Experimental evolution confirms signatures of sexual selection in genomic divergence.

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Abstract

Comparative genomics has contributed to the growing evidence that sexual selection is an important component of evolutionary divergence and speciation. Divergence by sexual selection is implicated in faster rates of divergence of the X chromosome and of genes thought to underlie sexually selected traits, including genes that are sex-biased in expression. However, accurately inferring the relative importance of complex and interacting forms of natural selection, demography and neutral processes which occurred in the evolutionary past is challenging. Experimental evolution provides an opportunity to apply controlled treatments for multiple generations and examine the consequences for genomic divergence. Here we altered sexual selection intensity, elevating sexual selection in polyandrous lines and eliminating it in monogamous lines, and examined patterns of divergence in the genome of *Drosophila pseudoobscura* after more than 160 generations of experimental evolution. Divergence is not uniform across the genome but concentrated in "islands", many of which contain candidate genes implicated in mating behaviours and other sexually selected phenotypes. These are more often seen on the X chromosome, which overall shows divergence above neutral expectations. There are also characteristic signatures of selection seen in these regions, with lower diversity and greater F_{st} on the X chromosome than the autosomes, and differences in diversity on the autosomes between selection regimes. Reduced Tajima's D implies that selective sweeps have occurred within the divergent regions, despite considerable recombination. These changes are associated with both differential gene expression between the lines and sex-biased gene expression within the lines. Our results are very similar to those thought to implicate sexual selection in divergence in natural populations, and hence provide experimental confirmation of the likely role of sexual selection in driving such types of genetic divergence, but also illustrate how variable outcomes can be for different genomic regions.

Impact Summary

How does sexual selection contribute to the divergence of genomes? It is often thought that sexual selection is a potent force in evolutionary divergence, but finding 'signatures' of sexual selection in the genome is not straight-forward, and has been quite controversial recently. Here we used experimental evolution to allow replicate populations of fruit fly to evolve under relaxed or strengthened sexual selection for over 150 generations, then sequenced their genomes to see how they had diverged. The feature we find are very similar to those reported in populations of natural species thought to be under strong sexual selection. We found that genomic divergence was concentrated in small patches of the genome rather than widespread. These are more often seen on the X chromosome, which overall shows greatly elevated divergence. There are also characteristic signatures of selection seen in these regions, with lower genetic diversity and greater differences on the X chromosome than the autosomes. Selection was probably strong in these regions. The changes are associated with both differential gene expression between the lines and sex-biased gene expression within the lines. Many of the patches of divergence also contain candidate genes implicated in mating behaviours and other sexually selected phenotypes. Our results provide experimental confirmation of the likely role of sexual selection in driving such types of genetic divergence.

Introduction

The role of sexual selection in influencing evolutionary divergence and speciation is unclear (Panhuis *et al.*, 2001; Ritchie, 2007; Maan & Seehausen, 2011; Servedio & Boughman, 2017). Associations between species diversity and proxies of sexual selection such as sexual dimorphism or mating system variation often imply that sexual selection can accelerate divergence, especially when acting alongside natural selection (Arnqvist *et al.*, 2000; Gage *et al.*, 2002; Ellis & Oakley, 2016). However, different indicators of sexual selection give contrasting results in such comparative studies, and a consensus is not clear (Kraaijeveld *et al.*, 2011; Janicke *et al.*, 2018). One potentially compelling source of evidence that sexual selection is involved in divergence is coming from the increasing number of comparative genomic studies available across a range of organisms. Many descriptions of genomes, including those of species thought to have undergone strong sexual selection such as the Hawaiian *Drosophila* or African cichlids, have found that genes associated with mating behaviour or sensory perception potentially involved in sexual communication are often outliers in measures of divergence (e.g. Mattersdorfer *et al.*, 2012; Kang *et al.*, 2016). It has also been known for some time that genes which diverge particularly rapidly and show stronger signatures of positive divergent selection are often sex-biased in expression (Pröschel *et al.*, 2006; Ellegren & Parsch, 2007; Zhang *et al.*, 2007). Sex-biased gene expression itself, especially male-biased expression, evolves rapidly and is associated with indicators of sexual selection such as increased sexual dimorphism in birds (Harrison *et al.*, 2015; Wright *et al.*, 2019). Additionally, divergence of sex chromosomes between species is usually much greater than autosomes, sometimes dramatically so (Counterman *et al.*, 2004; Ellegren *et al.*, 2012).

However, such patterns of divergence are not necessarily driven by elevated sexual selection on these genes or genomic regions. The evolution of sex-biased gene expression is often due to sexually antagonistic selection on gene expression, which is often an important factor in sexual selection. However, it is complicated by additional factors including dosage compensation, as well as turnover of sex-biased expression and resolution of conflict via sex-linkage or sex-limited expression (Mank *et al.*, 2010a; Wright *et al.*, 2019). The increased divergence of sex chromosomes is potentially influenced by many factors, only one of which is sexual selection. These include a greater role of genetic drift due to a smaller effective population size on X chromosomes compared to autosomes, dominance effects, and other consequences of sex-linkage such as dosage compensation (Vicoso & Charlesworth, 2006; Ellegren, 2009; Mank *et al.*, 2010b). Hemizygosity results in a lower effective population size (N_e) on the X (N_{eX}) than on autosomes (N_{eA}). Under random mating the ratio is expected to be

3:4 and this should reduce neutral diversity and increase between-species divergence by the same proportion (Vicoso & Charlesworth, 2006). Hemizygosity should also result in an increased efficacy of selection for partially recessive beneficial mutations and against recessive deleterious mutations on the X. Finally, because of the inheritance patterns of X-linked loci (males transmit them only to daughters while females transmit them to both daughters and sons), sex-specific selection as well as sexual selection will influence their divergence (Mank *et al.*, 2010a; Corl & Ellegren, 2012).

It is very difficult to infer the historical role of different evolutionary processes from patterns of contemporary divergence between populations and species, because they can result in similar genomic signals (Butlin *et al.*, 2012). One way of directly addressing the role of sexual selection or mating system variation in genomic divergence is to examine the genomic consequences of experimental evolution under manipulated sexual selection regimes in the laboratory. A great advantage of this approach is that there are potentially fewer confounding variables involved than when making comparisons across species or natural populations. However, a disadvantage is that the time scale over which divergence can be studied is typically much shorter than evolutionary time-scales in nature. Studies of experimental evolution and speciation are in their infancy, and general conclusions are, as yet, difficult to draw (White *et al.*, 2020). Enforcing monogamy in otherwise polyandrous species will lead to both changes in the intensity of sexual selection and the balance of sexual conflict, as it effectively eliminates sexual selection and sexually antagonistic selection. A classic example of such manipulation is where *D. melanogaster* were kept under enforced monogamy for about 50 generations (Holland & Rice, 1999). Females from the monogamy treatment had reduced longevity compared to ancestral females, when exposed to ancestral males. This was expected because the reduction of conflict should favour less harmful males and females that are less resistant to male harm. Other experimental evolution studies under altered mating systems have been performed in dung flies (Hosken *et al.*, 2001; Hosken & Ward, 2001; Martin & Hosken, 2003), different species of fruit flies (*D. melanogaster*; (Gerrard *et al.*, 2013; Hollis *et al.*, 2014; Innocenti *et al.*, 2014; Perry *et al.*, 2016); *D. pseudoobscura*; (Crudgington *et al.*, 2005), seed beetles (McNamara *et al.*, 2020) and hermaphroditic flatworms (Janicke *et al.*, 2016). Though aspects of the treatments differ amongst such experiments, some common patterns have emerged. Gene expression changes are seen, especially of genes that are initially sex-biased, though the details can vary between studies (Hollis *et al.*, 2014; Veltsos *et al.*, 2017). Moreover, gene expression changes can be more pronounced for genes expressed in

reproductive tissues (Innocenti *et al.*, 2014) , and genes involved in the post-mating physiological manipulation of female egg-laying and re-mating rates (Perry *et al.*, 2016).

A feature emerging from genomic comparisons between diverging species is that details of genomic architecture complicate the assessment of patterns of divergence across chromosomes. Whole chromosomal regions can show correlated responses due to reduced recombination and hitchhiking effects, especially in species with segregating inversions. Early studies of species differences interpreted "islands" of divergence in the genome as resulting from divergent selection on genes within these regions with gene flow homogenising the genetic background (Turner *et al.*, 2005; Nosil *et al.*, 2009). More recently it has been appreciated that chromosomal inversions and other regions of low recombination or diversity can accentuate such clustered divergence (Noor & Bennett, 2009; Cruickshank & Hahn, 2014; Wolf & Ellegren, 2016; Ravinet *et al.*, 2017). "Barrier loci", genomic regions under divergent selection that restrict gene flow (Butlin & Smadja, 2018), may occur within such clusters but the lack of recombination makes them difficult to localise precisely. In experimental evolution the amount of recombination will be determined by both genomic architecture and the number of generations completed during the study, which is often modest in studies of eukaryotes. Also, in experimental evolution the lines can be kept effectively allopatric, so homogenising gene flow in regions not experiencing selection should be absent. The genomic divergence which occurs during experimental evolution is usually extensive, with widespread differences dispersed throughout the genome (Kawecki *et al.*, 2012; Tobler *et al.*, 2014; Michalak *et al.*, 2019).

Here we directly test the influence of sexual selection on genomic divergence. We examine replicated experimentally evolved lines of *D. pseudoobscura* in which sexual selection has been manipulated for over 160 generations. One set of 4 replicate lines were raised under enforced monogamy (M lines), which should eliminate both sexual selection and conflict. Another 4 replicates were reared under elevated polyandry (E lines), with 6 males per female. Polyandry mediates the strength of both intra- and intersexual selection and sexual conflict (Pizzari & Wedell, 2013) and elevated polyandry will increase both pre- and post- copulatory sexual selection via female choice and sperm competition (Snook, 2014). Previous studies of these lines have found divergence in some, but not all, of the types of traits predicted to diverge under sexual selection. Sperm morphology and heteromorphism, and testis mass did not diverge, but E males had larger accessory glands and a greater mating capacity (Crudgington *et al.*, 2009), were more competitive in mating encounters (Debelle *et al.*, 2016), and produced more attractive courtship song than M males (Debelle *et al.*, 2017). Coe-

volutionary changes have occurred in female song preferences (Debelle *et al.*, 2014). Sexu-
174 ally dimorphic cuticular hydrocarbons have also diverged between the lines (Hunt *et al.*, 2012).

Patterns of gene expression have changed between the lines. E females show an in-
177 crease in expression of genes normally enriched in ovaries (Immonen *et al.*, 2014). Sex-biased genes responded more strongly to the sexual selection treatment, but the direction of gene expression changes differed between sexes, tissues, and according to courtship experi-
180 ence (Veltsos *et al.*, 2017). In most cases, the transcriptome was "feminised" under polyandry (i.e. female-biased genes were up-regulated or male-biased downregulated in E lines), in a striking contrast to a similar study with *D. melanogaster* (Hollis *et al.*, 2014). Males changed
183 in patterns of gene expression in the testes and accessory glands, and changes in gene expression in females following mating also diverged, especially in the female reproductive tract (Veltsos et al. *in prep*.).

186 Here we examine genomic divergence between these lines using a pool-sequence approach after more than 160 generations of experimental evolution. The relatively long timescale of this study should reduce linkage effects on allele frequency changes. We identify al-
189 leles that changed in frequency consistently across the replicates, to help reduce the potentially confounding effects of drift or replicate-specific selection. We find that divergent SNPs are not distributed randomly across the genome, but occur in distinct, obvious clusters. We
192 examine what genes are involved and find several with mutant phenotypes related to mating and courtship behaviours. We found that the X chromosome has accumulated more divergence than the autosomes and explore if divergence is associated with recombination rate or
195 changes in gene expression between the experimental lines.

Methods

198 *Experimental Evolution*

A full description of the experimental evolution methods is available elsewhere (Crudgington *et al.*, 2005). Briefly, a population of *D. pseudoobscura* was established from
201 50 wild caught females, bred in the laboratory for four years then four independent monogamy (M) and elevated polyandry (E) lines were established. M females were housed with a single male and E females with 6 males, with females typically mating with two or
204 three males. The effective population size was maintained around 120 (Snook *et al.*, 2009) for both treatments. At each generation, offspring were collected and pooled together for each replicate line, and a random sample used to constitute the next generation in the appropriate

sex ratio, thus reflecting the differential offspring production across families (Crudgington *et al.*, 2005; Crudgington *et al.*, 2009). Enforced monogamy is expected to eliminate sexual selection and sexual conflict while elevated polyandry increases both pre- and postmating sexual selection and sexual conflict (Crudgington *et al.*, 2005; Bacigalupe *et al.*, 2007; Crudgington *et al.*, 2009).

Sequencing and Mapping

Sequencing was carried out after ca. 160 generations of selection (specifically, 164 for replicate 1, 163 for replicate 2, 162 for replicate 3, and generation 160 for replicate 4). Two pools of 40 females (one E and one M) were taken from each replicate line and genomic DNA extracted using a standard Phenol-Chloroform extraction protocol. Each pool was sequenced across two lanes on a Illumina HiSeq platform at the Center for Genomic Research (CGR) at the University of Liverpool. Details of coverage are provided in the supplementary material. Reads from each sequenced pool were mapped to the *D. pseudoobscura* reference genome (FlyBase v3.1 February 2013) using BWA mem (v. 0.7.7; Li, 2013). Alignments were filtered to remove duplicate reads, reads with a mapping quality < 30, and any reads which were not properly paired, using samtools (v 1.3; Li *et al.*, 2009 following Schlotterer *et al.*, 2014). Reads were locally re-aligned around indels using GATK (v3.7.0; McKenna *et al.*, 2010; DePristo *et al.*, 2011). The .bam files for each line were then merged using bamtools (Barnett *et al.*, 2011) and the genome-wide coverage calculated from these merged files with bedtools (v. 2.26; Quinlan & Hall, 2010). SNPs were called using a heuristic SNP calling algorithm (PoolSNP; Kapun *et al.*, 2020). Sites were considered only if the total coverage at the site was > 17 and < the 95th percentile for each contig or chromosome. An allele was only called if the count for that allele across all pools was > 16. Nearly 2 million SNPs were called and used in downstream analyses (see Supplementary Material).

Genomic Analyses

Identifying Consistent Allele Frequency Differences

Many evolve and resequence studies of *Drosophila* find that a multitude of SNPs have diverged, perhaps tens of thousands (Michalak *et al.*, 2019). The number is inflated upwards at least in part due to segregating inversions and other areas of low recombination, and hitchiking (Barghi & Schlotterer, 2019). In order to focus on the loci most likely to have diverged due to the treatment, we only considered as significant SNPs which diverged consistently across all 4 replicate pairs of lines. We identified these using quasibinomial

Generalised Linear Models, which are less prone than other statistical approaches to be influenced by strong divergence in only some replicates (Wiberg *et al.*, 2017). The model structure applied was;

$y \sim treatment + e$

where y is the allele frequency, *treatment* is the experimental evolution treatment regime, and e is a quasibinomially distributed error term. +1 was added to zero counts in any population. P-values were converted to q-values using the "qvalues" R package (v. 2.16.0; Storey & Tibshirani, 2003). A threshold of 0.05 was chosen to control the false discovery rate (FDR), thus we define "top SNPs" as those with q-value < 0.05 and the rest are referred to as "background" SNPs.

Genetic Diversity and Differentiation

We calculated genome-wide genetic diversity statistics (π and Tajima's D) for windows of 50kb (with a 10kb overlap) using available python scripts (Kapun *et al.*, 2020). Similarly, we computed pairwise F_{st} estimates between E and M line pairs for each SNP using the R package "poolfstat" (v. 0.0.1; Hivert *et al.*, 2018), averaged in windows of 50kb (with a 10kb overlap between windows). Comparisons of parameters between selection regimes and genomic regions were tested using non-parametric Wilcoxon tests. Additionally, we estimated the F_{st} expected from drift and differences in effective population sizes on X chromosomes (F_X) as in (Machado *et al.*, 2016) using the equations of (Ramachandran *et al.*, 2004) (equation 8), F_X is given by:

$$F_X = 1 - \left\{ \frac{\left(9(z+1)(1-F_A)\right)}{\left(8(2z+1) - (1-F_A)(7z-1)\right)} \right\}$$

where, z is the ratio of the number of breeding males to females and F_A is the observed F_{st} on autosomes. We assumed z to be either 1 or 6 to represent extreme possibilities based on the mating system manipulation. For each E-M pairwise comparison, we calculated mean F_{st} across each chromosome type and converted to F_X. We used a bootstrapping approach to obtain a random distribution of F_X for each replicate. For each of 1,000 bootstrap iterations we sampled, with replacement, a number of windows equal to the total number across all autosomes from the set of all windows, then we calculated mean F_{st} across all sampled windows and converted to F_X using the equation above. Additionally, we computed a value of

273 F_{st} and Tajima's D for each annotated *D. pseudoobscura* gene by taking the mean value across all 50kb windows that spanned a gene.

276 *Linkage Disequilibrium (LDx)*

Although haplotype information is not available from pool-seq data, short range linkage information is available from paired reads. We used LDx (Feder *et al.*, 2012) to first compute
279 the r^2 of SNPs located on the same read pairs. We only used SNPs with a minor allele frequency > 0.1, a minimum coverage of 10, a maximum read coverage of 400, and a phred score > 20. We binned pairs of SNPs into distance classes and then computed mean r^2 per
282 distance class. We only used distance classes with a minimum of 5 SNPs. We estimated the decay of r^2 as a function of distance by fitting a linear model of r^2 as a function of the log of the distance between the SNPs. Thus, the slope measures the decay rate of linkage due to
285 recombination (Feder *et al.*, 2012), giving an indication of the distance over which LD is present. In regions of low recombination one would expect high overall values of r^2 but a weakly negative slope as LD is maintained over relatively longer regions of the genome.
288 Comparing the slope parameter across different genomic regions gives an indication of differences in the recombination rate (or extent of selective sweeps). This was performed for each chromosome, as well as for different regions on the 3rd chromosome (see below).

291

Functional Genomics

In analyses of gene function and regulation we used the *D. pseudoobscura* annotation
294 and a dataset of regulatory long non-coding RNAs (lncRNAs; Nyberg & Machado, 2016). We identified genes or lncRNAs within a distance of 10kb up- or downstream of focal SNPs with bedtools (Quinlan & Hall, 2010) intersect (keeping any potential ties). Enhancer regions,
297 transcription factor binding sites, and other regulatory regions can occur up to 1 Mb up- or downstream from a target gene in other species (e.g. Maston *et al.*, 2006; Chan *et al.*, 2010; Werner *et al.*, 2010; Pennacchio *et al.*, 2013) but typically lie within 2kb of a gene region in
300 *D. melanogaster* (Arnosti, 2003), 10kb thus represents a compromise. We submitted the implicated genes to ModPhEA (Weng & Liao, 2017) for phenotypic enrichment analysis. We combined the phenotypic classes "courtship behavior defective" (FBcv:0000399) and
303 "mating rhythm defective" (FBcv:0000401) into one phenotype group and also tested the phenotypic class "stress response defective" (FBcv:0000408) for enrichment. We chose these classes *a priori* because they were most likely to be involved in phenotypic differences
306 between the treatments related to mating or courtship behaviour and responses.

We also took advantage of recent sex- and tissue-specific expression data from the same experimental evolution lines (Veltsos *et al.*, 2017; Veltsos et al., *in prep.*) to ask if SNPs
309 co-localised with genes that are differentially expressed between the lines and if these also show different levels of diversity (Tajima's D) or differentiation (F_{st}) between E and M lines. Full details of the differential expression analyses are given elsewhere (Veltsos *et al.*, 2017
312 Veltsos et al., *in prep.*). Briefly, gene expression was measured in different tissues, and for individuals of different mating status (virgin or mated). For simplicity here we consider a gene to be differentially expressed if it shows significant differences between E and M lines
315 in any of the tissues examined; male abdomens, male heads, testes, accessory glands, female abdomens, female heads, reproductive tracts of mated or virgin females, and ovaries of mated or virgin females. We used a bootstrapping approach, sampling genes (without replacement)
318 from the *D. pseudoobscura* annotation, to determine the amount of overlap with the DE genes that is expected by chance. For each bootstrap sample, we picked 428 genes from the annotation, which is the same size as the set of genes near top SNPs (see Results). We then
321 calculated the proportion of these genes that also occur in the DE gene sets and repeated this procedure 1,000 times to build a distribution of expected overlap between bootstrap samples and the DE gene sets. If the empirical set of genes near top SNPs had a proportion >= the 95th
324 percentile of the bootstrap distribution it was deemed a "significant" overlap. Furthermore, using the values of Tajima's D and F_{st} computed for each gene (see above) we also asked whether there was any evidence of different levels of diversity or divergence between DE
327 genes in any set (N = 3,173) and non-DE genes (N = 13,583). For Tajima's D we contrast DE and non-DE genes separately for each chromosome type (autosomes, X-chromosome left arm, X-chromosome right arm), and each experimental evolution treatment (E and M; 6
330 contrasts in total), using Wilcoxon rank sum tests. For F_{st} we contrast DE genes and non-DE genes separately for each chromosome type (3 contrasts), testing for differences with Wilcoxon rank sum tests. In both cases, the mean value for non-DE genes was used as a
333 single value against which to compare DE genes, which reduces the effect of the enormous sample size for the non-DE genes on the significance of the test. Finally, we asked whether the changes in sex-biased expression (data from Veltsos *et al.*, 2017) between E and M
336 treatments (ΔSB_{EM}) was related to diversity (Tajima's D) within either E or M lines. Sex-bias in expression was assessed for two tissues, head and abdomen, in both courted or virgin data combined. Within each tissue, sex-bias was computed as the log(fold change) in expression
339 between males and females in E and M lines separately, after which ΔSB_{EM} is calculated as $\log(FC)_E - \log(FC)_M$. Thus, positive values of ΔSB_{EM} correspond to greater male-bias in

expression in the E lines, while negative values correspond to greater male-bias in the M lines. ΔSB_{EM} was then related to values of Tajima's D in either E ($TajD_E$) or M ($TajD_M$) lines. For each tissue (head and abdomen) we performed an ANCOVA with chromosome (autosome, X-chromosome right arm, and X-chromosome left arm) as a co-factor, as well as mean Tajima's D across E lines and mean Tajima's D across M lines as co-variates. We also included the interactions between Tajima's D and chromosome. The full model is:

$$\Delta SB_{EM} \sim chromosome + TajD_E + TajD_M + TajD_E{:}chromosome + TajD_M{:}chromosome$$

We further extracted the 30bp up- and down-stream of each SNP from the reference genome using gffread from the Cufflinks package (v2.2.1; (Trapnell *et al.*, 2010) and tested for enrichment of TF binding site motifs around divergent SNPs with the AME routine from the MEME package (v. 4.10.2; (McLeay & Bailey, 2010). GO term enrichment analysis was performed with GOwinda (v. 1.12; (Kofler & Schlotterer, 2012). We considered SNPs to be associated with genes if they occurred within 10kb up or downstream of an annotated gene. An empirical p-value distribution was produced from 1 million simulated SNP sets.

All statistical analyses were made with R (v. 3.6.3; R Development Core Team 2020) except where otherwise stated. Figures were drawn using the "ggplot2" package (v. 2.2.1; (Wickham, 2009) and associated packages (table S1).

Results

Consistent Allele Frequency Differences

In total, 480 SNPs show significant consistent allele frequency differences due to the experimental evolution treatment (hereafter the "top SNPs"). These occur on all of the main chromosomes but many show striking co-occurrence into a few clusters of highly differentiated SNPs (figure 1A). The distribution of the top SNPs across the genome is not random, with a significant excess on the 3rd chromosome and both arms of the X chromosome (table S3). In particular, a large cluster of differentiated SNPs are observed at the end of the right arm of chromosome 3 (figure 1A). Other large clusters occur on both arms of the X chromosome (figure 1A). If all top SNPs within 50kb of others are grouped into clusters, this produces 70 distinct clusters throughout the genome (figure 1A). The majority of SNPs (72.9%) occur in only 6 clusters with > 10 SNPs. The clusters do not correspond to known inversions in *D. pseudoobscura.* In particular, the large cluster on chromosome 3 containing many (N = 199, 41.5%) top SNPs does not correspond to the most common inversions that

375 have shaped the evolution of this chromosome in the wild (Wallace et al., 2011; Wallace et al., 2013). Allele frequencies in E and M lines for the top 100 SNPs are shown in figure S2. More than half of these (57%) are fixed differences in all replicates. Across all the top SNPs,
378 12% are fixed differences between the E and M lines in all replicates, with all of these occurring on the X chromosomes

381 *Genetic diversity*

The observed discrete clusters of divergent SNPs are unusual in evolve and resequence studies. A possible explanation is strongly localised selective sweeps, which would reduce
384 Tajima's D. Within E lines, Tajima's D is actually slightly higher within the clusters containing top SNPs (-0.03) than outside these clusters (-0.05; Wilcoxon signed rank test: V = 17623, p-value = 0.04). Within M lines there is no statistically significant difference between
387 clusters (-0.07) and outside clusters (-0.6; V = 13390, p-value = 0.3). However, patterns of Tajima's D are very variable. The most differentiated region on chromosome 3 shows reductions in Tajimas's D within replicates from the E treatment compared to the M treatment
390 (figure 1B), as would be expected following selective sweeps. Similar patterns are seen for some peaks on the X chromosome (figure S3). In a few cases, there are reductions of Tajima's D associated with regions containing top SNPs within M lines compared to E lines
393 (figure 1B and figure S3). However, many of these regions are quite small and consequently estimates of Tajima's D may be unreliable (figure S3).

Nucleotide diversity across the chromosomes was estimated as π (figure S4). Diversity
396 is lower overall in E lines than in M lines (figure 2A). Lowest diversity (in both treatments) is seen on the more differentiated chromosomes (X and 3; figure 2A) and mean π is marginally non-significantly lower within the clusters of M (V = 12471, p = 0.052), but not E (V =
399 13843, p = 0.19), lines. The ratio of diversity between the sex chromosome and autosomes is lower in E lines than in M lines, though this is variable across replicates (figure 2B). Overall, it seems like there is greater evidence for selective sweeps in E lines, especially for the X.

402 F_{st} between the E and M lines is always higher on the X chromosome than on autosomes (figure 3A). Moreover, average F_{st} on the X is greater than expected when compared to the autosomes, even after accounting for the effects of drift and expected
405 differences in effective population sizes when compared to F_{st} on the autosomes (figure 3B) and the X:A ratio of F_{st} is always > 1 (figure 3C). As expected, F_{st} was higher within peak regions than outside peak regions (0.64 vs. 0.59; Wilcoxon signed rank test: V = 15309, p-
408 value < 0.001, Figure 3D).

Linkage Disequilibrium

411 A combination of hitchhiking and selective sweeps could lead to clustered genomic divergence, often with low diversity, especially in regions of low recombination such as telomeric regions. We examined patterns of linkage disequilibrium in the clusters and if this 414 varied with treatment. Throughout the genome, the decay rate (*a* parameter) of LD is generally shallower (i.e. less negative) in the E treatment (figure 4A). This is seen for chromosome 3 as well as both arms of the X chromosome (figure 4A). A lower decay rate is 417 indicative of more LD, due to less recombination and/or a potential for greater hitchhiking under positive selection. Contrary to predictions, we found a steeper rate of decay (less LD) within the differentiated region of chromosome 3 than outside it, especially in E lines (figure 420 4B and C). Although statistically significant ($F_{(2,13)} = 4.6$, $p < 0.001$), these differences are slight. The most striking pattern overall is greater overall LD on chromosome 3.

423 *Gene functions and expression variation*

The top SNPs were not significantly enriched in any GO terms after correcting for multiple testing, even at a 10% FDR (table S4). Similarly, there was no enrichment of genes 426 with annotations for mating behaviour or stress response phenotypic classes. However, several genes within 10kb of a top SNP are potentially interesting candidate genes for traits evolving under sexual selection based on described functions (table S4). For example, the 429 genes *Odorant-binding protein 47a* (*Obp47*), *pickpocket 6* (*ppk6*), and *Accessory gland protein 53C14c* (*Acp53C14c*) all occur within 10kb of a top SNP and are genes potentially underlying sexually selected behaviours or traits. Two of these genes (*ACP53C14c* and 432 *Obp47a*) are within the region of highly differentiated SNPs on the 3rd chromosomes, which also includes several additional accessory gland proteins (*Acp53Ea, Acp53C14b, Acp53C14a*), and other genes (table S4), all of which are thought to influence mating and 435 courtship behaviours or phenotypes based on known functions of similar genes in *D. melanogaster*.

Previous studies have shown that there is divergence in gene expression patterns 438 between E and M lines (Immonen *et al.*, 2014; Veltsos *et al.*, 2017; Veltsos et al., *in prep.*). We therefore asked if these expression differences were associated with the top SNPs. Genes within 10kb (N = 428) of the top SNPs show a significantly greater overlap with genes that 441 are differentially expressed (DE) in ovaries and testes between E and M lines than expected by chance (figure S5 and table S3). This pattern also holds for genes within 1Mb (N = 7,045;

figure S6). Also, there is evidence that F_{st} between E and M lines is higher for genes that are
444 DE between the lines, especially for X-linked genes (figure 5A; Wilcoxon rank sum tests, Autosomes - V = 1026000, p = 0.03; X-chromosome right arm - V = 89067, p = 0.005; X-chromosome left arm – V = 59623, p = 0.04). There is no evidence that Tajima's D is
447 different between DE and non-DE genes (Wilcoxon rank sum test; all p > 0.05; figure 5B). There is some evidence that the degree to which sex-biased expression of a gene changes between E and M lines is associated with Tajima's D in M lines, but only on the X-
450 chromosome and only within abdominal tissues (figure 5C). Specifically, as the change in sex-bias becomes more negative (i.e. more female-biased expression in M lines), Tajima's D also becomes more negative (interaction of Tajima's D in M lines and chromosome type:
453 $F_{(11189,11191)} = 4.4$, p = 0.013).

The regions immediately up- or down-stream of top SNPs are not enriched for TF binding motifs or lncRNAs, after correction for multiple testing, so there were no obvious
456 differences between treatments in regions governing gene expression.

Discussion

459 There is much debate about the influence of sexual selection and sexually antagonistic selection on patterns of genomic variation (Mank, 2017; Sayadi *et al.*, 2019) and how this may influence divergence between species (Wolf & Ellegren, 2016). Sex-biased gene
462 expression, especially male-bias, evolves quickly and is related to phenotypic sexual dimorphism (Wright *et al.*, 2019). Outliers in genome scans often implicate sexual selection as a diversifying force (Andres *et al.*, 2008; Blankers *et al.*, 2018). Sexual antagonism may be
465 associated with genomic signatures of selective sweeps or balancing selection (Cheng & Kirkpatrick, 2016; Wright *et al.*, 2019) and may be promoted by strong sexual selection. However, inferences of the sources of selection on natural variation in genomic divergence
468 are usually indirect and ambiguous, because multiple forces act in concert to produce variation seen at the genomic level in nature. Here we used experimental evolution to alter sexual selection intensity, elevating sexual selection in polyandrous lines and eliminating it in
471 monogamous lines, and examined patterns of divergence in the genome after more than 160 generations of experimental evolution.

Many of the results we found recapitulate patterns seen in natural populations and
474 between species. Divergence is not uniform across the genome but clustered in "islands" of divergence, some of which contain candidate genes for an involvement in mating success. These clusters are more often seen on the X chromosome, which is a "hot spot" for

477 divergence. There are signatures of selection within the islands of divergence, with marginally lower diversity (π) within clusters than the rest of the genome, but only in M lines. Moreover, F_{st} between E and M lines is greater within clusters. F_{st} is also greater on the X
480 than autosomes, and differences in diversity are seen in the autosomes between selection regimes. Tajima's D implies selective sweeps have occurred, but only within some of the divergent regions. These patterns of diversity and divergence are associated with changes in
483 both differential gene expression between the lines and sex-biased genes. Overall, F_{st} between the lines is high in all replicates, probably due to low overall effective population sizes, though effective population sizes are similar between E and M lines (Snook *et al.*, 2009).

486 The concept of "islands" of divergence originated from omparisons of genomic divergence between species has given rise to (Nosil *et al.*, 2009; Ravinet *et al.*, 2017). These are usually thought to have arisen due to the combination of strong selection on barrier loci
489 and genetic hitchhiking within genomic regions, with background gene flow reducing divergence outside of the islands. Here we find distinct clustered divergence akin to the islands seen in natural systems. Our system is effectively allopatric, so there was no
492 background gene flow counteracting divergence outside of these clusters, which therefore must have arisen due to strong localised divergent selection across all replicates. Although *D. pseudoobscura* has relatively well-characterised inversion polymorphisms (Sturtevant &
495 Dobzhansky, 1936; Dobzhansky & Sturtevant, 1938; Wallace *et al.*, 2011), the clusters we describe do not correspond to the most common inversions known for this species, which are often very large. Our short-read sequencing approach allowed some examination of LD and
498 there was no suggestion of reduced LD within the clusters. In fact, the large peak at the right end of chromosome 3 (figure 4) surprisingly seems to be within a region of high recombination (which is often suppressed at telomeric regions). Interestingly, recombination
501 is higher within this peak than the chromosome-wide rate, but also differs between the treatments, being greater in the M lines. Perhaps selection against recombination was reduced in monogamous individuals because of epistatic interactions in the region which were
504 important in sexual selection or sexual conflict. There was no obvious difference in LD in the other clusters but their smaller size and hence "noisier" estimates makes robust inferences from pool-sequence data difficult. Indeed, the estimates of LD within the cluster on
507 chromosome 3 also rely on relatively few SNPs at longer ranges compared to the rest of the chromosome, so inferences should be taken with caution.

The lack of background gene flow or stronger linkage disequilibrium within the clusters
510 suggests that they have arisen primarily through localised strong selection which is consistent

across all replicates. In support of this, we see lower Tajima's D in some of the larger clusters. However, these patterns are very variable with lower Tajima's D in separate clusters for the E and M lines. Thus, overall, there is no significant difference between E and M lines. The genes contained within the clusters are not enriched for genes of particular functional categories, however, they do include strong candidate genes for an involvement in mating system evolution. For example, the large region on chromosome 3 contains numerous accessory gland proteins. In *D. melanogaster* these are well known to influence male reproductive success, exert antagonistic effects on female fecundity and lifespan, and play a role in sperm competitive success (Chapman *et al.*, 1995; Ram & Wolfner, 2007). Some of the evolutionary response in E lines is antagonistic, because M females have a higher fecundity when mated with E males. Moreover, when mated to E males, the reproductive schedule of M females is manipulated to the males benefit (Crudgington *et al.*, 2010). Accessory gland proteins show accelerated coding sequence and expression evolution across species (Swanson & Vacquier, 2002; Begun & Lindfors, 2005). Other genes within the clusters are involved in sexual chemical communication, which is also often implicated in outlier analyses in genome comparisons between species (Smadja & Butlin, 2009). For example, mutants of members of the pickpocket family in *D. melanogaster* show aberrant male mating success because of their involvement in the detection of female pheromones (Thistle *et al.*, 2012; Toda *et al.*, 2012). E males, subject to both intra- and intersexual selection, have diverged in aspects of courtship behaviour, such as time until initiation of courtship, have a higher intensity courtship song and have a higher competitive mating success than M males (Debelle *et al.*, 2016; Debelle *et al.*, 2017).

If strong selection has driven this clustered genomic divergence, an interesting question is whether the responses to selection are stronger in the E or M lines. Imposing monogamy on a naturally polyandrous species probably leads to relaxed selection on many genes involved in intra-or intersexual competition. Therefore, the response is likely to involve changes in both the intensity and direction of selection on some loci. Thus, perhaps the variation in signals of selection we see in Tajima's D and changes in LD are to be expected. Overall, we see stronger reductions in divergence in E lines, perhaps suggesting that directional selection was stronger when sexual selection was strengthened.

One pattern very commonly seen in studies of natural populations and species is more rapid divergence of the X chromosome (Vicoso & Charlesworth, 2006). We also see this here, the X having a higher prevalence of divergent clustered regions and higher F_{st} between the lines. Remarkably, all SNPs with fixed differences between the lines occurred on the X.

Faster X evolution can occur for many reasons, including greater genetic drift due to its
546 smaller effective population size. We calculated expected X/A divergence ratios under a range of plausible sex ratios and the observed X/A divergence exceeded all of them, suggesting the accelerated X divergence is not due to drift effects alone, and that either selection or a
549 combination of effects are involved. Genes under sexual selection are potentially more likely to be sex-linked, due to antagonistic, or sex-specific selection, or if sexually selected loci show dominance effects (Reinhold, 1998; Kirkpatrick & Hall, 2004; Grieshop & Arnqvist,
552 2018).

Links between genomic parameters and gene expression variation have been a somewhat contentious source of evidence of sexual selection, especially antagonistic forms of
555 sexual selection (Kasimatis *et al.*, 2019; Cheng & Kirkpatrick, 2020; Mank *et al.*, 2020). Genes that are male-biased in expression show accelerated divergence between species and sex-biased gene expression shows rapid evolution and turnover (Pröschel *et al.*, 2006;
558 Harrison *et al.*, 2015). Whether sex-biased expression is expected to be related to sex-specific F_{st} or signatures of balancing selection such as Tajima's D is open to debate, partly because of the potential resolution of antagonistic selection by the strengthening of sex-biased
561 expression. Previously we found that gene expression differences have evolved between the lines, especially in sex-biased genes (Veltsos *et al.*, 2017). Here we show that there is significant overlap between these genes and genes and the regions of genomic divergence of
564 the lines found here. Thus, the expression divergence is associated with the broad patterns of genomic divergence. Also, genetic differentiation (F_{st}) is greater for the differentially expressed genes, once again recapitulating patterns from natural systems (sex-biased genes
567 here are not more likely to be sex-linked, so this is independent of the large X effect seen). We find no general difference in Tajima's D between these DE loci. However, there is one very intriguing pattern where the magnitude of change in sex-biased gene expression is
570 related to Tajima's D. As ΔSB increases (more male-biased expression in E lines) Tajima's D in these lines becomes more negative. This pattern is potentially consistent with more resolved sexual conflict in the M lines, because males in M lines are released from sexual
573 selection, and selection driving female-beneficial alleles to fixation or high frequency could result in sweeps and/or reduced balancing selection.

In conclusion, we have examined genomic divergence following >160 generations of
576 experimental evolution under altered mating systems. We find that genomic divergence between the experimental lines is highly clustered in the genome, much greater on the X and is associated with changes in gene expression between the experimental lines. Associations

with LD and population genetic parameters indicative of selective sweeps or balancing selection are also observed, but are very variable. This raises the possibility that selection has been strong in both M and E lines, but differs in nature (relaxed in M, directional in E), complicating predictions of responses. Overall, our main results confirm those seen in natural populations, providing an elegant demonstration of the power of experimental evolution to aid the interpretation of complex patterns of natural variation.

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Author contributions

RAWW performed the data analysis. PV contributed data. The experiment was designed by MGR and RRS. All authors contributed to writing the MS

Data Accessibility

Raw reads have been deposited in the short read archive (SRA) of NCBI under the BioProject PRJNA661678

606 **Figures and Figure Legends**

Figure 1. A) Manhattan plot of log10(q-values) for each SNP from a quasibinomial GLM
609 with treatment as a predictor. Red points denote SNPs with a q-value < 0.05 and the horizontal red dashed line indicates the q < 0.05 cutoff. Grey bars give the locations and span of the 70 divergent regions (see text). **B)** Mean (± SE) Tajima's D across replicates for the
612 three most divergent regions (see text), red points denote SNPs with a q-value < 0.05, all have been plotted at the same value on the y-axis for convenience.

615 **Figure 2. A)** Levels of genetic diversity (π) on each chromosome in E and M lines. π is estimated in overlapping windows of 50kb, then averaged across the chromosomes. Boxplots show the distribution of π on each chromosome across replicate experimental evolution lines.
618 **B)** The X chromosome to autosome ratio of π in the replicates of E and M lines and overall.

Figure 3. A) F_{st} between E and M treatment lines on the main chromosome arms for each
621 replicate. F_{st} is calculated for each SNP then averaged within overlapping 50kb windows on each chromosomal segment. **B)** Observed F_{st} on the autosomes and on the X chromosome as well as the expected F_{st} on the X chromosomes (see Methods), error bars represent bootstrap
624 95% confidence intervals. **C)** The X:autosome ratio of F_{st} within each replicate line. The error bars are bootstrap 95% confidence intervals.

627 **Figure 4. A)** Slope coefficients from the model $r^2 \sim a + \log(bp)$ where bp is the distance between pairs of SNPs and r^2 is the average measure of LD between SNPs. Distributions are shown for average values of each of the main chromosomes as well as X chromosomes
630 across replicates in E and M lines. **B)** Decay in LD as a function of distance between SNPs with the chromosome 3 peak region (see figure 3) and outside the peak region for E and M lines. **C)** The distribution of slope parameters for SNPs within the chromosome 3 peak and
633 outside the peak region.

Figure 5. A) F_{st} at DE vs. non-DE genes for different chromosome types. Asterisks indicate
636 significant differences **B)** Tajima's D at DE vs. non-DE genes for different chromosome types. **C)** relationship between change in sex-bias between E and M lines and Tajima's D in M lines.

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Figure 1

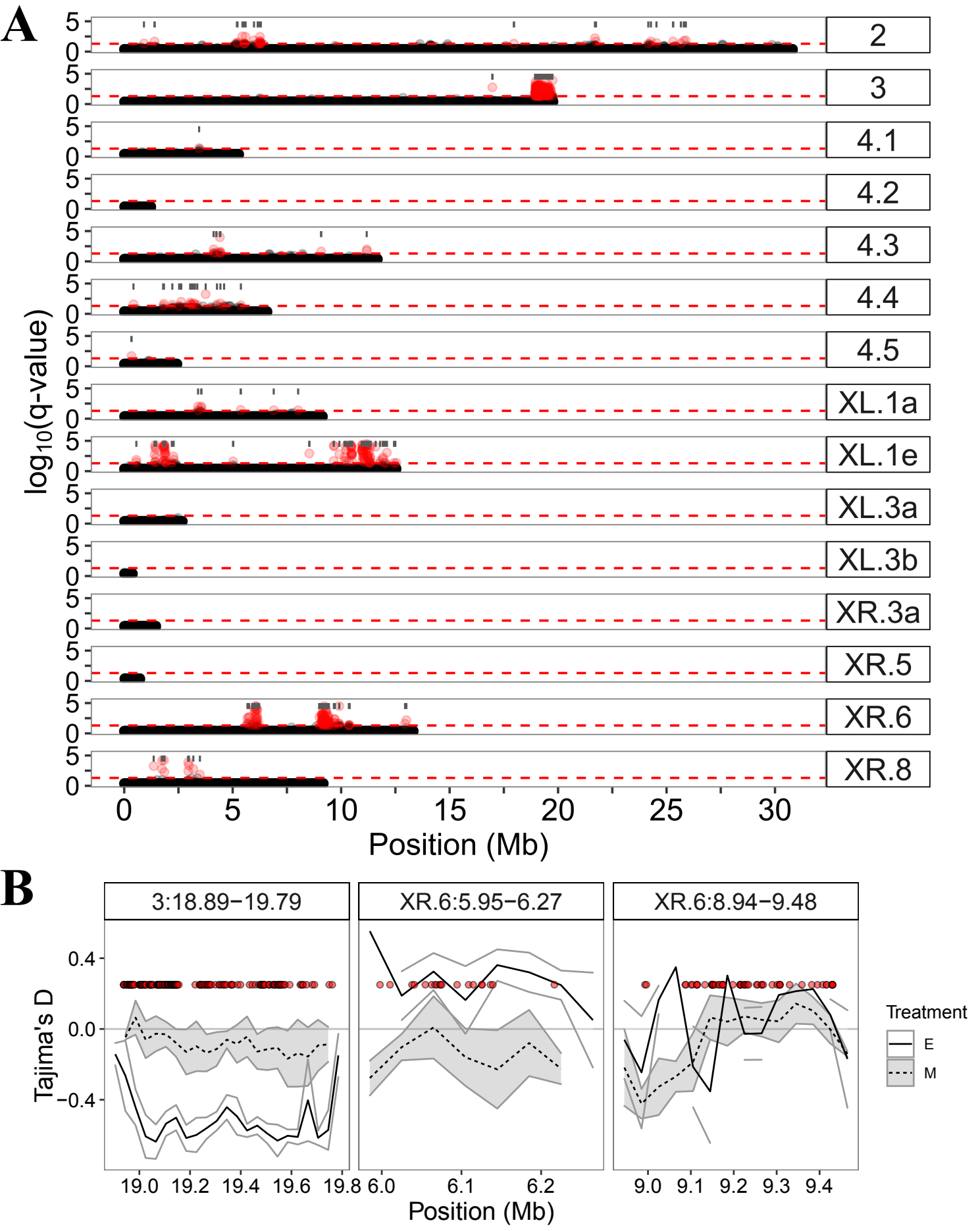

A
log10(q-value)
2
3
4.1
4.2
4.3
4.4
4.5
XL.1a
XL.1e
XL.3a
XL.3b
XR.3a
XR.5
XR.6
XR.8
Position (Mb)
B
3:18.89−19.79
XR.6:5.95−6.27
XR.6:8.94−9.48
Tajima's D
Position (Mb)
Treatment
E
M

Figure 2

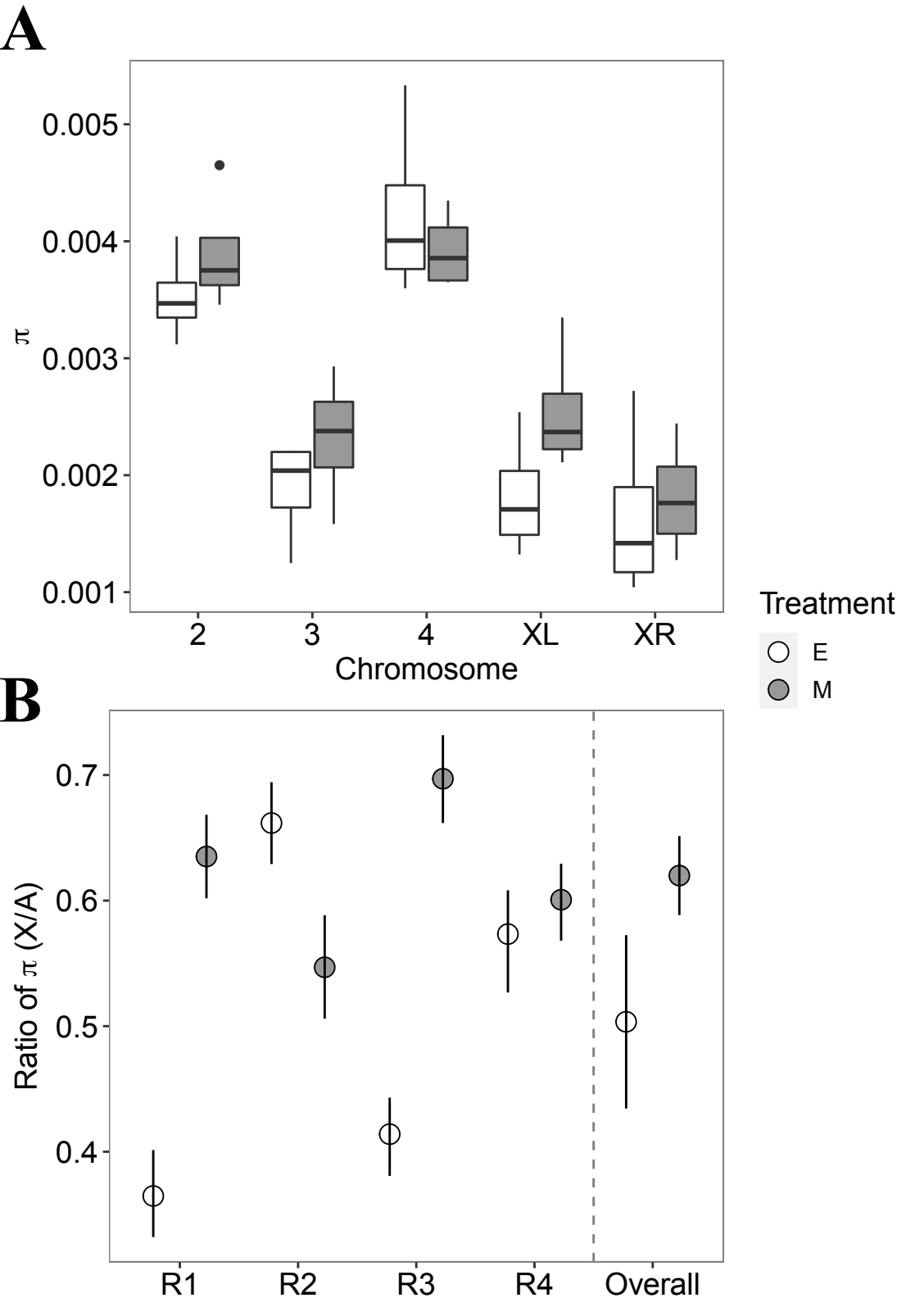

A
0.005
0.004
0.003
0.002
0.001
π
2
3
4
XL
XR
Chromosome
Treatment
E
M
B
0.7
0.6
0.5
0.4
Ratio of π (X/A)
R1
R2
R3
R4
Overall

Figure 3

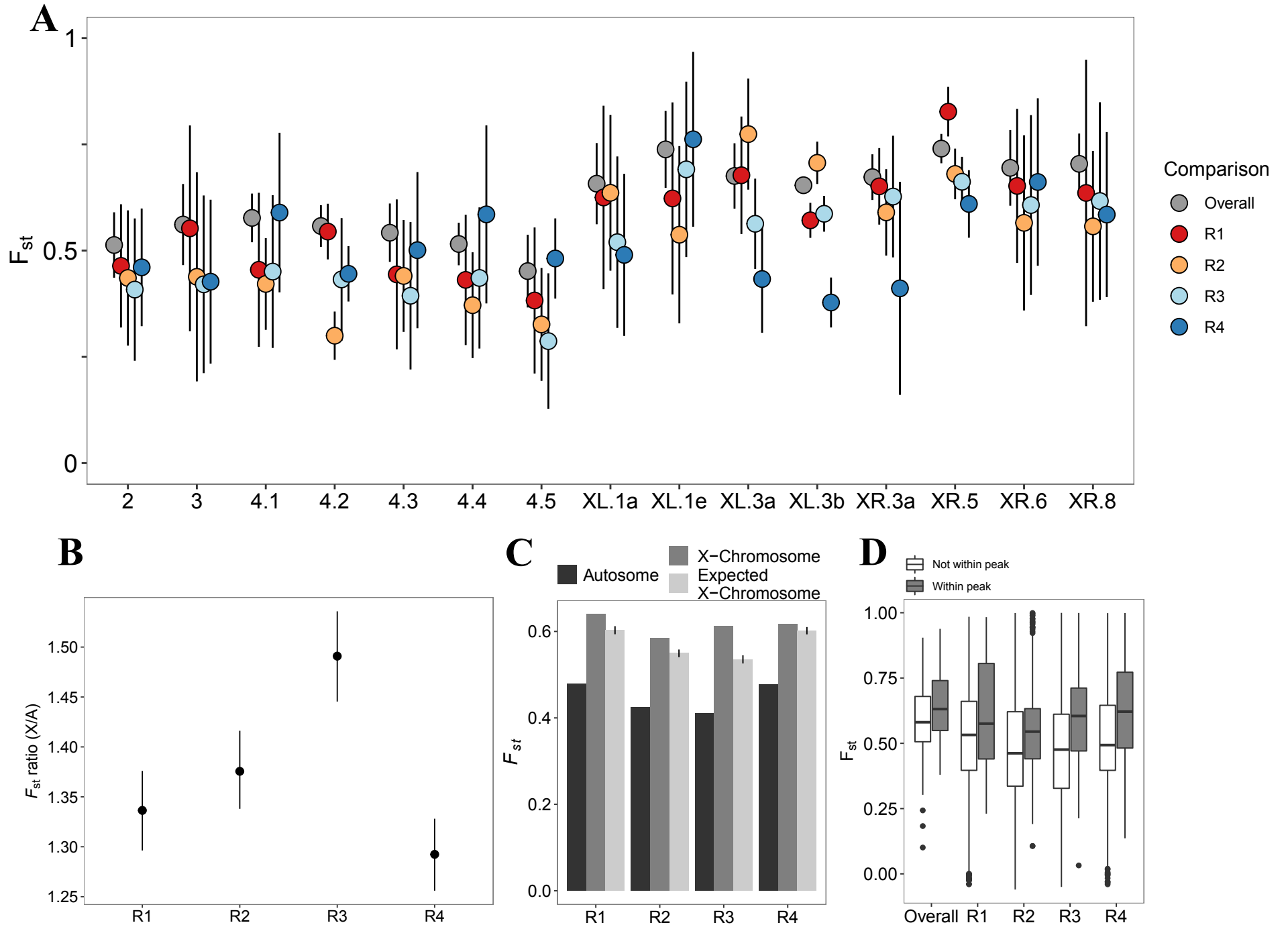

A
1
0.5
0
F_{st}
2
3
4.1
4.2
4.3
4.4
4.5
XL.1a
XL.1e
XL.3a
XL.3b
XR.3a
XR.5
XR.6
XR.8
Comparison
Overall
R1
R2
R3
R4
B
1.50
1.45
1.40
1.35
1.30
1.25
F_{st} ratio (X/A)
R1
R2
R3
R4
C
Autosome
X−Chromosome
Expected X−Chromosome
0.6
0.4
0.2
0.0
F_{st}
R1
R2
R3
R4
D
Not within peak
Within peak
1.00
0.75
0.50
0.25
0.00
F_{st}
Overall
R1
R2
R3
R4

Figure 4

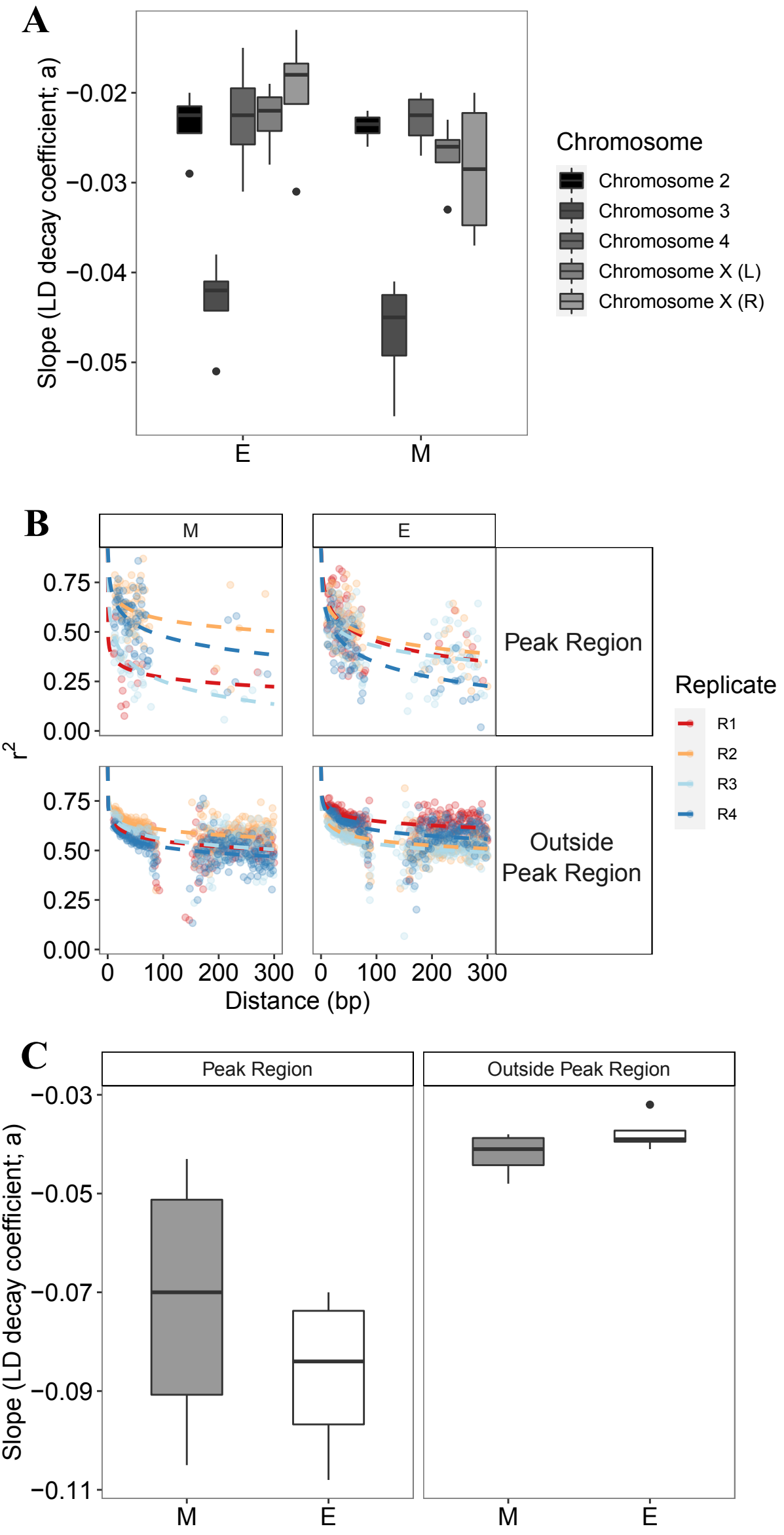
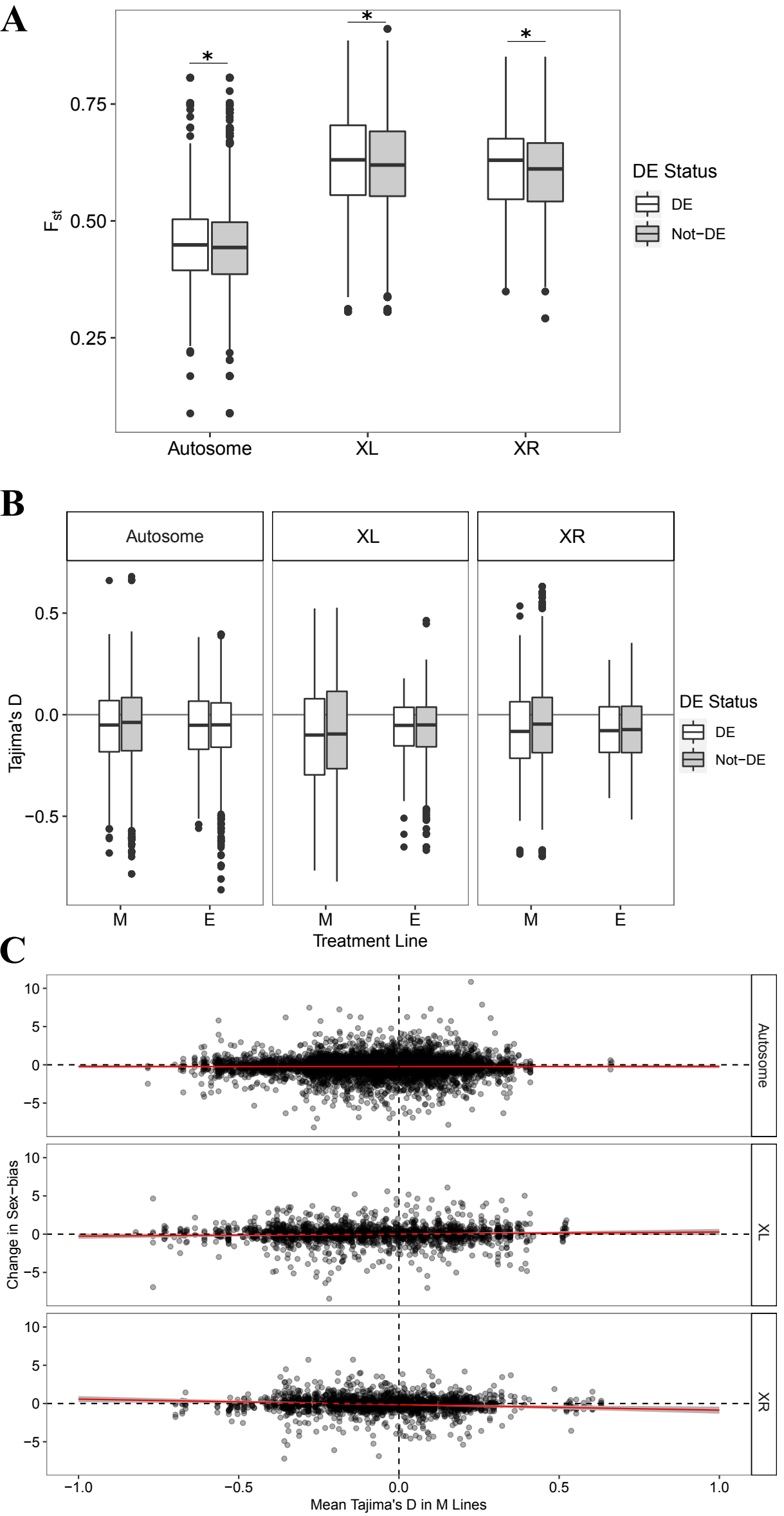

A
Slope (LD decay coefficient; a)
−0.02
−0.03
−0.04
−0.05
E
M
Chromosome
Chromosome 2
Chromosome 3
Chromosome 4
Chromosome X (L)
Chromosome X (R)
B
M
E
Peak Region
Outside Peak Region
r2
0.75
0.50
0.25
0.00
0
100
200
300
Distance (bp)
Replicate
R1
R2
R3
R4
C
Peak Region
Outside Peak Region
Slope (LD decay coefficient; a)
−0.03
−0.05
−0.07
−0.09
−0.11
M
E
M
E

Figure 5


A
*
*
*
0.75
0.50
0.25
F_{st}
Autosome
XL
XR
DE Status
DE
Not−DE
B
Autosome
XL
XR
0.5
0.0
−0.5
Tajima's D
M
E
M
E
M
E
Treatment Line
DE Status
DE
Not−DE
C
10
5
0
−5
Autosome
XL
XR
Change in Sex−bias
−1.0
−0.5
0.0
0.5
1.0
Mean Tajima's D in M Lines