

Nucleotide Diversity Loss on a Plant Y Chromosome Following Recent Recombination Suppression

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ABSTRACT X and Y chromosomes differ in effective population size (N_e), rates of recombination, and exposure to natural selection, all of which can affect levels of genetic variability within the genome. On Y chromosomes with suppressed recombination, selection is expected to remove linked neutral variation and reduce the N_e of Y compared to X chromosomes or autosomes. However, non-selective factors including female biased sex ratios and high variance in male reproductive success can also reduce Y-linked N_e , making it difficult to infer the causes of low Y diversity. Here, we investigate the factors affecting X- and Y-linked neutral polymorphism during plant sex chromosome evolution in *Rumex hastatulus* (Polygonaceae), a dioecious annual with recently evolved sex chromosomes. Strikingly, we find that neutral diversity for genes on the Y is on average ~2.1% of the value for their homologues on the X, corresponding to a chromosome-wide reduction of ~93% compared to the neutral expectation. We demonstrate that the magnitude of this diversity loss is inconsistent with a reduced male N_e caused by neutral processes including female-biased sex ratios and high variance in male reproductive success. Rather, using forward population genetic simulations and estimates of the distribution of fitness effects of deleterious mutations, we show that Y diversity can be explained by purifying selection removing linked neutral variation, although selective sweeps may have also contributed. Given the recent origin of *R. hastatulus* sex chromosomes, our study suggests that Y chromosome degeneration in the early stages occurs through strong selective interference among many linked sites rather than primarily through Y-inactivation followed by neutral genetic drift.

KEYWORDS Sex Chromosome Evolution; Nucleotide Diversity; Recombination; Deleterious Mutations

Introduction

Morphologically distinct sex chromosomes have evolved multiple times independently in both plant and animal kingdoms (Westergaard 1958; Ohno 1967; Bull 1983; Charlesworth 1991). Despite clear biological differences between these kingdoms, X and Y chromosomes in both lineages have undergone similar genetic changes. For example, in both groups the loss of recombination between X and Y chromosomes has been associated with an accumulation of deleterious mutations and a gradual loss of genes from the Y chromosome (Hough *et al.* 2014; Bergero *et al.* 2015; Bachtrog 2013), and in some species, the genetic degeneration of the Y has

led to the evolution dosage compensation of the X chromosome (Charlesworth 1996; Muyle *et al.* 2012; Mank 2013; Papadopoulos *et al.* 2015). The independent evolution of these phenomena in such taxonomically distant species suggest that general evolutionary mechanisms may be involved, but inferring the causes of molecular evolution and patterns of polymorphism in genomic regions that lack recombination is a longstanding challenge for both theoreticians and experimentalists (Charlesworth 1978; Feldman *et al.* 1980; Barton 1995; Charlesworth 1996; Otto and Feldman 1997; Charlesworth and Charlesworth 2000; McVean and Charlesworth 2000a).

One fundamental difference between the X and Y chromosomes is that there are 1/3 as many Y-linked gene copies as X-linked ones in a diploid population. Because of this, the effective population size (N_e) of the Y chromosome is expected to be 1/4 that of the autosomes, whereas the N_e for the X chromosome should be 3/4 that of autosomes (assuming an equal number of reproducing females and males). The lowered N_e of

the Y chromosome implies that deleterious mutations are more likely to rise to high frequency because of genetic drift (Nei and Tajima 1981). Moreover, this loss of recombination on the Y implies that genes on this chromosome will experience strong linkage disequilibrium, making them vulnerable to "Hill-Robertson Interference", in which the genetic associations between selected alleles and their genetic backgrounds cause a reduction in the efficacy of selection (Fisher 1930; Muller 1964; Hill and Robertson 1966; McVean and Charlesworth 2000b). This suggests that Y chromosomes should harbor a lower amount of neutral genetic variability than predicted based on the number of Y chromosomes in a population (proportional to the product of N_e and the mutation rate, μ , at equilibrium), though the extent to which such interference should affect chromosome-wide levels of nucleotide diversity is not known. Given that a large number of sites are probably under weak purifying selection (refs), the "interference" effect on Y chromosomes with suppressed recombination might alone cause a substantial diversity loss. Indeed, simulations suggest that the effects of HRI on 100,000 linked sites can reduce polymorphism levels by ~30 % compared to neutral expectations (McVean and Charlesworth 2000b), suggesting that HRI is likely important for the evolution of Y chromosomes.

In species with female-biased reproductive sex ratios or extensive male-male competition, high variance in male reproductive success can also reduce the N_e of genes on the Y chromosome (Caballero 1995; Charlesworth 2001; Laporte and Charlesworth 2002; Pool and Nielsen 2007; Ellegren 2009), which suggests that inferences about the effects of HRI (including the effects of purifying and positive selection) on the Y need to be distinguished from neutral models that include sex ratio variation as well as sex differences in reproductive success. Because variance in male reproductive success reduces both Y-linked N_e and autosomal N_e (Kimura and Crow 1964; Nomura 2002), evidence for this effect can be therefore obtained by comparing levels of neutral diversity on X and Y chromosomes relative to values on autosomes. For example, high variance in male reproductive success causes a reduction in the Y/A diversity ratio, but an increase in the X/A ratio. Based on such comparisons, studies in humans have suggested examples of historical sex-differences in dispersal during human migration (Wilkins 2006), though the inflated X/A ratio has also been attributed to a historical excess of breeding females over males (Hammer *et al.* 2008) (and see (Bustamante and Ramachandran 2009; Hammer *et al.* 2010; Cotter *et al.* 2016))

Despite widespread interest in determining the evolutionary factors affecting neutral diversity on both X and Y chromosomes (Ellegren 2011; Bachtrog 2013), we still know very little about the influence of demography or selection on patterns of sex-linked diversity in more recently evolved sex chromosomes, such as in dioecious plants. This information is important, however, as it highlights the relevant time scales over which effects such as linked selection are likely to be important. In humans, estimates of Y-linked diversity are considerably lower than predicted under models of neutral evolution, with simulations showing that current levels of Y-diversity are consistent with a combination of very low N_m and a history of strong purifying selection (Wilson Sayres *et al.* 2014). However, given that human sex chromosomes evolved from autosomes ~200 million years ago (MYA), is not immediately obvious whether such strong effects of purifying selection on diversity should also apply to sex chromosomes that evolved *de*

novi from autosomes over much more recent evolutionary time (e.g., within the last 20 MYA in the case of most dioecious plants (Charlesworth 2015)). Y-linked diversity loss due to purifying selection might on the one hand be expected to be lower in such systems due to recombination having been suppressed for a shorter length of time. Contrary to this intuition, however, simulations have shown that two key types of HRI - background selection and selective sweeps - both have strongest effects during the earliest stages of sex chromosome evolution, before Y-chromosomes have lost the majority of their genes (Bachtrog 2008). Indeed, this is consistent with studies of Y-degeneration in young plant sex chromosome systems, which have found evidence for rapid genetic degeneration (Hough *et al.* 2014; Papadopoulos *et al.* 2015; Charlesworth 2016). In these cases, if Y-degeneration involved widespread Y-linked gene inactivation (silencing), as predicted by (Orr and Kim 1998), and discovered on the neo-Y in *Drosophila miranda* (Zhou and Bachtrog 2012), then linked selection might in fact play a less important role than theory would predict, with the early stages of Y-degeneration instead being primarily driven by gene-silencing followed by neutral genetic drift (Bachtrog 2013).

To investigate the factors affecting nucleotide diversity in the early stages of sex chromosome evolution, we analyzed neutral polymorphism levels on X, Y, and autosomal chromosomes in the plant *R. hastatulus* (Polygonaceae). This species is a dioecious annual with highly heteromorphic X and Y chromosomes that are estimated to have evolved approximately 15 MYA (Quesada del Bosque *et al.* 2011; Grabowska-Joachimik *et al.* 2015; Navajas-Pérez *et al.* 2005), making sex chromosomes in this species over 100 million years younger than the well-studied mammalian sex chromosomes (Lahn and Page 1999; Ross *et al.* 2005). *Rumex hastatulus* has also received particular attention because of the unique occurrence of an intraspecific polymorphism in sex chromosome system, in which both XY and XY₁XY₂ males occur in geographically distinct populations ('chromosomal races') (Smith 1963). The XY₁XY₂ sex chromosome system in this species (the North Carolina race) is thought to have originated through an X-autosome fusion, with the XY system (the Texas race) maintaining the ancestral chromosome complement (Smith 1964). Despite the recent origin of sex chromosomes in both races, there is evidence that both Y's have undergone significant gene loss and functional deterioration (Hough *et al.* 2014). Here, to simplify our comparison of polymorphism levels on X, Y, and autosomes, we focus only on the ancestral Y chromosome, which occurs in both sex chromosome races.

Of particular relevance to our study, *R. hastatulus* populations have been found to consistently exhibit female-biased reproductive sex ratios, with a mean sex ratio of $N_f / (N_m + N_f) = 0.6$ (Pickup and Barrett 2013). Female-biased sex ratios in dioecious plants with heteromorphic X and Y chromosomes are not uncommon (refs) (Hough *et al.* 2013), and their occurrence in *R. hastatulus* provides an excellent opportunity to study both the demographic and selective factors contributing to sex-linked variability. Using estimates of neutral polymorphism on sex chromosomes and autosomes, together with forward population genetic simulations, our study tests whether sex chromosome evolution in the early stages is driven primarily by neutral processes, or whether linked selection has played a significant role.

Materials and Methods

Population Samples and Sex-Linked Genes

We analyzed sex-linked and autosomal genes identified from Illumina RNA sequence data from 12 males and 12 females (1 male and 1 female from each of 6 populations). Samples were collected in 2010 from throughout the native range of *R. hastatulus* (locations in Table S1), and plants were grown in the glasshouse at the University of Toronto from seeds collected from open-pollinated females. We extracted RNA from leaf tissue using Spectrum Plant Total RNA kits (Sigma-Aldrich). Isolation of mRNA and cDNA synthesis was conducted according to standard Illumina RNAseq procedures, and sequencing was conducted on two Illumina HiSeq lanes with 150-bp end reads at the Genome Quebec Innovation Center. Reads from these samples were mapped to the *R. hastatulus* reference transcriptome (Hough *et al.* 2014) using the BurrowsWheeler Aligner (Li and Durbin 2010), followed by Stampy (Lunter and Goodson 2011). We used Picard tools (<http://picard.sourceforge.net>) to process mapping alignments for the Genome Analysis Toolkit (McKenna *et al.* 2010) variant calling software, and subsequently removed genes with low coverage (<10x) and low Phred Quality Scores <20. The population samples analyzed here were previously reported in Hough *et al.* (2014), where they were used to validate the ascertainment of sex-linked genes identified through segregation analysis, and raw sequences are available from the GenBank Short Read Archive under accession no. SRP041588. Here, to consider sex linked genes that were identified in all of our sequenced population samples, we focused on the previously described set of 460 X/Y genes for which a Y homolog was found in both the Texas and North Carolina races (i.e., X/Y genes where the Y copy was inferred to be on the Y_1 chromosome).

Autosomal Genes

In evaluating evidence for nucleotide diversity differences between X and Y chromosomes, it is important to distinguish between reduced Y-linked diversity, and the possibility that X-linked diversity is elevated above the level predicted from a neutral model. To do this, we normalized our sex-linked diversity estimates by autosomal diversity, and compared empirical X/A and Y/A nucleotide diversity ratios to those predicted from neutral models and from simulations (described below). Because the criteria for ascertaining autosomal loci in Hough *et al.* (2014) were based on identifying four segregating SNPs per locus, and since this set of genes is likely to be higher in diversity than the average autosomal gene, here we instead used the larger set of all non-sex linked (putatively autosomal) genes as our autosomal reference. We filtered genes in this set to remove any genes that may have been sex-linked but were not identified as such by Hough *et al.*'s conservative ascertainment criteria. In particular, we removed: (i) any genes in which there was evidence for at least one SNP with a sex-linked segregation pattern, (ii) any genes where SNPs showed fixed heterozygosity in males and fixed homozygosity in females, (iii) genes with less than 10X coverage or greater than 100X coverage from independently obtained genomic coverage data (to filter out duplicates or genes with highly repetitive sequences), and (iv) any genes containing SNPs with large (>0.4) allele frequency differences between males and females. Finally, we removed genes with fewer than 100 synonymous sites to avoid biasing our results toward genes that may have been particularly short due to assembly problems. This filtering resulted in a final set of 12,356

autosomal genes.

Phasing X and Y alleles

To estimate polymorphism for X and Y sequences separately, it is necessary to infer the phase of SNPs in sex-linked transcripts in males. In previous work, phasing alleles on *R. hastatulus* sex chromosomes was achieved using segregation analysis from a genetic cross. Here, to phase SNPs from population samples where such segregation data was unavailable, we used HAPCUT (Bansal and Bafna 2008), a maximum-cut based algorithm that reconstructs haplotypes using sequenced fragments (Illumina read data) from the two homologous chromosomes to output a list of phased haplotype blocks containing the SNP variants on each chromosome. Because the resulting haplotype blocks produced by HAPCUT contained SNPs that were phased relative to each other, but not designated to either the X or Y chromosome, we assigned individual variants to X or Y by independently identifying fixed X-Y differences within each haplotype block (i.e., sites where all females were homozygous, and all males were heterozygous). Identifying such fixed differences within phased haplotype blocks enabled us to then infer the correct phase (X or Y) of the polymorphisms from HAPCUT's output. In particular, this was done by matching the phase of fixed X-Y differences with their neighboring polymorphic sites: when a fixed X-Y difference occurred in the same phased haplotype block as a polymorphic site, then the polymorphic variants in that block were assigned to either X or Y based on the known phase of the fixed difference with which they were matched. SNPs that were identified outside of phased blocks, or in blocks without fixed X-Y differences, were recorded as missing data. Finally, we filtered out SNPs with coverage > 60, QUAL score > 60, and those within a distance of 10bp or less from indels. This filtering procedure resulted in fasta-formatted alignments of X and Y sequences for 372 sex-linked genes.

We further validated the results of HAPCUT's allele phasing by comparing the accuracy of this method with the phasing-by-segregation method that was conducted in Hough *et al.* (2014). To do this, we first phased the sequence data from parents and their progeny using HAPCUT's algorithm (using the same parameters as for the population data), and then identified cases where SNPs were inferred on the Y chromosome by HAPCUT, but where the true level polymorphism, obtained from the genetic cross, was zero. We identified 7 % of sex-linked genes that either had phasing errors of this kind genotyping errors. This corresponds to a SNP error rate estimate of 1.7×10^{-4} . Note that this rate is very low relative to population-based estimates of polymorphism on the X and autosomes (Table 1), and therefore should have minimal effects on our estimation of the X/A ratio. However, because this rate is high relative to the expected level of polymorphism on the Y chromosome, we further filtered genes in which we found evidence for false-positive SNP calls arising from: (i) phasing errors caused by gene duplicates (more than two haplotypes), (ii) polymorphisms around indels, and (iii) genotyping errors caused by low Y-expression. This final filtering was conducted by manually checking each individual putative polymorphism on the Y chromosome using IGV (Robinson *et al.* 2011).

Estimating nucleotide diversity on sex chromosomes and autosomes

For each locus in our analysis, we calculated Wattersons (1975) estimator of the population parameter $\theta = 4N_e\mu$, where N_e is the effective population size, and μ is the mutation rate (Watterson 1975), using a modified version of the Perl program Polymorphura (Bachtrog and Andolfatto 2006). To compare sex-linked and autosomal loci, we calculated the average value of θ , weighted by the number of synonymous sites in each gene (Figure 2; Table 1). We obtained 95 percent confidence intervals for our estimates of the X/A and Y/A diversity ratios by bootstrapping per gene using the BCa method (Efron and Tibshirani 1994) implemented in the Boot package in R (Canty and Ripley 2012), and calculating X/A and Y/A diversity on each iteration for 20000 replicates each. Bootstrapping was conducted on the final filtered set of 173 sex-linked, and 12355 autosomal genes. Note that the lack of recombination on the Y chromosome implies that assumptions about independence across loci are violated, suggesting that the true uncertainty in our Y/A diversity estimate may be wider than implied by bootstrapping. To address this, we also used a maximum likelihood approach, implemented in a modified version of the MLHKA software (Wright and Charlesworth 2004), to independently estimate a credibility interval for the Y/A ratio (Figure S1). Because of the thousands of genes involved, a likelihood method incorporating divergence to control for heterogeneity in mutation rate was not feasible, as this would require maximizing the likelihood estimate of the mutation rate for each locus independently. Therefore, we assumed no heterogeneity in mutation rate, no recombination between Y-linked genes, and free recombination between autosomal loci. Our model thus had two parameters: $\theta_{autosomal}$ and Y/A. We varied both parameters and evaluated the likelihood for Y/A from 0.001 to 1, and $\theta_{autosomal}$ from 0.001 to 0.01.

Neutral predictions and the effect of sex ratio bias on diversity

To test whether the levels of diversity we observed on X, Y and autosomal chromosomes could be explained by the occurrence of biased reproductive sex ratios or high variance in reproductive success, we compared our empirical diversity estimates to predictions from a neutral model that considers these two effects (Kimura and Crow 1964). In particular, we calculated the effect of variance in reproductive success on N_e as:

$$N_{e_i} = \frac{N_i k_i - 1}{k_i - 1 + \frac{V_{k_i}}{\bar{k}}} \quad (1)$$

where k_i and V_{k_i} are the mean and variance in offspring number, respectively, for the i th sex and \bar{k} is the total mean number of progeny (Kimura and Crow 1964). Using sex-specific values of k and V_{k_i} to calculate N_m and N_f , the expected N_e for autosomes and sex chromosomes is then obtained using equations from (Wright 1931):

$$N_{eA} = \frac{4N_m N_f}{N_m + N_f} \quad (2)$$

$$N_{eX} = \frac{9N_m N_f}{4N_m + 2N_f} \quad (3)$$

$$N_{eY} = \frac{N_m}{2} \quad (4)$$

Note that with equal sex ratios, $N_{eX}/N_{eA} = 0.75$, and $N_{eY}/N_{eA} = 0.25$. Figure 1 shows the expected X/A and Y/A ratios as a function of the reproductive sex ratio across a range of values for the variance in male reproductive success. We therefore compared our empirical diversity ratios to these predictions (Figure 2) using a sex ratio of 0.6 as estimated in *R. hastatulus*

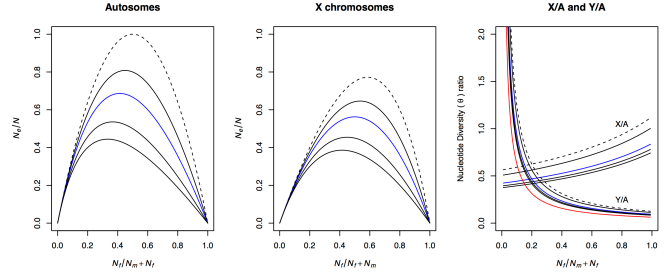


Figure 1 The relation between relative effective population size and sex ratio bias for genes on autosomes (A) and sex chromosomes (B) (Y chromosome in red). The sex ratio is shown as the proportion of males, $N_m/(N_f + N_m)$, where N_m and N_f are the effective number of breeding males and females, respectively, plotted against N_e/N , where $N = N_m + N_f$ and the N_e for sex chromosomes and autosomes is given in equations 1-3. Dotted curves show predictions in the standard neutral model, where both males and females produce Poisson-distributed offspring numbers and the chromosomal N_e 's are given by equations 4-6 (Wright 1931). Solid curves correspond to increasing levels of variance in male reproductive success (Nomura 2002) (see Methods). Assuming $\theta = 4N_e\mu$ and equal neutral mutation rates among genes, the predicted N_e 's are used to generate null predictions for X/A and Y/A ratios of diversity.

Simulations of purifying selection

To test whether our observed level of Y-chromosome diversity could be explained by the effects of linked purifying selection, we followed the approach used in (Wilson Sayres et al. 2014) and conducted forward-time simulations of a non-recombining Y chromosome using the software SFSCODE (Hernandez 2008) and compared our empirical diversity estimates on the Y with those from simulations. This approach was implemented rather than using analytical predictions of background selection primarily because the equilibrium background selection model over-predicts the reduction in diversity when there are many linked sites under selection (Kaiser and Charlesworth 2009), as is expected to be the case for large Y chromosomes that lack crossing over.

To parameterize the forward simulations, we estimated the distribution of fitness effects of deleterious mutations (DFE) using our polymorphism data on the X-chromosome with the program DFE-alpha (Keightley and Eyre-Walker ref). This program fits a gamma distribution of selection coefficients to the observed frequency distribution of nonsynonymous and synonymous polymorphism from the population genetic analyses above. We used this estimated gamma distribution in our forward simulations. To examine the expected reduction in diversity, we varied the number of sites under selection, and obtained an approximate likelihood surface for our observed data

based on the proportion of simulations that matched the observed estimate of $\pi_{\text{synonymous}}$. To make the simulation output comparable to our data, we initialized the simulations with our empirically estimated autosomal θ , adjusted for the expected neutral reduction in effective population size on the Y chromosome under a sex ratio of $N_f/(N_f+N_m)=0.6$, and we sampled 6 haploid chromosomes per simulation. Simulated sequences contained 45,331 of linked neutral sequence from which we calculated diversity, matching the number of synonymous sites sampled in our empirical analyses on the Y chromosome (see Supporting Information).

Results and Discussion

Extensive loss of Y-chromosome diversity

Our analysis reveals that diversity on the *R. hastatulus* Y chromosome is significantly lower than expected under neutrality, with estimates indicating $Y/A=0.02$, ...which is 12.5 fold lower than the standard neutral prediction of $Y/A = 0.25$ ($P<0.0001$). We also observe that the Y chromosome shows a 40-fold lower than mean diversity on the X chromosome (Table 1). Note that by normalizing X and Y diversity by autosomal diversity, our results indicate that the X-Y difference we observed was not due to an elevation of X chromosome diversity, but rather a Y-specific reduction. Conceivably, such low diversity on the Y could arise from a low Y-linked mutation rate, or a lower mutation rate in males compared to females. However, these possibilities are unlikely because the number of synonymous mutations in X and Y lineages, estimated by both parsimony and maximum likelihood, are not significantly different (Hough *et al.* 2014). Similarly, our estimates of weighted average synonymous substitution rate between *R. hastatulus* and the out-group *R. bucephalophorus* reveal similar levels of synonymous divergence at sex-linked (0.2016) and autosomal genes (0.219) (Hough *et al.* 2014).

In contrast to the X chromosome, however, our data indicate a strong and consistent diversity reduction on the Y chromosome: an approximately 50 fold reduction compared to the mean θ_{Aut} . We next consider several possible models - neutral and selective - that might explain this reduction.

Female biased sex ratio and high variance in male fitness

The occurrence of female-biased sex ratios in *R. hastatulus* is expected to lower Y diversity through lowering male N_e and therefore the N_e of the Y chromosome. In addition, the N_{eY} might be further reduced due to high variance in male reproductive success (Figure 1), which is not unusual in annual wind-pollinated plants such as *R. hastatulus* that exhibit extensive phenotypic plasticity in plant size and flower production (Harper 1977). Given that male plants in this species produce large amounts of pollen, and female flowers are uniovulate, there is reason to believe that there could be strong competition among males.

In common with most flowering plants we do not have marker-based estimates of the variance in male reproductive success in *R. hastatulus*. However, by comparing our empirical estimates of diversity to predictions from models that jointly predict the effects on diversity of sex ratio bias and male reproductive variance, we evaluated whether these effects could jointly explain the level of Y/A diversity that we observed (see Methods). Conditioning on estimates of sex ratio bias in *R. hastatulus*, ranging from $N_m/(N_m + N_f) = 0.4$ to $N_m/(N_m +$

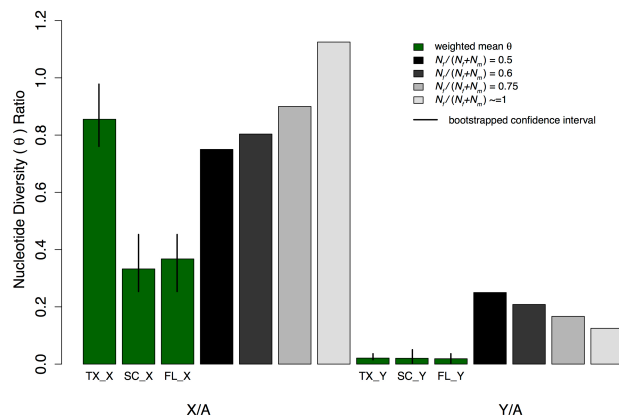


Figure 2 The relation between relative effective population size and sex ratio bias for genes on autosomes (A) and sex chromosomes (B) (Y chromosome in red).

$N_f) = 0.35$ (Pickup and Barrett 2013), the predicted Y/A diversity ratio is approximately 0.2 (Table1, Figure 3). This is significantly lower than our estimated mean Y/A ratio of 0.02 ($P < 0.0001$), and remains significant even if we consider an upper bound estimate obtained from our likelihood based estimated of the confidence interval. This suggests that the sex ratio effect alone is insufficient to explain our data under the standard sex ratio model.

Assuming that there is extensive variance in male reproductive success (σ), the predicted ratios of Y/A diversity (supp) were also significantly higher than our estimates for the empirically estimated sex ratio (table). We also find, however, that purely neutral models in which the sex ratio was highly female-biased (σ), with level of variance in male reproductive success on the order of (σ), predicted a Y/A ratio that could not be rejected (σ). However, because a highly females biased sex ratio is expected to increase the X/A ratio as well, these models simultaneously predicted a range of X/A ratios that were significantly different from what we observed (Figure). Thus, our results indicate that the combined effects of sex ratio bias and variance in reproductive success cannot jointly explain our observed levels of X, Y, and autosomal diversity.

Background Selection and Selective Sweeps

Conclusions

The observation of widespread degeneration and diversity loss on Y chromosomes illustrates the importance of recombination for maintaining fitness and the genetic variability needed for adaptation (Maynard Smith 1978; Kondrashov 1993; Barton Charlesworth 1998). The extensive loss of diversity on a young plant Y chromosome revealed by our study provides a clear example of the a large genomic regions that lack recombination.

Acknowledgments

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