in the *S. latifolia* short haplotype group plus *S. dioica* (Supplementary Figure 2, summarized in Supplementary Table 2). There were no fixed differences between the sequences of the two species, but 15 sites have polymorphisms exclusive to *S. dioica*.

Linkage disequilibrium among sites polymorphic in the X-linked alleles

As expected, recombination is detected in the *S. latifolia SlX9* alleles (Table 3). Among the 37 single-nucleotide polymorphism sites, 9 pairs have significant linkage disequilibrium in our sample of alleles (Fisher's exact test; $P < 0.05$, after Bonferroni correction for multiple comparisons). Eight of these pairs were <100 bp apart from each other, in or near exon 3, whereas one pair of sites is separated by 602 bp (Supplementary Table 5, Supplementary Figure 2). The LD results do not strongly indicate introgression, as opposed to ancestral polymorphism.

However, ancestral polymorphism leaves the high diversity unexplained. Balancing selection in the common ancestor of the two species, and maintained in *S. latifolia* could explain this, but predicts that many variants should be at higher frequencies than expected under neutrality, resulting in a positive Tajima's *D* statistic. However, in our sample, Tajima's *D* for *SlX9* was negative, although nonsignificant, as are other tests of selection on *SlX9* (Table 3), except for Fu's *F*, which is very sensitive to a frequency spectrum bias towards rare polymorphisms.

Discussion

Causes of low Y diversity relative to the X

Like other known genes on the *S. latifolia* sex chromosomes, the *SlY9* diversity is lower than expected purely from the lower Y effective population size assuming a 1:1 sex ratio and equal variance of offspring numbers in the two sexes. A reduced mutation rate on the Y cannot account for the diversity difference, because rates in *S. latifolia* either do not differ between the X and Y copies (as we found here), or are significantly higher for the Y-linked copies (Filatov and Charlesworth, 2002; Filatov, 2005; Nicolas *et al*., 2005).

Population structure can increase species-wide diversity, but subdivision should affect diversity on the Y chromosome more than that of the X, even if pollen and seed dispersal rates are the same (Laporte and Charlesworth, 2002). Strong population structure has been found in *S. latifolia* for the *SlX4/Y4* and *DD44-X/Y* genes (Laporte *et al*., 2005) and *SlX1/Y1* (Atanassov *et al*., 2001; Ironside and Filatov, 2005), in all cases much more markedly for the Y than the X, because of the low Y diversity. Our small within-population samples are not suitable for tests for subdivision, but we found modest subdivision at a larger geographic scale for the X,

Table 3 Tests of neutrality and recombination for *SlX9* and *SlY9*

Gene	*Tajima's D*	*Fu and Li's D**	*Fu and Li's F**	*Fu's F*	*Strobeck's S statistic*	*Fay and Wu's H*	*$R=3N_er$ (adjacent sites)*
SlX9	−0.32	−0.25	−0.32	−7.63	1.000	−1.23	0.14
	NS	NS	NS	$P<0.05$		NS	
SlY9	−1.19307	−1.74431	−1.84007	−0.515	0.836	0.46377	—
	$P<0.1$	$P<0.1$	$P<0.1$	NS		NS	

Abbreviation: NS, nonsignificant.

consistent with results for other X-linked loci. We conclude that diversity on the Y is reduced, rather than elevated on the X, and that subdivision or local adaptation probably cannot explain the high X diversity.

The low diversity is therefore probably because of hitch-hiking processes in the *S. latifolia* Y chromosome's large non-recombining region. This chromosome probably contains many active genes. There is as yet no adequate estimate of the proportion of X-linked genes that have copies on the Y, or of the absolute number of functional genes on the Y. Only 1 of the 11 genes so far identified on the X, the recently added *SlCyt* gene (Kaiser *et al*., 2009), has a missing or truncated Y copy, and none has a Y-linked allele with any evident loss-of-function mutation, although several of these genes have lower expression than their X-linked alleles, and molecular evolutionary analysis suggests some loss of adaptation, relative to the X-linked alleles (Marais *et al*., 2008). However, many of these genes were ascertained by Y-linkage of genes discovered from EST sequences, which will preferentially detect functional Y-linked genes. At present, therefore, one can conclude only that the *S. latifolia* Y carries several functional genes.

Hitch-hiking should produce an excess of low-frequency variants. However, with the small sample of genes whose diversity has been studied in this species, and the small number of variants in the Y-linked alleles, it is difficult to compare the frequency spectra for X- and Y-linked genes, or even to estimate Tajima's *D* for Y-linked genes. The X-linked genes so far studied, *SlssX*, *SlCypX* and *SlCyt*, have negative Tajima's *D* values (Bergero *et al*., 2008; Kaiser *et al*., 2009), so it is likely that recent demographic history may have produced a genome-wide excess of low-frequency variants, which will impede interpretation of the frequency spectrum of Y-linked variants. Previous studies of three Y-linked genes (*SlY*1, *SlY*4 and *DD44Y*) did not find significantly negative Tajima's *D* values, but pointed out that population subdivision, with local fixation of different Y haplotypes, could cause a deficit of rare variants, obscuring the effects of sweeps (Atanassov *et al*., 2001; Laporte *et al*., 2005). However, strong Y chromosome differentiation among populations (Ironside and Filatov, 2005) argues against recent species-wide selective sweeps, but is consistent with background selection, or geographically localized sweeps (Ironside and Filatov, 2005). The positive (though nonsignificant) Fay and Wu's *H* for *SlY9* also argues against a selective sweep on the Y chromosome.

Our results are, however, consistent with purifying selection, and thus with the suggestion that Muller's ratchet and/or background selection should be most important early in Y chromosome evolution, when the number of functional genes that can undergo detrimental mutations is still very large (Bachtrog, 2008). Selective sweeps may become more important after the Y has lost many genes; there is then a higher chance that beneficial mutations can occur on chromosomes not carrying many deleterious mutations.

Causes of high X diversity in *S. latifolia*

To estimate the diversity for X-linked genes relative to homologues on the Y (or to estimate X/autosome diversity ratio), it is essential to have reliable diversity estimates for genes on the different chromosomes, and introgression from a different species will make this difficult and could increase the estimated diversity (Sweigart and Willis, 2003). We argued above that subdivision is not extreme for *SlX9*, and does not explain the high diversity observed.

The shared short intron 2 structure, and shared single-nucleotide polymorphisms are consistent with introgression from *S. dioica*. Evidence for introgression between *S. dioica* and *S. latifolia* has also been reported for *DD44X* and *SlX4* (Laporte *et al*., 2005). For *SlX*4, a size variant in a *S. latifolia* intron matched an intron size variant in *S. dioica*, similar to our observation for *SlX9* (Laporte *et al*., 2005); for *SlX1*, almost all shared polymorphisms were within the first 1755 bp, and fixed differences were found only at the 3′ end of the gene (Atanassov *et al*., 2001), whereas, for *DD44X*, the sequences sampled from the two species shared 14 polymorphic sites and had no fixed differences (Laporte *et al*., 2005). These results suggest that the introgressed regions can be very localized (Laporte *et al*., 2005), and thus that introgression may be infrequent, and that introgressed regions are often eliminated after recombination (presumably because of selection). For the diversity analysis of *SlX9*, exon 1 and intron 1 were not included, so that our diversity value might be an over-estimate if the region surveyed coincides with a region of the gene in which *SdX9* sequence has been introgressed.

Introgression between *S. latifolia* and *S. dioica* has also been detected using AFLP markers and (maternally inherited) chloroplast markers: out of 209 markers studied by Minder *et al*. (2007), only 7 were species-specific, and 5 out of 7 chloroplast haplotypes in *S. latifolia* were also present in *S. dioica* (Prentice *et al*., 2008). Allopatric populations of *S. dioica* and *S. latifolia* in Switzerland separated by small distances were found to be more distinct than sympatric ones (Minder and Widmer, 2008), as expected if hybridization occurs locally, but the introgressed regions are usually eliminated, rather than persisting.

We find both of the *SlX9* haplotypes in plants from the Mediterranean region, in which *S. dioica* is absent. This argues against a hypothesis of recent introgression creating the *S. latifolia* short intron 2 X-linked haplotypes.

As *S. latifolia* sequences with the longer X-linked intron 2 also share polymorphisms with *S. dioica*, recombination must probably have occurred after introgression, to yield a group of haplotypes with the short intron 2 characteristic of the *S. dioica* X, but with variants derived from the long *S. latifolia* haplotype.

We find slightly higher diversity in *SlX9* than in *SdX9*, suggesting that introgression of X-linked sequences occurred mainly from *S. dioica* into *S. latifolia*. This is consistent with the distribution of chloroplast versus genomic markers, which suggested that hybridization events mainly involve *S. dioica* as the pollen donor (Minder *et al*., 2007). However, this conclusion is not definitive, in the absence of extensive sampling of the chloroplast genome and its variants in both species, because ancestral polymorphisms may exist. Moreover, experiments with equal amounts of pollen from the two species yielded progeny from *S. latifolia* recipients in which <20% were hybrids, compared with 50% with *S. dioica* recipients (Rahme *et al*., 2009). We can detect no *S. dioica* Y-linked allele of *SdX9*, which implies that *SlY9* does not introgress into *S. dioica*. Our results also exclude *S. dioica* Y chromosome introgression into *S. latifolia*. If such introgression occurs, and if the *S. dioica* Y has no copy of the gene, we should find individuals within *S. latifolia* lacking *Y9*, but all 46 *S. latifolia* males yielded *SlY9* sequences. This lack of Y chromosome introgression supports results from other Y-linked genes (Laporte *et al*., 2005).

Is introgression the sole cause of the unexpectedly high X diversity? If introgression of the X occurred recently, it might be possible to remove the introgressed alleles and estimate X diversity for comparison with the Y. However, if introgression is not recent, introgressed alleles cannot be recognized. In an attempt to analyze diversity in *S. latifolia* that is unlikely to be due to introgression, we excluded from the *SlX9* data set all polymorphisms that are shared with our *S. dioica* sequences. The X–Y difference in diversity remained unchanged, suggesting that introgression is not the sole factor causing higher X than Y diversity (HKA test: $\chi^2 = 4.19$, $P < 0.05$). If most introgressed sequences are selectively eliminated, and only certain small introgressed regions remain, these will be difficult to distinguish from shared ancestral polymorphisms for such closely related species. In *Mimulus guttatus*, recent introgression from *Mimulus nasutus* was detectable because high diversity was found only in sympatric *M. guttatus* populations, and also because *M. nasutus* sequences were clearly distinguishable from *M. guttatus* (Sweigart and Willis, 2003), unlike the situation for our species, and so we cannot rule out that ancestral polymorphism has contributed to some of the observed pattern in *S. latifolia*.

Conflict of interest

The authors declare no conflict of interest.

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