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Report

Evidence for Degeneration of the Y Chromosome in the Dioecious Plant *Silene latifolia*

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Summary

The human Y—probably because of its nonrecombining nature—has lost 97% of its genes since X and Y chromosomes started to diverge [1, 2]. There are clear signs of degeneration in the *Drosophila miranda* neoY chromosome (an autosome fused to the Y chromosome), with neoY genes showing faster protein evolution [3–6], accumulation of unpreferred codons [6], more insertions of transposable elements [5, 7], and lower levels of expression [8] than neoX genes. In the many other taxa with sex chromosomes, Y degeneration has hardly been studied. In plants, many genes are expressed in pollen [9], and strong pollen selection may oppose the degeneration of plant Y chromosomes [10]. *Silene latifolia* is a dioecious plant with young heteromorphic sex chromosomes [11, 12]. Here we test whether the *S. latifolia* Y chromosome is undergoing genetic degeneration by analyzing seven sex-linked genes. *S. latifolia* Y-linked genes tend to evolve faster at the protein level than their X-linked homologs, and they have lower expression levels. Several Y gene introns have increased in length, with evidence for transposable-element accumulation. We detect signs of degeneration in most of the Y-linked gene sequences analyzed, similar to those of animal Y-linked and neo-Y chromosome genes.

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Results

Y Protein Evolution

We studied seven genes (all nonduplicated *Silene* sex-linked genes so far identified, including three recently identified XY pairs [13, 14]) and sequenced additional species to obtain orthologs of these genes in *S. dioica* and *S. diclinis* and nondioecious outgroups (*S. conica*, *S. vulgaris*, and *S. noctiflora*; see Table S1 available online).

We first used phylogenetic analysis by maximum likelihood (PAML) to perform branch-model analyses to compare d_N/d_S ratios (nonsynonymous/synonymous substitution rates, or ω) in the X and Y lineages (Supplemental Data, Methods 1). Both X and Y ω are below 1, suggesting that both copies evolve under purifying selection (this was confirmed by a site-model analysis, see Table S3). However, four Y-linked genes show significantly faster protein evolution than their X-linked counterparts (Table 1), which is consistent with previous work based on smaller datasets [13, 15]. This suggests that the purifying selection may not be preventing Y genes degenerating, i.e., that their high ω values are due to fixation of slightly deleterious mutations in the Y copies.

However, the branch-model analysis gives only global ω values for the X and Y lineages. In some Y genes, some codons may also have switched from purifying selection to positive selection, increasing the global ω. To distinguish between positive selection versus lower efficacy of natural selection, we therefore did a branch-site analysis (Supplemental Data, Methods 1). Table 2 gives the percentages of sites estimated by PAML to have switched from purifying selection to neutral evolution (degenerating sites), and from purifying selection to positive selection, in the different Y copies. In *SlY1*, *SlCypY*, *SlY7*, and *SlY4*, the estimated percentages of degenerating sites are between 4% and 14%; no adaptively evolving sites were detected. The Y copies of these four genes thus seem to be degenerating. No degenerating sites are detected in *SlssY*, *DD44Y*, or *SlY3*, but 4%–9% of sites are detected as evolving adaptively, specifically in the Y lineages, suggesting possible Y-specific positive selection (however, the ω values of the codons evolving under Y-specific positive selection differ significantly from 1 only for *SlssY* and *DD44Y*).

We also did branch-site analysis with Fitmodel [16], which can estimate the numbers of codons suggesting either degeneration or positive selection, for both X and Y sequences (Supplemental Data, Methods 1). For three genes (*SlX3/Y3*, *SlX4/Y4*, and *DD44X/Y*) a simple site model, M2a, differs significantly from a branch-site model, M2a + S1 (Table 2). *DD44* has an excess of codons supporting Y-specific positive selection, fully confirming this gene's PAML branch-site results, whereas *SlX3/Y3* and *SlX4/Y4* showed excesses of codons supporting Y

Table 1. Evidence for Higher d_N/d_S for Y- Than X-Linked Genes

Genes (Number of Codons)	Branch-Model Results ω_X	ω_Y	
SlX1/Y1 (458)	0	0.11	X < Y***
SlCypX/Y (519)	0.14	0.14	X = Y
SlssX/Y (251)	0.18	0.23	X < Y ns
DD44X/Y (217)	0.13	0.90	X < Y**
SlX3/Y3 (318)	0.04	0.13	X < Y*
SlX7/Y7 (246)	0.08	0.11	X < Y ns
SlX4/Y4 (362)	0.11	0.25	X < Y*

$\omega = d_N/d_S$. ω values were estimated with PAML, and statistical significance was assessed by likelihood-ratio tests (LRT) and is indicated as follows: ns = nonsignificant, *p < 0.05, **p < 0.005, ***p < 0.0005. Note that PAML assigns a value of 0.001 to ω when ω is 0, and it was this value that was used for the LRTs.

degeneration and also codons supporting Y-specific positive selection (consistent with these genes' PAML results).

Y Introns and Transposable Elements

For most genes, the total intron lengths in X- and Y-linked genes in *S. latifolia* are similar (Figure S2), including the newly obtained complete *SlX4* and *SlY4* genomic sequences, contrary to previous reports [17], and the partial *SlCyp* and *Slss* sequences (with only some of the introns).

For *DD44X/Y* and *SlX3/Y3*, the total Y intron length exceeds that for the X copies (Figure S2). Use of *S. vulgaris* as an outgroup indicates that these intron size differences are due to increases in the Y copies of about 5 to 6 Kb in the largest X and *S. vulgaris* introns (Figure 1, Figure S3); thus TEs might be involved. Although large introns often contain regulatory elements for gene expression [18–20], TE insertions are likely to affect splicing efficiency less in long than in short introns. Detailed examination of the intron sequences indeed detected transposable elements (TEs). Intron 2 of *DD44Y* contains a LTR retrotransposon (Figure 1A), similar to the Sabrina element of maize, and also direct repeats and similarities to the Perere-3 *Schistosoma mansoni* element, a non-long terminal repeat (LTR) retrotransposon. The first intron of *SlY3* contains many inverted repeats (Figure 1B), the footprints of DNA transposon insertions [21], and also weak similarities with proteins of plant TEs.

We also aligned the *SlX3/Y3* gene pair's noncoding sequences between the closely related dioecious species *S. latifolia*, *S dioica*, and *S. diclinis* to search for Y-specific indels that are in the *S. latifolia* and *S. dioica* Y-linked alleles but are absent in *S. diclinis*, indicating recent TE insertions in the Y (and similarly for X-specific insertions). In both *S. dioica* and *S. latifolia*, insertions (including those > 100 bp) are more frequent in *SlY3* than in *SlX3* (Table S4).

Y Expression Levels

The estimated expression levels of Y-linked genes were considerably and significantly lower than for their X-linked counterparts for most of the pairs (Figure 2).

Table 2. Evidence for Y Degeneration from Branch-Site d_N/d_S Analysis

Genes (Number of Codons)	Branch-Site Analysis Methods	Codons Consistent with Y Degeneration	Codons Consistent with Y-Specific Positive Selection	Switching Significance
SlX1/Y1 (458)				
	PAML	6%	0%	
	Fitmodel			No significant switching (p > 0.05)
SlCypX/Y (519)				
	PAML	10%	0%	
	Fitmodel			No significant switching (p > 0.05)
SlssX/Y (251)				
	PAML	0%	9% (ω = 5.5)*	
	Fitmodel			No significant switching (p > 0.05)
DD44X/Y (217)				
	PAML	0%	5.5% (ω = 14.8)**	
	Fitmodel	0 sites	8Y > 4X	Significant switching*
SlX3/Y3 (318)				
	PAML	0%	4% (ω = 3.5)ns	
	Fitmodel	12Y > 3X	3Y > 0X	Significant switching**
SlX7/Y7 (246)				
	PAML	4%	0%	
	Fitmodel			No significant switching (p > 0.05)
SlX4/Y4 (362)				
	PAML	14%	0%	
	Fitmodel	22Y > 8X	9Y > 4X	Significant switching**

ω stands for d_N/d_S. For the PAML results, the table shows the percentages of codons consistent with Y degeneration (ω < 1 in X copies and outgroups, and ω = 1 in Y copies) and with Y-specific positive selection ($\omega \leq 1$ in X copies and outgroups and ω > 1 in Y copies). For Fitmodel, two models were compared: a model without switching of codons and a model with switching from one form of evolution (ω < 1, ω = 1, or ω > 1) to another (ω < 1, ω = 1, or ω > 1). The left-hand column shows the number of codons consistent with Y or X degeneration (for Y degeneration, ω < 1 or ω > 1 in X and the outgroups, and ω = 1 in Y; for X degeneration, ω < 1 or ω > 1 in Y and outgroups, and ω = 1 in X; for example, for the *SlX4/Y4* gene pair, 22 Y codons suggest Y degeneration, and eight X codons suggest X degeneration). The right-hand column shows the number of codons suggesting Y- or X-specific positive selection (respectively, ω < 1 or ω = 1 in X and outgroups, and ω > 1 in Y, or ω < 1 or ω = 1 in Y and outgroups, and ω > 1 in X). The significance of each LRT is indicated as follows: ns = nonsignificant, *p < 0.05, **p < 0.005, ***p < 0.0005.

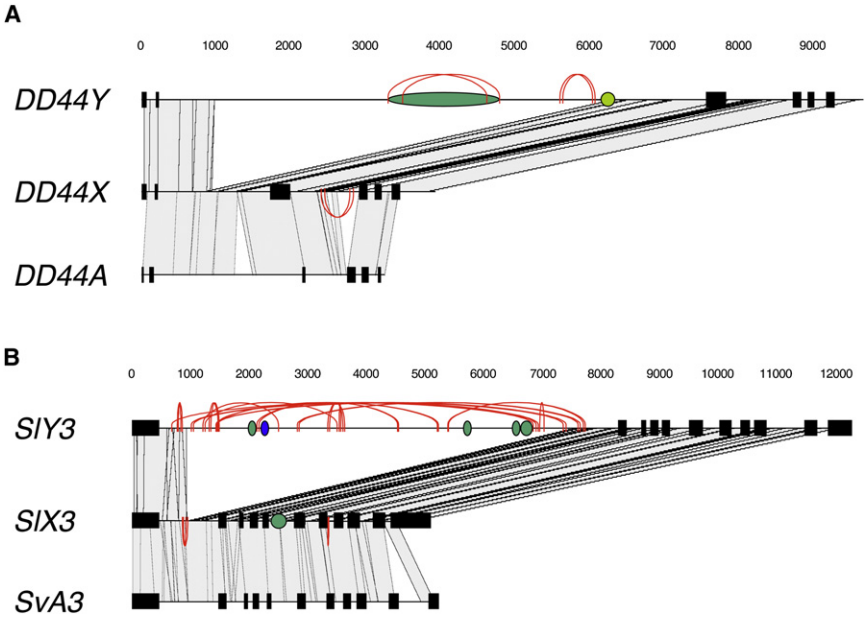

Figure 1. Intron Sizes and Transposable-Element Content in *DD44X/Y* and *SlX3/Y3*

DD44X/Y is shown in (A), and *SlX3/Y3* is shown in (B). Exons are shown as black boxes, and introns as black lines. Blocks of similarity between the X, Y, and non-sex-linked copies are shown in gray. Similarities with transposable elements are indicated by oval symbols (blue, DNA transposons; dark green, LRT retrotransposons; light green, non-LRT retrotransposons). Direct and inverted repeats are indicated by red lines. The sex-linked sequences are from *S. latifolia*, and the autosomal ones are from *S. vulgaris*.

Discussion

Degeneration and Adaptive Evolution of Y Genes

Y-linked genes show a clear tendency to have lower expression levels than their X counterparts. Although it is not yet certain that the difference represents reduced Y-linked expression (because expression data from outgroups are not yet available), and although degeneration of Y-linked genes might not always cause lower expression (because deleterious mutations could alter expression in either direction), the consistently lower expression of Y-linked genes is unlikely to be adaptive, but suggests functional degeneration.

This is consistent with our sequence analyses suggesting that most Y genes accumulate slightly deleterious mutations. There are some caveats, given that differences between sequences from different species may be within-species polymorphisms, not fixed differences, and it is not yet known how PAML or Fitmodel analyses are affected by polymorphisms. However, polymorphism seems unlikely to falsely suggest Y degeneration (Supplemental Data, Method 1). Because X sequences are much more polymorphic than Y ones [22–24], some X-Y differences could be slightly deleterious X-linked mutations at low frequencies, which would reduce X-Y differences in d_N/d_S; thus our tests should be conservative. The PAML and Fitmodel analyses should, however, in the future be supplemented with independent tests using within-species diversity, which can potentially detect differences in selection, and/or its effectiveness, in the X and Y lineages [6].

Rapidly evolving genes on the Y are not necessarily degenerating. Y-specific positive selection has been found in humans [25] and *Drosophila* [26, 27]. Our tests to distinguish lower efficacy of selection from positive selection find some evidence of codons under Y-specific positive selection, perhaps suggesting that selective sweeps contribute to Y degeneration in *Silene*. Polymorphism is greatly reduced in *S. latifolia* Y-linked genes, relative to the X, but data from *SlY1*, *DD44Y*, and *SlY4* do not specifically indicate recent selective sweeps [22–24]. However, adaptive events in Y genes may be ancient and no longer detectable in polymorphism data. Another possibility is that the positively selected codons detected by PAML in some of our genes are not truly evolving under adaptive evolution. Indeed, all these genes are housekeeping genes [13, 17, 28, 29], and Y-specific positive selection is unexpected.

Effects of Transposable Elements on Y Evolution

In *S. latifolia* and *S. dioica*, the Y chromosome is about 50% bigger than the X ([11, 30], M.N., unpublished data), unlike animals, whose Y chromosomes are often smaller than the X. Our data support the suggestion based on chromosome painting [31, 32] that this large Y size may be due to accumulation of repetitive sequences on the Y, probably including TEs. Many of the sequences detected in *S. latifolia* Y-linked genes are incomplete TEs, and some are not identifiable as known families, but merely as sequences likely to derive from TE activity. Plant TE families are highly diverse [33, 34], and it is difficult to investigate TE dynamics in a species, such as *S. latifolia*, in which only a few TEs are known [35–37]. Thus we have probably underestimated the contributions of TEs to the additional material in the Y genes' introns, especially if old insertions have been partially deleted, or the sequences of inactive elements are too changed to be recognizable.

The mechanisms for reduced expression of Y genes are not known. TEs could contribute to this [8], but the TE insertions in the *S. latifolia* Y genes so far investigated show no relationship with expression levels (Figure S4). However, we surveyed only introns, and not regions such as intergenic DNA, in which insertions might be more likely to affect gene expression. Data from Y and X BAC sequences should help test this question further.

The Time Scale of Y Degeneration in *Silene*

In the evolution of several animal and plant sex chromosomes, recombination was suppressed gradually [1, 38–41]. In the human Y chromosome, the regions that have been nonrecombining longest indeed, as expected, have few intact genes, relative to the number on the X chromosome (5/734 genes, on the basis of [2] and Ensembl version 47). In *S. latifolia*, the different aspects of Y degeneration studied here are not correlated with X-Y divergence times (estimated with synonymous site divergence, Figure S4).

TE insertion does not correlate with time of X-Y divergence (Figure S4), though too few genes are available for any patterns to emerge other than very strong ones. TE insertions may occur very fast once recombination ceases, as observed in the *Drosophila miranda* neo-Y [5, 7, 42] and the small sex-determining regions of the stickleback fish [43] and the plant

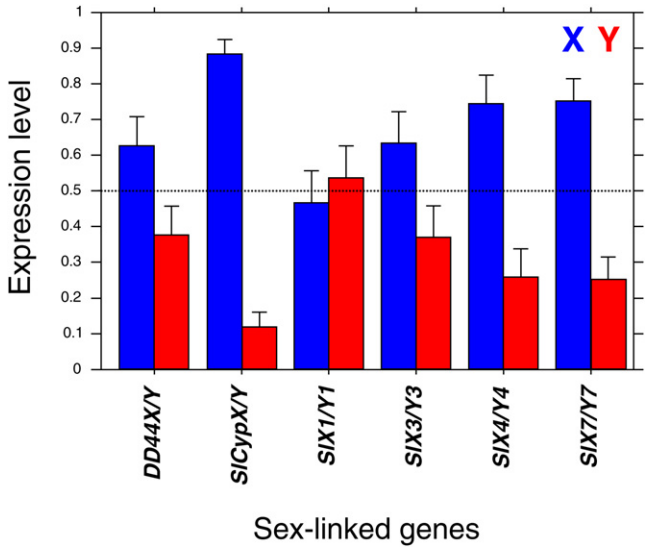

Figure 2. Expression Levels of X and Y Copies in Male Flowers

Expression levels were quantified as the percentages of X and Y clones in cDNA prepared from *S. latifolia* male flowers, determined with sequence variants distinguishing the X and Y sequences of the study plants (see Experimental Procedures). One gene, Slss [13], was not studied because paralogs have been found in *S. latifolia* [14]. Error bars indicate standard errors assuming a binomial distribution. Overall, the differences between X and Y copies are statistically significant, via the nonparametric Mann-Whitney U test ($p = 0.008$).

species *Carica papaya* [44]. In *S. latifolia*, the *DD44Y* gene, which is only moderately diverged from its X allele, has acquired TEs in its introns to the same extent as the much more diverged *SlY3* gene. The absence of insertions in some genes with the highest X-Y divergence suggests that TE insertion is a random process, and that 10 million years may be too little time for all genes to gain insertions.

There is also no correlation between higher ω_Y/ω_X ratios and lower Y/X expression ratios in our set of genes. The variance of ω estimates for genes with low X-Y divergence is high, and further tests using many genes will be needed to accurately estimate ω for genes with low X-Y divergence, but this result is compatible with reduced expression due to transposable-element insertions, and with random inactivation, as proposed for *Drosophila miranda*, in which silencing of neo-Y-linked alleles seems to be unrelated to whether a gene is degenerated, and even genes encoding potentially functional proteins can be silenced [8].

Y Degeneration in Plants versus Animals

The same trends (faster protein evolution, insertions of transposable elements, and low expression) are found in *Silene* Y genes as in animal Y or neo-Y genes. Our results suggest that the *Silene* Y genes have ω_Y/ω_X values very similar to those for the *D. miranda* neo-Y (the value estimated by PAML for *S. latifolia*, *S. dioica*, and *S. diclinis* is 2.9, excluding the *SlX1/Y1* gene pair, which is too little diverged to provide a reliable estimate, and the *D. miranda* value is 2.0, from [5]). In our set of functional *Silene* Y-linked genes, the effect of expression in pollen has therefore not detectably slowed the accumulation of amino acid changes. Future work should test more loci, including ones known to be pollen expressed, and obtain BAC sequences from the *S. latifolia* sex chromosomes, to better estimate the proportion of Y genes that have been lost and compare this with values from animal sex or neo-sex chromosomes.

Experimental Procedures

Species and Genes

Details of the dataset (including the accession numbers and the sequences that were newly obtained for this study) are in Table S1.

Alignments

Coding sequences were aligned with MUSCLE via the Seaview software ([45], http://pbil.univ-lyon1.fr/). Genomic sequences were aligned with L-Fasta and the LaInview interface ([46], http://pbil.univ-lyon1.fr/). Multiple alignments of genomic sequences (*SlX3* for *S. latifolia*, *S. diocia*, and *S. diclinis* + *SlY3* for *S. latifolia*, *S. diocia*, and *S. diclinis*) used MAVID [47].

Sequence-Divergence Analysis

We ran PAML (http://abacus.gene.ucl.ac.uk/software/paml.html) and Fitmodel on the coding-sequence alignments of the seven genes in our sample, plus a phylogenetic tree. Because phylogenetic trees with only six species are unreliable, especially when evolutionary rates may differ (e.g., between X and Y sequences), the phylogenetic relationships between our set of species were based on internal transcribed spacer (ITS) and a multispecies alignment from [12]. For all genes except *SlX1/Y1*, we modified the ITS tree by introducing two distinct clades for X and Y copies, in agreement with the phylogenetic trees used for individual genes ([41] and unpublished data; Figure S1B). For *SlX1/Y1*, we used the published tree [41] in which the X and Y of each dioecious species cluster together (Figure S1A). The topology of this tree is not affected by shared polymorphisms between *S. dioica*, *S. diclinis*, and *S. latifolia* [41]. Details of the parameters used for the PAML and Fitmodel analyses are in the Supplemental Data, Methods 1.

Transposable-Element Identification

We searched for transposable elements in the introns of two sex-linked genes, *DD44X/Y* and *SlX3/Y3*, via several strategies, initially using blastn to compare the introns against themselves to detect repeats, then blastn against GenBank to find similarities with known TEs, and blastx against a set of TE proteins from *Arabidopsis thaliana* and *Silene* species to search for similarities with known transposable-element sequences. RepeatMasker (http://repeatmasker.org) was used to search for plant TEs.

Expression Analysis

Expression data were obtained by RT-PCR on male inflorescences from *S. latifolia* at different developmental stages. For each sex-linked locus, we designed pairs of primers perfectly matching both the X and Y copies, in order to amplify both types of genes with equal efficiency. RT-PCR was performed with these primers. To quantify, for each sex-linked locus, the level of expression of X and Y genes, we picked at least 30 clones at random. They were identified as X- or Y-derived via a restriction-enzyme screen or full sequencing (Supplemental Data, Methods 2). Slss was not analyzed because it belongs to a multigene family [14]. Data for *SlCypX/Y* and *SlX7/Y7* were compared with results from Pyrosequencing and were very similar (data not shown).

Supplemental Data

Additional Experimental Procedures, four figures, and three tables are available at http://www.current-biology.com/cgi/content/full/18/7/545/DC1/.

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