

Research



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Sex chromosome degeneration, turnover, and sex-biased expression of sex-linked transcripts in African clawed frogs (*Xenopus*)

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The tempo of sex chromosome evolution—how quickly, in what order, why and how their particular characteristics emerge during evolution—remains poorly understood. To understand this further, we studied three closely related species of African clawed frog (genus *Xenopus*), that each has independently evolved sex chromosomes. We identified population polymorphism in the extent of sex chromosome differentiation in wild-caught *Xenopus borealis* that corresponds to a large, previously identified region of recombination suppression. This large sex-linked region of *X. borealis* has an extreme concentration of genes that encode transcripts with sex-biased expression, and we recovered similar findings in the smaller sex-linked regions of *Xenopus laevis* and *Xenopus tropicalis*. In two of these species, strong skews in expression (mostly female-biased in *X. borealis*, mostly male-biased in *X. tropicalis*) are consistent with expectations associated with recombination suppression, and in *X. borealis*, we hypothesize that a degenerate ancestral Y-chromosome transitioned into its contemporary Z-chromosome. These findings indicate that *Xenopus* species are tolerant of differences between the sexes in dosage of the products of multiple genes, and offer insights into how evolutionary transformations of ancestral sex chromosomes carry forward to affect the function of new sex chromosomes.

This article is part of the theme issue 'Challenging the paradigm in sex chromosome evolution: empirical and theoretical insights with a focus on vertebrates (Part I)'.

1. Introduction

Many vertebrates have separate male and female individuals rather than hermaphrodites, with sex-specific traits in each sex being regulated by transcripts with sex-biased expression. In species with genetic sex determination, DNA variation that triggers sexual differentiation is present on sex chromosomes. This is important because sex-specific genetic variation establishes a genomic compartment—which may expand—that has unique features as compared to autosomes (copy number, mode of inheritance, rates of mutation and recombination) that strongly influence evolution in important ways [1,2]. For example, compared to autosomes, non-recombining portions of

sex chromosomes tend to have a higher abundance of repetitive elements and more extensive pseudogenization [3], and distinctive evolutionary dynamics of non-neutral mutations that depend on the degree of dominance [4,5]. Sex chromosomes evolve from autosomes and, in theory, genetic recombination near the genomic region that contains the master regulator of sex determination can decline or become absent owing to several non-exclusive factors. For example, it is conceivable that neutral processes may drive recombination suppression [4]. It is also possible that inheritance (or non-inheritance) of a non-recombined genomic region may be advantageous in order to avoid sterile progeny [6], genetic linkage to a sex-determining locus may resolve genomic conflict associated with mutations with opposite fitness effects in each sex [5,7], or recombination suppression could be favoured to maintain heterozygosity, especially in inbred populations [8]. Mechanistically, recombination suppression may be achieved by inversion or translocation [9,10], or potentially without genomic rearrangement [11] owing to genetic mutations that alter recombination (recombination modifiers [12]).

There are costs of recombination suppression in sex-linked regions that are associated with Hill-Robertson effects, which reduce the efficacy of natural selection [13]. Some of these costs may be curtailed by the origin of and gene conversion among duplicates [14]. Benefits of recombination suppression also exist, which are related to sex-specific or sex-biased modes of inheritance. This can help resolve genomic conflict, and these regions can also foster the elaboration of sex-specific genetic functions, such as the many genes that are crucial for spermatogenesis on the male-specific portion of the human Y-chromosome [14]. Gene loss on the sex-specific sex chromosome (the Y- or W-) creates hemizygous genotypes, allowing natural selection to respond more efficiently to recessive alleles in the heterogametic sex (XY males or WZ females); the associated rapid evolution is known as the faster-X (or faster-Z) effect [2,4]. One of the consequences of their unique evolution is that sex chromosomes play an oversized role in the establishment of reproductive incompatibilities among species [2,4,15].

(a) Signs of rapid sex chromosome evolution

In species that have separate sexes, sexual differentiation is usually a necessary prelude to reproduction, and disruption of this developmental process can have dire fitness consequences. Consequently, one might expect purifying selection on sex-determination pathways—including the trigger for sex determination—to be strong. Contrary to this expectation, components of the developmental cascade that govern sexual differentiation actually vary considerably among species, including and especially the triggers for sex determination and the chromosomes on which they reside. This variation is sometimes evidenced by a known trigger for sex determination that is present in one lineage but not another. An example of this is the *Sry* locus, which triggers male differentiation in most placental and marsupial mammals, but not in monotremes [16]. Another line of evidence for rapid evolution comes from studies that document variation among species or populations in whether females or males are heterogametic. In Diptera, for instance, the heterogametic sex in some species is male but female in others, and sex-linked regions in several species are not homologous [17].

Another example of rapid evolution involves dramatic differences in the extent of sex chromosome heteromorphy—microscopic differences in the size of the sex chromosomes or genomic differences in the magnitude and genomic extent of differentiation between sex chromosomes—with some species having non-diverged (homomorphic) sex chromosomes and others with heteromorphic sex chromosomes. These differences are owing to variation in the genomic extent of recombination suppression surrounding a trigger for sex determination; an example of this variation is found in livebearing teleost fishes [18]. Some aspects of rapid evolution eventually impede further change. For example, sex chromosome hemizygosity associated with loss of alleles on the non-recombining portion of the sex-specific sex chromosome creates imbalances in the stoichiometries of interacting sex-linked and autosomal genes. This has the potential to arrest sex chromosome divergence [19], favour the origin of new sex chromosomes [20] or spur the evolution of systems for dosage compensation [21]. Over time, the origin of systems for dosage compensation and sex-specific viability alleles may act to dampen the evolutionary meanderings of sex chromosomes [22].

(b) Young sex chromosomes of *Xenopus* frogs

How can we make sense of the diverse and sometimes correlated or counterposed drivers that choreograph the evolutionary fates of sex chromosomes? One approach is to examine young sex chromosomes with an eye on identifying early steps in evolution before they become blurred or confounded by subsequent change. To this end, the sex chromosomes of African clawed frogs (genus *Xenopus*)—and particularly the Marsabit clawed frog, *Xenopus borealis*—provide compelling subjects. Within *Xenopus*, all of the examples of rapid sex chromosome evolution discussed above are present (figure 1). Variation in heterogamy exists within *Xenopus tropicalis*, with a variation on chromosome 7 (hereafter chr7) defining W-, Y- and Z-sex chromosomes [23]. In *Xenopus laevis* and several other closely related species, a recently evolved genetic trigger for female sex determination called *dmw* established chromosome 2L (chr2L) as the sex chromosomes [24]. *Xenopus tropicalis* diverged before *dmw* arose [25], whereas *X. borealis* lost *dmw* and evolved new sex chromosomes on chromosome 8L (chr8L; [26]). Though all *Xenopus* species have homomorphic sex chromosomes [27], there exists considerable variation in recombination suppression around the sex-linked portions of sex chromosomes in different *Xenopus*. *Xenopus laevis* and *X. tropicalis* have a very small region of recombination suppression, whereas this region in *X. borealis* comprises almost half of the sex chromosomes; the other half of chr8L undergoes recombination in both sexes, and, therefore, is a pseudoautosomal region [28,29].

Xenopus borealis is an allotetraploid species, meaning that it was formed via the fusion of two diploid ancestral species (figure 1; reviewed in [30]). For this reason, its genome contains autosomal chromosomes that are homeologous to the sex chromosomes (chromosome 8S; chr8S). The closely related species *X. laevis* is also allotetraploid, shares a most recent common allotetraploid ancestor with *X. borealis* approximately 18–34 million years ago (Ma) [31] or older [32] and has autosomal chromosomes that are orthologous (chr8L) and homeologous (chr8S) to the sex chromosomes

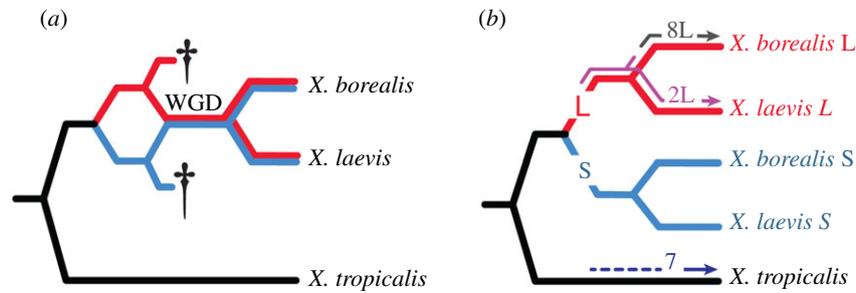


Figure 1. Evolutionary relationships among focal species (a) and their orthologues and homeologues (b) in this study. Allopolyploidization involved whole-genome duplication (WGD) via the merger of two extinct ancestral species (daggers) that generated two subgenomes (L and S, red and blue) in the tetraploid species *X. borealis* and *X. laevis*. Sex chromosomes in each species are indicated on the right above branches in grey, pink or blue. The pink line corresponds with the origin of the female-determining gene *dm-w* on chr2L. A dashed blue line indicates the unknown timing of the origin of the *X. tropicalis* sex chromosomes; branches not to scale. (Online version in colour.)

of *X. borealis*. Likewise, the sex chromosomes of *X. laevis* are orthologous and homeologous to autosomes of *X. borealis* (chr2L and chr2S, respectively). *Xenopus tropicalis*, a diploid, has autosomal chromosomes (chr8 and chr2) that are each, respectively, orthologous to the sex chromosomes of *X. borealis* and of *X. laevis*, and the sex chromosomes of *X. tropicalis* (chr7) are orthologous to pairs of homeologous autosomes in *X. borealis* and also in *X. laevis* (chr7L and chr7S—both of these homeologous chromosomes are in both of these allotetraploid species). *Xenopus tropicalis*, *X. laevis* and *X. borealis* share a most recent common ancestor approximately 60 Ma [32]. Together, these three sets of alternatively sex-linked, orthologous and/or homeologous chromosomes in these frogs provide informative contrasts with which to consider the evolution of sex-linked regions (figure 1). We follow the *X. laevis* chromosome nomenclature of [33] by postfixing an 'L' to the name of chromosome pairs from the 'long' subgenome and an 'S' to the names of chromosome pairs from the 'short' subgenome; these postfixes thus refer to entire chromosome pairs and not to the long or short arms of individual chromosomes. For *X. tropicalis*, we follow the chromosomal nomenclature of [34] which uses the same numbers for orthologous chromosomes as [33] except for *X. tropicalis* chr9 and chr10 which are orthologous to fused chromosomes in *X. laevis* and *X. borealis* (chr9_10L, chr9_10S).

With an aim of better understanding the evolutionary dynamics of sex chromosomes, we used genome-wide approaches to test for population structure and variation in sex chromosome differentiation in natural populations of *X. borealis* sampled in their native range in Kenya. We then evaluated whether and how the expression of genes in the sex-linked region of the sex chromosomes differs from the rest of the genome, and when these changes emerged during *Xenopus* diversification. We considered whole transcriptome data from *X. borealis* adult liver and gonad/mesonephros tissues from two tadpole stages during the onset of gonadal differentiation, and we also contextualize findings in *X. borealis* with similar comparisons with adult liver transcriptomes of *X. laevis* and *X. tropicalis*.

2. Material and methods

(a) Samples, analyses of population structure in *Xenopus borealis*

Xenopus borealis naturally occurs in Kenya and northern Tanzania. We analysed a total of 93 wild-caught *X. borealis*

specimens that were sampled through the central portion of its range in Kenya in 2018. Information on the sampling localities, voucher specimens and sex of these samples is provided in the electronic supplementary material, table S1; figure 2; specimens from this collection, including genetic samples, are accessioned at the Museum of Comparative Zoology at Harvard University, USA, and the National Museums of Kenya in Nairobi, Kenya. As described in the electronic supplementary material, we performed phylogenetic analysis on a portion of mitochondrial DNA from a subset of these samples (84 specimens) and also two laboratory specimens. We also collected and analysed reduced representation genome sequences (RRGS, [35]) from a subset of these samples (51 specimens, 26 females, 25 males) combined with whole-genome sequencing data from the two laboratory specimens. These analyses allowed us to discern population structure and sex chromosome differentiation within *X. borealis*.

(b) Comparative transcriptomics of *Xenopus borealis*, *Xenopus laevis*, and *Xenopus tropicalis*

We sequenced and analysed transcriptomes from gonads at two juvenile developmental stages and adult liver from our laboratory strain of *X. borealis*. In order to gain an evolutionary perspective on these transcriptomes, we also analysed transcriptomes from adult liver from *X. laevis* and *X. tropicalis*. *Xenopus borealis* and *X. laevis* specimens were obtained directly or were offspring of animals that were purchased from Xenopus Express (Brooksville, Florida, USA) and the *X. tropicalis* adult liver data were obtained from the NCBI-SRA (from two males: SRR5412273 and SRR5412274, and two females: SRR5412275 and SRR5412276). Details of RNA extraction and sequencing, and the transcriptome assembly and analysis of sex-biased expression are provided in the electronic supplementary material.

Genomic locations of assembled transcripts from each tetraploid species (*X. borealis* and *X. laevis*) were assessed in the *X. laevis* genome assembly version 9.2 [31], which were obtained from Xenbase [36], using the splice-aware aligner GMAP [37] with parameters: -A -B 5 -t 20 -f samse. The *X. tropicalis* transcripts were mapped to the v. 10 *X. tropicalis* genome assembly (NCBI BioProject AAMC00000000.4; GenBank Assembly submission GCA_000004195.4) using the same approach. The parameter cross-species was used for the *X. borealis* transcripts to accommodate divergence from the *X. laevis* reference genome.

In our laboratory strain of *X. borealis*, the sex-linked region of the sex chromosomes corresponds to positions 4 605 306 to 56 690 925 base pairs (bp) on chr8L of the *X. laevis* genome assembly v. 9.2 [29]. For this study, we assume that the sex-linked region starts from the beginning of chr8L because no RRGS data from [29] mapped to less than 4 605 306 bp on chr8L. In order to establish approximate margins of regions of orthology and homeology for sex-linked regions in each of our

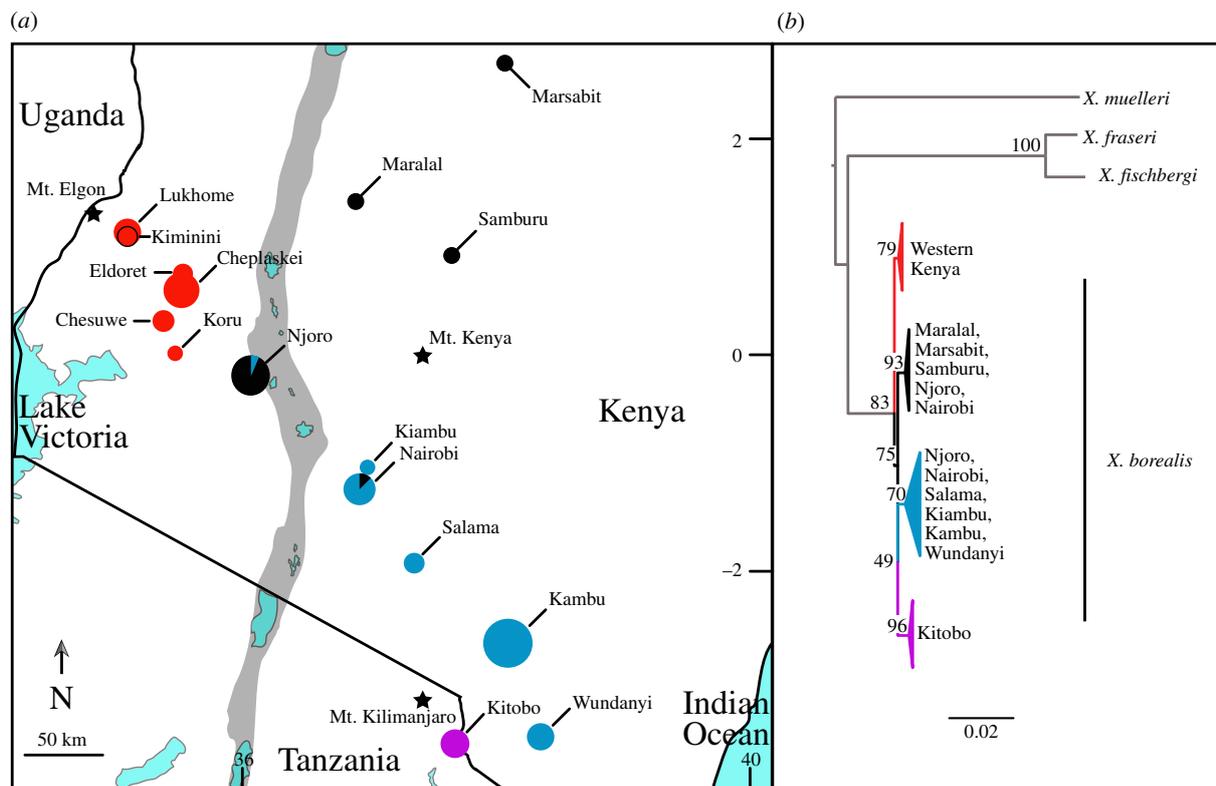


Figure 2. Sampling locations for *X. borealis* (a) and estimated relationships among four mitochondrial clades in *X. borealis* with respect to other species in the *muelleri* species group (b); bootstrap values are above branches and a scale bar indicates substitutions per site. The size of the circles corresponds to the number of mitochondrial sequences analysed from each locality; pie charts from two localities indicate the proportions of each clade sampled with colours matching the phylogeny. Stars indicate locations of major mountains and grey shading indicates location of the eastern arm of the Great Rift Valley. (Online version in colour.)

three focal species, we generated dot plots from chromosomes in the genome assemblies of *X. laevis* and *X. tropicalis* using Gopard version 1.40 [38] and re-DOTable v. 1.0 [39]. Based on these plots, we concluded that the sex-linked region of *X. borealis* is homeologous to 16–45 and 51–68 Mb of *X. laevis* chr8S, and orthologous to 0–12 and 20–73 Mb of the *X. tropicalis* chromosome 8 (e.g. electronic supplementary material, figure S1). The sex-linked region of the *X. laevis* sex chromosomes is above 178 Mb on chr2L [29], and based on dotplots, this region is homeologous to 154–158 Mb on *X. laevis* chr2S and orthologous to 172–177 Mb on chr2 of *X. tropicalis*. The sex-linked region of the *X. tropicalis* sex chromosome is less than 11 Mb on chr7 [28,40]. This region is orthologous to less than 9 Mb and less than 6 Mb on *X. laevis* chr7L and chr7S, respectively. This information allows us to compare homologous (i.e. either orthologous or homeologous) genomic regions that are and that are not sex-linked.

For each tetraploid transcriptome, we used a generalized linear model to test whether a binary response variable—whether or not a transcript was significantly differentially expressed (1) or not (0) according to false detection rate (FDR) corrected *p*-values from edgeR (*significance*)—was affected by the interaction between two predictor variables: whether a transcript was from the L (1) or S (0) subgenome (*subgenome*), and whether a transcript was encoded by a gene in the sex-linked region or the region that is homeologous to the sex-linked region (1), or by a gene from elsewhere in the genome (0) (*SL_homeologous*):

$$\text{significance} \sim \text{SL_homeologous} * \text{subgenome}.$$

We had a one-sided expectation that there would be a positive interaction between *subgenome* and *SL_homeologous*, which would indicate that odds of sex-biased expression are higher for transcripts encoded by genes in the sex-linked portion of

chr8L compared to the rest of the genome, after controlling for the subgenome effects. It is important to consider subgenome effects because in several *Xenopus* allotetraploids the S subgenome has more rearrangements, shorter coding regions, and more pseudogenization than the L subgenome, and transcripts from the S subgenome tend to be more lowly expressed than homeologous transcripts from the L subgenome [31,41]. For the diploid transcriptome of *X. tropicalis*, there was no subgenome parameter, and the model simplifies to a logistic regression with one predictor variable (*sex_linked*)—that indicates whether or not a transcript was from a sex-linked (1) or non-sex-linked gene (0).

For juvenile tissues from *X. borealis*, we were only able to make intraspecific comparisons, for example between the sex-linked portion of chr8L and the homeologous portion of chr8S (the latter of which is autosomal). For adult liver tissues, we had comparable data from all three focal species. In order to compare evolutionary trends across these species for adult liver, we performed three different logistic regressions, each with sex-linked and homeologous or orthologous to sex-linked regions across each species defined to match each one of our three focal species. For example, when the adult liver of *X. borealis* was the focal species, *SL_homeologous* and *sex_linked* were set to a value of one for transcripts encoded by genes in the sex-linked, homeologous and orthologous regions of chr8L, chr8S and chr8 for transcriptome data from each of the three focal species (even though this region is only sex-linked in *X. borealis*), and transcripts encoded by genes in the rest of each of these three genomes were set to zero. The same was done for models in each species for chr2L, chr2S and chr2 (when *X. laevis* was the focal species) or chr7L, chr7S and chr7 (when *X. tropicalis* was the focal species). These tests allowed us to test whether sex-linked changes in one species arose concurrently with sex linkage or whether they were present ancestrally. More specifically, if the sex-biased expression of transcripts encoded by sex-linked genes evolved

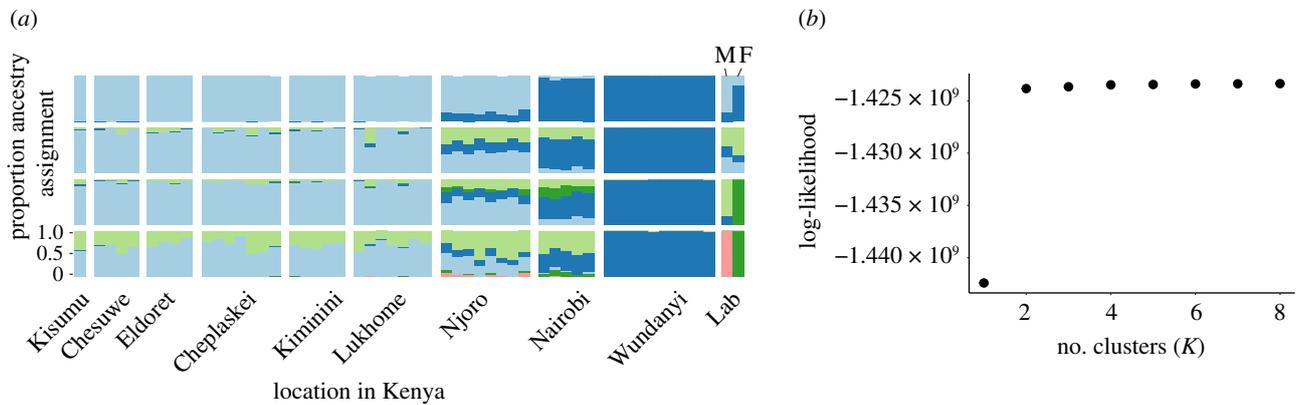


Figure 3. Genetic cluster analysis of RRGs data from wild-caught specimens and homologous WGS data from two laboratory individuals. (a) Ancestry components of wild *X. borealis* from Kenya with K clusters from two (top) to five (bottom) exhibit geographical structure, and genetic affinities of a male (M) and female (F) laboratory animal (Lab, right side) suggest origins from central Kenya (Njoro, Nairobi). (b) log-likelihood of values of K from 1–8. (Online version in colour.)

independently in the different sex-linked regions of each species, we expected a significant coefficient when the *SL_homeologous* or *sex_linked* matched their sex-linked region of the focal species but not the non-focal species. Alternatively, if the sex-biased expression was present in an ancestor prior to a region becoming sex-linked, we expected significant coefficients for some non-focal species even when the *SL_homeologous* and *sex_linked* parameters did not match their sex-linked region.

Analysis of individual transcripts allowed us to evaluate the sex-biased expression of splice variants or possibly individual alleles. We also analysed differential expression at the gene level based on summed normalized expression levels of all transcripts from individual genes. For this gene-level analysis, expression levels were summed for transcripts that mapped within or overlapped with (by at least 200 bp) annotated genes in v. 9.2 of the *X. laevis* (for *X. borealis* and *X. laevis* in this study) and version 10 of the *X. tropicalis* genome (for *X. tropicalis* in this study). We also included in this analysis the summed expression levels of transcripts in unannotated portions of these genomes that overlapped by at least 200 bp.

3. Results

(a) Population variation in sex chromosome differentiation in *Xenopus borealis*

Partial mitochondrial DNA sequences from wild-caught *X. borealis* cluster into four closely related, geographically structured clades. Some of these clades have only modest bootstrap support, and relationships among them are not well resolved (figure 2). At two localities in central Kenya (Njoro and Nairobi), two of these four clades were present; at all other localities, only one clade was present in our sample. In Kenya, a geographical boundary between the most diverged intraspecific mitochondrial lineages of *X. borealis* may roughly correspond to the eastern arm of the Great Rift Valley. The two sequences from our laboratory strain clustered in different clades: the male (BJE3896) clustered in the black clade in figure 2, whereas the female (BJE3897) clustered in the blue clade, with no divergent sites compared to four other sequences, all of which were from Nairobi specimens. To our knowledge, sequences from the western (red) clade and the Kitobo (purple) clade have not been previously reported. The black and blue clades (figure 2) have been previously reported and are estimated to have diverged from one another about 2 Ma [33,41].

Although samples from Kitobo, which has a unique mitochondrial lineage, were not included in the RRGs data, available information from other samples is consistent with the findings from mitochondrial DNA in terms of evidencing geographical structure and also admixture between Nairobi and Njoro (figure 3). With two ancestry components, the geographical structure is evident, with specimens from the central Kenya localities of Nairobi and Njoro having a mixture of two ancestry components, specimens from Wundanyi (in the east) assigned to one ancestry component, and other specimens (all from west Kenya) assigned to another. With three ancestry components, the Wundanyi population is further distinguished from the others, and a gradient of ancestry components is still evident in the Nairobi and Njoro populations.

Using RRGs data, we calculated F_{ST} between females and males for the wild samples of *X. borealis*. For the autosomes and pseudoautosomal regions, this statistic is expected to be influenced by polymorphism and differences in the number of males and females in different subdivided populations should increase F_{ST} between males and females across the sample). For the sex chromosomes, F_{ST} should be additionally influenced by the divergence between the W- and Z-sex chromosomes. We detected substantial differentiation in the region of recombination suppression on the sex chromosomes (chr8L, [27]) of the *X. borealis* specimens from central (Nairobi) and eastern (Wundanyi) Kenya, but not elsewhere on this chromosome (figure 4). In populations from western Kenya, there was no strong evidence of differentiation between the sexes in any substantially sized genomic region, including the sex-linked region of chr8L (figure 4). In both of these populations or when all samples were considered, there were no other substantially sized regions where F_{ST} was substantially elevated beyond the 95% confidence interval for the non-sex-linked portion of the genome (data not shown). In the sex-linked region of *X. borealis* specimens from central and eastern Kenya, there was a dip in F_{ST} around 20 Mb (figure 4); we suspect this could be because RRGs data from *X. borealis* chr8S inappropriately mapped to this part of chr8L in the *X. laevis* reference genome.

Using Sanger sequencing, we checked whether two previously studied genes with sex-linked variation in our laboratory *X. borealis* [29] had sex-linked variation in the

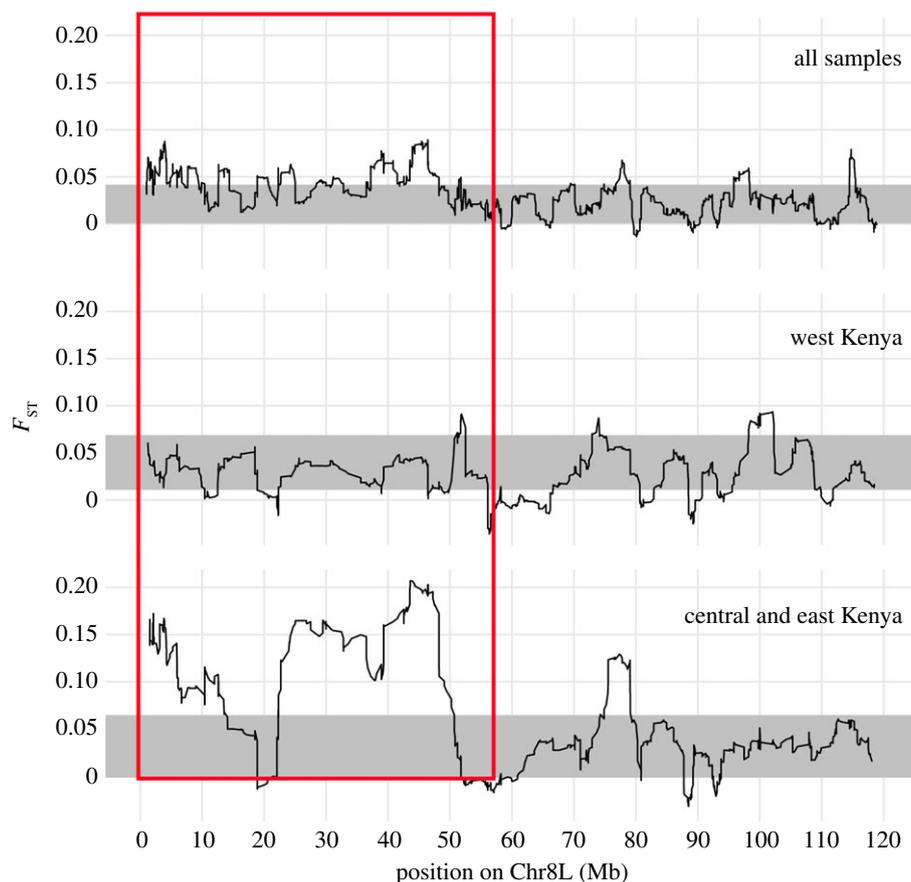


Figure 4. F_{ST} between males and females is substantial on the sex-linked portion (red box) of the sex chromosomes (Chr8L) for the samples from central and east Kenya (bottom; nine females, six males) but not west Kenya (middle; 17 females, 19 males) and is slightly elevated when all samples are considered (top). Grey boxes indicate the 95% confidence interval for F_{ST} values in the non-sex-linked portion of the genome. (Online version in colour.)

wild samples of *X. borealis*. One was *SOX3-L*, which is position approximately 34 Mb on *X. laevis* chr8L; the other is *NR5A1*, which is approximately 10 Mb on chr8L. We found that both had female-specific heterozygous sites in individuals from Wundanyi (*SOX3*: three females, two males; *NR5A1*: three females, four males), but not central or west Kenya for *SOX3* (29 females, 19 males). For *NR5A1*, there also were heterozygous sites that were female-specific in central Kenya (Njoro, Nairobi and Kiambu; 13 females, six males) and almost female-specific in Kambu (present in four females and one male, not present in five other males), but no sex-specific variation was present in west Kenya (13 females, 10 males).

(b) The genomic landscape of genes with sex-biased expression in *Xenopus borealis*

The transcriptome assembly for *X. borealis* tadpoles (a combined assembly for gonad/mesonephros from stages 46 and 48) had 523 313 transcripts; the assembly for *X. borealis* adult liver had 562 550, transcripts; independent assemblies for *X. laevis* adult liver and *X. tropicalis* adult liver had 391 563 and 207 184 transcripts, respectively. These transcripts include biological complexity stemming from splice variants and allelic variation and technical variation owing to incompletely assembled transcripts and assembly errors. We performed analyses of individual transcripts and also of groups of transcripts that were binned by genomic location into putative genes.

In the analysis of *X. borealis* transcripts from tadpole stages 46 and 48 gonad/mesonephros and adult liver, a

total of 453, 763 and 614 transcripts, respectively, were significantly sex-biased (table 1). In all three of these *X. borealis* transcriptomes, there were significant and substantially higher densities of genes in the sex-linked region of the *X. borealis* sex chromosome that encoded sex-biased transcripts compared to (i) the homeologous region of chromosome 8S in *X. borealis* and (ii) the rest of the genome of *X. borealis* ($p \leq 0.001$ for all comparisons, binomial tests, table 1 and figure 5). More specifically, between 31 and 50% of all of the sex-biased transcripts that mapped to chromosomes in these three *X. borealis* transcriptomes were encoded by genes in the sex-linked region of chr8L, which comprises only approximately 2% of the genome. With *X. borealis* as the focal species, the interaction terms from the logistic regression were significant for each *X. borealis* transcriptome ($p \ll 0.001$ for all three tests). The interaction coefficient of these models provides an estimate of the ratio of odds ratios of the probability a transcript is significantly differently expressed in the sex-linked region of chr8L compared to the rest of the L subgenome, divided by the probability that a transcript is significantly differently expressed in the region of chr8S that is homologous to the sex-linked region of chr8L compared to the rest of the S subgenome. Exponentiating the 95% confidence intervals for these interaction coefficients from each model indicates that the odds of significant sex-biased expression in the sex-linked region of chr8L is 2.9–8.6, 3.9–8.7 or 2.4–5.6 times higher than the rest of the genome after controlling for subgenome effects (for the gonad/mesonephros tadpole stages 46 and 48 and adult liver, respectively). In the *X. borealis* transcriptomes, the

Table 1. The number of significantly sex-biased transcripts by chromosome in *X. borealis* tadpole stages 46 and 48 gonad/mesonephros (46, 48) and adult liver (L), including female-biased (FB), female-specific (FS), male-biased (MB) and male-specific (MS) transcripts; with biased transcripts being expressed in both sexes but significantly higher in one, and specific transcripts being expressed exclusively in one sex. (For the sex chromosome (chr8L), numbers in parentheses refer to the number of differentially expressed transcripts in *X. borealis* that are encoded by genes within the sex-linked region. For the chromosome that is homologous to the sex chromosome (chr8S), numbers in parentheses indicate differentially expressed transcripts that are encoded by genes within the portion of this chromosome that is homologous to the sex chromosome. For four other chromosomes, numbers in parentheses indicate the number of differentially expressed transcripts in *X. borealis* that are encoded by genes in genomic regions that are homologous to sex-linked regions of *X. laevis* (chr2L, chr2S) and *X. tropicalis* (chr7L, chr7S), respectively. Some sex-biased transcripts mapped to scaffolds whose chromosomal locations are unknown (scaffolds) or did not map to the *X. laevis* reference genome (unmapped).)

chr	FB46	FS46	MB46	MS46	FB48	FS48	MB48	MS48	FBL	FSL	MBL	MSL
chr1L	4	6	8	16	3	6	9	3	7	9	5	4
chr1S	2	3	6	1	0	4	0	1	2	1	6	5
chr2L	1(0)	3(0)	1(0)	1(0)	0(0)	5(0)	6(0)	3(0)	7(0)	10(0)	3(0)	1(0)
chr2S	0(0)	4(0)	0(0)	3(0)	2(0)	6(0)	5(0)	0(0)	2(0)	12(0)	3(0)	3(0)
chr3L	4	8	4	7	6	6	9	2	4	9	1	1
chr3S	1	2	0	2	8	13	7	3	5	1	3	0
chr4L	2	5	2	2	3	3	9	3	11	6	2	3
chr4S	2	2	1	1	4	4	2	2	6	2	3	1
chr5L	3	1	2	4	4	7	9	2	12	22	10	8
chr5S	1	3	3	1	5	3	1	2	3	3	4	6
chr6L	3	3	2	1	3	3	1	4	5	4	3	1
chr6S	0	3	1	0	4	3	7	0	2	4	4	7
chr7L	1(0)	1(0)	2(0)	0(0)	1(0)	5(0)	5(1)	1(0)	7(0)	2(0)	1(0)	5(0)
chr7S	0(0)	2(0)	1(0)	0(0)	2(0)	0(0)	3(1)	1(0)	2(0)	3(0)	2(1)	0(0)
chr8L	53(52)	99(95)	10(9)	12(9)	74(69)	206(195)	31(28)	14(11)	41(34)	123(102)	12(12)	16(14)
chr8S	4(3)	18(13)	2(2)	1(1)	12(7)	29(25)	6(3)	0(0)	8(6)	28(19)	2(1)	3(2)
chr9_10L	1	0	1	1	2	3	5	7	0	5	11	4
chr9_10S	2	4	5	4	3	3	2	2	4	8	3	4
scaffolds	12	34	4	6	0	48	14	8	12	21	6	15
unmapped	11	19	2	4	15	40	13	13	6	22	1	1

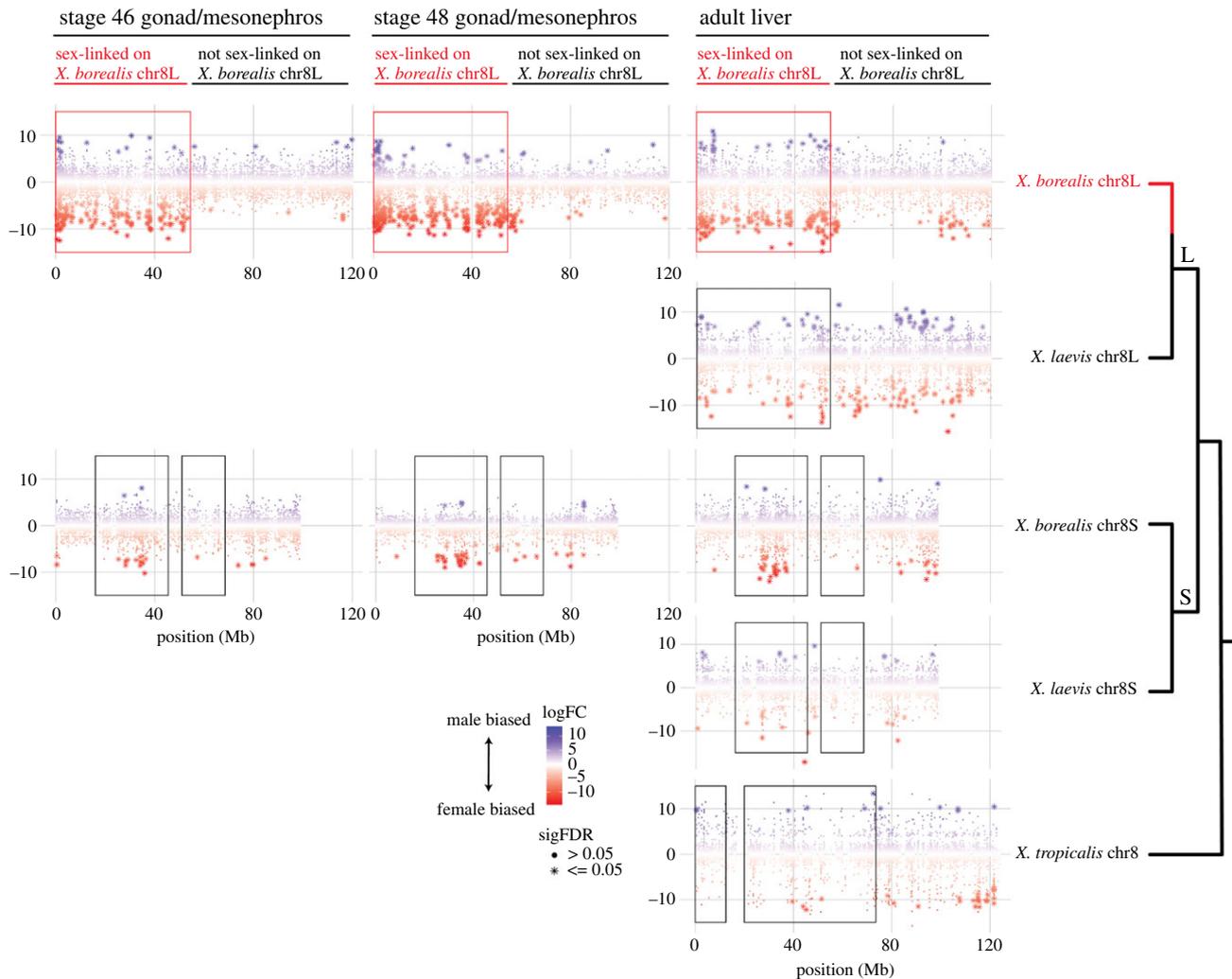


Figure 5. Comparative transcriptomics illustrates that female-biased expression (negative log(male/female fold change) on the y-axis, red asterisks) evolved contemporaneously with sex linkage in the new sex chromosomes (chr8L) of *X. borealis*. Results of analyses of differential expression for gonad/mesonephros tissue are presented for tadpole stages 46 (left) and 48 (centre) and adult liver (right). A high number of sex-biased transcripts are encoded by genes in the sex-linked portion of the *X. borealis* sex chromosomes (chr8L) (red boxes) but not in homeologous regions of *X. borealis* and *X. laevis* chr8S or orthologous regions of *X. laevis* chr8L or of *X. tropicalis* chr8 (black boxes). These differences indicate that these changes evolved recently in *X. borealis*, after allotetraploidization and speciation from *X. laevis*. On the right, the phylogeny depicts relationships among homeologues and orthologues as detailed in figure 1; branches corresponding to the L and S subgenomes are labelled. Plots of all chromosomes for each transcriptome and species in these analyses are provided in the electronic supplementary material, figures S2–S6. (Online version in colour.)

effect of subgenome was not significant in any of these models ($p = 0.90, 0.12$ and 0.85 , respectively), and the results were essentially the same when the subgenome predictor and interaction term were not included in each model (data not shown).

In the non-sex-linked portion of the *X. borealis* genome, the proportions of the sex-biased transcripts that mapped to chromosomes that were female-biased were fairly similar in all three transcriptomes (53%, 59% and 66%, respectively, for tadpole stages 46 and 48 gonad/mesonephros and adult liver), as was the density of sex-biased genes (0.07, 0.10 and 0.12 sex-biased transcripts Mb⁻¹, respectively, assuming that the non-sex-linked portion of the genome is approximately 3.0 Gb). However, in the sex-linked region, the proportions of sex-biased transcripts with female-biased expression were uniformly higher than the rest of the genome (89%, 87% and 84% in tadpole stages 46 and 48 and adult liver, respectively) and the densities of sex-biased transcripts in the sex-linked region were 23–52 fold higher than the rest of the genome (2.89, 5.32 and 2.84 sex-biased transcripts Mb⁻¹,

respectively, assuming the sex-linked region of the *X. borealis* sex chromosomes is approximately 57 Mb in size [29]). This large disparity is qualitatively obvious when expression divergence of transcripts is visualized relative to the genomic position (red asterisks denote significantly negative log(-male/female) fold change—indicating female-biased expression—in figure 5). It is also apparent when densities are calculated relative to the number of annotated genes in each region instead of Mb (electronic supplementary material, Results). Although the proportions of male-biased transcripts were lower in the sex-linked region of *X. borealis* compared to other genomic regions (owing to so many female-biased transcripts that are encoded by genes in this region), there was still a 10–16 times higher density of male-biased transcripts relative to the non-sex-linked part of the genome. Thus, the sex-linked region is enriched in female-biased as well as male-biased transcripts, but more so for the female-biased transcripts.

For adult liver, additional comparisons to orthologous transcriptomes of *X. laevis* and *X. tropicalis* were possible.

These comparisons illustrate that the proportion of genes with differentially expressed transcripts in the sex-linked portion of the *X. borealis* sex chromosomes is higher than (i) the orthologous region of chr8L in *X. laevis*, (ii) the homeologous regions chr8S in *X. laevis*, and (iii) the orthologous region of chr8 in *X. tropicalis*. In contrast with the findings from *X. borealis*, the interaction term from the logistic regression was not significant for the *X. laevis* adult liver transcriptome when the sex-linked and homeologous regions were defined to match *X. borealis* (i.e. when *X. laevis* is a non-focal species and *X. borealis* is the focal species; $p=0.82$). Likewise, in *X. tropicalis*, the logistic regression also found no significant increase in the odds of differential expression for transcripts in the genomic region that is orthologous to the sex-linked region of *X. borealis* as compared to other genomic regions (i.e. when *X. tropicalis* is a non-focal species and *X. borealis* is the focal species; $p=0.80$). Previous work has demonstrated that the sex chromosomes of *X. borealis* evolved after divergence from the most recent common ancestor with *X. laevis* (figure 1, [27]). Thus, these results from *X. laevis* and *X. tropicalis* together with intraspecific comparisons within *X. borealis* demonstrate that rapid regulatory evolution of the sex-linked region of *X. borealis* corresponded in time with this region becoming sex-linked (i.e. after divergence from the most recent common ancestor with *X. laevis*), as opposed to this region of chr8L encoding a high number of differentially expressed transcripts in an ancestor prior to the origin of the new sex chromosomes of *X. borealis*. These transcriptome results demonstrate that the *X. borealis* transcriptome evolved to have sex-specific expression owing to evolution in *cis*- of the regulatory regions of sex-linked genes on the sex chromosomes of *X. borealis*. This effect was pronounced (figure 5) and significant, but affects only the small proportion of the transcriptome that is encoded by sex-linked genes.

On the homeologous chr8S of *X. borealis*, we observed a lower density of sex-biased genes than the sex chromosome (chr8L) for all three transcriptomes, although this was still higher than the rest of the genome (electronic supplementary material, figures S2–S4). A probable explanation is that some of these sex-biased transcripts erroneously mapped to chr8S of the *X. laevis* reference genome, even though they actually are encoded by genes on chr8L in *X. borealis*. Thus, we suspect the female-biased expression of sex-linked transcripts is even higher than our data suggests. In some of the *X. borealis* transcriptomes, various non-sex-linked genomic regions had a high density of female-biased transcripts (e.g. <15 Mb on chr3S in the gonad/mesonephros tissue of tadpole stage 48 transcriptome and 130–155 Mb on chr5L in the adult liver transcriptome, electronic supplementary material, figures S3 and S4). However, these patches were much smaller and had far lower densities of sex-biased transcripts than the sex-linked region of the new sex chromosomes of *X. borealis*.

(c) The genomic landscapes of genes with sex-biased expression in *Xenopus laevis* and *Xenopus tropicalis*

Another question we were able to investigate is whether the sex-linked regions of *X. laevis* and *X. tropicalis* sex chromosomes also are enriched with transcripts with sex-biased expression (electronic supplementary material, figure S7). Similar to the findings in *X. borealis*, each of the independently evolved sex-linked regions of *X. laevis* and

Table 2. The number of differentially expressed transcripts by chromosome in *X. laevis* adult liver, with labelling following table 1. (For the sex chromosome (chr2L) and its homeologue (chr2S), numbers in parentheses refer to the number of differentially expressed transcripts that are encoded by genes within the genomic region that is sex-linked or homeologous to the sex-linked region, respectively, in *X. laevis*. For the orthologue to the *X. borealis* sex chromosome (chr8L), the homeologue to the *X. borealis* sex chromosome (chr8S) and both orthologues to the sex chromosome of *X. tropicalis* (chr7L, chr7S), numbers in parentheses indicate the number of differentially expressed transcripts in *X. laevis* that are encoded by genomic region that are homologous to the sex-linked regions of *X. borealis* or *X. tropicalis*, respectively. Numbers in parentheses comprise a large fraction of the total for the chromosome only for the sex chromosome of *X. laevis* (chr2L).)

chr	FB	FS	MB	MS
chr1L	45	97	28	33
chr1S	18	23	16	24
chr2L	21(18)	28(22)	8(7)	42(33)
chr2S	11(0)	55(2)	4(0)	30(1)
chr3L	34	63	29	87
chr3S	11	17	5	12
chr4L	63	76	13	47
chr4S	20	27	15	58
chr5L	13	12	4	15
chr5S	2	23	5	5
chr6L	23	27	7	13
chr6S	3	8	6	11
chr7L	44(1)	121(1)	18(1)	41(2)
chr7S	7(0)	18(0)	6(1)	21(2)
chr8L	44(19)	55(12)	15(6)	45(16)
chr8S	8(4)	10(5)	3(2)	10(3)
chr9_10L	20	13	11	6
chr9_10S	13	20	4	8
scaffolds	25	35	23	35
unmapped	1	3	0	4

X. tropicalis also was significantly enriched with sex-biased transcripts compared to other homeologous and/or orthologous genomic regions. In *X. laevis*, the density of sex-biased transcripts in the sex-linked region was 27 sex-biased transcripts Mb⁻¹, which is over 40 times the density elsewhere (0.57 sex-biased transcripts Mb⁻¹; table 2; electronic supplementary material, figure S7). When sex-linked and homeologous regions were defined to match the sex chromosomes of *X. laevis* (i.e. portions of chr2L and chr2S respectively and *X. laevis* is the focal species), the interaction term of the linear model for *X. laevis* was significant ($p \ll 0.001$) and the 95% confidence interval of the odds that a transcript encoded by genes in the sex-linked region of *X. laevis* would have sex-biased expression was 1.9–5.7 times higher than the odds for transcripts encoded by genes in the rest of the genome after controlling for subgenome effects. When logistic regressions were performed on *X. borealis* and *X. tropicalis* transcriptomes but defining orthologous and homeologous to the sex-linked region to match

Table 3. The number of differentially expressed transcripts by chromosome in *X. tropicalis* adult liver, with labelling following table 1. (For the sex chromosome (chr7), numbers in parentheses refer to the number of differentially expressed transcripts that are from genes within the genomic region that is sex-linked in *X. tropicalis*. For chr2 and chr8, numbers in parentheses refer to the number of differentially expressed transcripts in *X. tropicalis* that are encoded by genes within genomic regions that are orthologous to the sex-linked regions of *X. laevis* and *X. borealis*, respectively. There is a high number of male-biased genes in the sex-linked portion of the sex chromosome of *X. tropicalis* (chr7) even though this region is a small portion of this chromosome.)

chr	FB	FS	MB	MS
chr1	13	30	5	7
chr2	3(0)	4(0)	7(0)	1(0)
chr3	6	10	1	12
chr4	53	35	13	2
chr5	6	2	6	4
chr6	5	8	3	12
chr7	2(1)	1(0)	7(3)	24(17)
chr8	16(14)	12(10)	8(6)	7(5)
chr9	4	1	3	2
chr10	4	4	4	1
scaffolds	0	0	0	0
unmapped	1	0	0	0

X. laevis (i.e. these two species are the non-focal species and *X. laevis* is the focal species), there was no significant coefficient (for *X. borealis* for the interaction term ($p=0.953$) or for *X. tropicalis* for the *sex_linked* predictor variable ($p=0.951$)). These contrasts are consistent with the proposal that sex-biased expression of the portion of the *X. laevis* that is encoded by the sex-linked portion of chr2L arose after divergence from the most recent common ancestor with *X. tropicalis* and coincided with this region becoming sex-linked in *X. laevis* and then were lost in *X. borealis* after that species acquired a new sex chromosome (on chr8L).

There was also strong evidence for male-biased expression of transcripts encoded by the independently evolved sex-linked portion of chr7 in the genome of *X. tropicalis*. In this region, the density of sex-biased transcripts in the sex-linked portion of chr7 was 1.9 sex-biased transcripts Mb⁻¹, which is 10 times the density of transcripts that map to other portions of chromosomes (0.18 sex-biased transcripts Mb⁻¹; table 3; electronic supplementary material, figure S7). This enrichment is reflected by a significant coefficient in a logistic regression ($p \ll 0.001$) which indicates that the odds of sex-biased expression are 3.5–8.5 higher in the sex-linked region of chr7 than in other genomic regions (i.e. with *X. tropicalis* as the focal species). Although the comparison to *X. laevis* and *X. borealis* is complicated by genome duplication, we ran the linear models anyway, but defining the *SL_homeologous* predictor variable to match the sex-linked portions of *X. tropicalis* chr7 (i.e. with these two species as non-focal species and *X. tropicalis* as the focal species). In these analyses, there was no significant interaction between the *SL_homeologous* and *subgenome* predictor variables either for *X. laevis* or for *X. borealis* ($p > 0.05$ for both species).

These contrasts are consistent with the proposal that the sex-biased expression of the sex-linked portion of the *X. tropicalis* transcriptome evolved in association with sex linkage in this species as opposed to being an ancestral condition, and that this enrichment was either lost when new sex chromosomes emerged in *X. laevis* and *X. borealis* or that it was never present if the most recent common ancestor of these three species had a sex chromosome elsewhere in the genome.

In contrast with *X. borealis*, the sex-linked region of *X. tropicalis* has a higher proportion of male-biased or male-specific transcripts ($n=20$ out of 21 sex-biased transcripts; 95%) than the rest of the genome, where 38% of sex-biased transcripts are male-biased (table 3). In *X. laevis*, there is a more similar proportion of female- and male-biased transcripts in the sex-linked region (50% female-biased) compared to the rest of the genome (60% female-biased; table 2), than in *X. borealis*.

One unexpected finding was that non-sex-linked transcripts in the *X. laevis* adult liver transcriptome were more strongly sex-biased compared to *X. borealis* and *X. tropicalis* (electronic supplementary material, figure S5 compared to electronic supplementary material, figures S4 and S6). In the *X. laevis* adult liver transcriptome, we detected 4.7 times as many sex-biased expressed transcripts in the non-sex-linked portion of the genome ($n=1718$) compared to *X. borealis* ($n=368$), although this disparity is magnified slightly by the larger size of the non-sex-linked region of the *X. laevis* genome. Even after excluding the sex-linked region, there were significantly more sex-biased expressed transcripts encoded by genes from the L ($n=1181$) than the S subgenome in *X. laevis* ($n=537$, $p \ll 0.0001$, binomial test). After controlling for sex linkage, the partial effect of the *subgenome* predictor was significant in logistic regression for *X. laevis* when this species was the focal species (i.e. with *SL_homeologous* set to 1 for the ends of chr2L and chr2S, see Methods; $p \ll 0.001$). Exponentiating the interaction coefficient indicates that the 95% confidence interval for the odds of sex-biased expression of transcripts encoded by genes in the L subgenome is 1.63–2.03 higher than for those encoded by genes in the S subgenome. Thus, not only are genes more highly expressed in the L subgenome [32], but it appears that they are more likely to encode transcripts with sex-biased expression, even after controlling for the effects of sex linkage on chr2L. One important caveat is that subgenome and significance are conflated because differential expression is statistically easier to detect for highly expressed transcripts (on the L), which violates assumptions of the linear model. Other caveats of this and other transcriptome comparisons are discussed in the electronic supplementary material.

Another unexpected observation relates to the gonad/mesonephros transcriptomes from tadpole stage 48 and adult liver, where the high density of sex-biased transcripts extends slightly beyond the sex-linked region identified by [29]. This was not the case in the corresponding transcriptome from tadpole stage 46 (figure 5). At this time, we do not have an explanation for this observation.

(d) Sex-biased expression is less pronounced in gene-level than transcript-level analyses

When expression levels of transcripts that mapped to the same genomic region were summed into putative genes,

analysis of the resulting genic expression recovered a less substantial or non-significant enrichment of sex-biased expression in the sex-linked region of these three frog species. With *X. borealis* as the focal species (i.e. when the *SL_homeologous* parameter defined to match the sex-linked and homeologous regions of *X. borealis*), the interaction term of the linear models was significant for *X. borealis* gonad/mesonephros tadpole stage 48 ($p = 0.01$) and adult liver ($p = 0.003$), but not gonad/mesonephros tadpole stage 46 ($p = 0.16$). Thus, after controlling for subgenome effects, the odds of sex-biased expression at the gene level were 1.6–20.4 and 1.5–8.1 times higher in the sex-linked region as compared to elsewhere in *X. borealis* gonad/mesonephros tadpole stage 48 and adult liver, respectively. When summed transcripts from *X. laevis* and *X. tropicalis* were analysed with each of these species as the focal species, the interaction coefficient for the *X. laevis* data was not significant, and the coefficient for sex linkage in *X. tropicalis* also was not significant ($p > 0.05$ for all coefficients). These results are consistent with plots of gene-level expression which illustrate that the summed (gene-level) sex-biased expression in the sex-linked regions is less distinguished from the rest of the respective genomes of these three focal species (electronic supplementary material, figures S8–S12) as compared to the analysis of individual transcripts (electronic supplementary material, figures S2–S4). Thus, it appears that much of the regulatory evolution that occurred on the sex-linked region influenced expression at the level of splice variants and alleles, as opposed to concurrently governing expression of all transcripts encoded by sex-linked genes.

4. Discussion

(a) Among population variation of the sex chromosomes of *Xenopus borealis*

To help disentangle concurrent and confounding drivers of sex chromosome evolution, we scrutinized the independently evolved sex chromosomes of three closely related frog species: the Marsabit clawed frog *X. borealis*, the African clawed frog *X. laevis* and the western clawed frog *X. tropicalis*. Using field-collected animals and genomic and transcriptomic approaches, we demonstrate that: (i) *X. borealis* has a modest but significant population structure that is relatively recently evolved; (ii) there exists variation among *X. borealis* populations in the extent of sex chromosome differentiation that corresponds to a previously identified region of recombination suppression [27]; (iii) the sex-linked portion of the *X. borealis* sex chromosomes (chr8L) carry genes that encode a high number of transcripts with sex-biased expression, and especially female-biased expression; and (iv) sex-biased regulatory evolution evolved in *X. borealis* independently after divergence from *X. laevis*, and in association with the origin of sex linkage on the new sex chromosomes of this species. In the independently evolved sex-linked regions of *X. laevis* (chr2L) and *X. tropicalis* (chr7), we also identified a high density of sex-biased transcripts encoded by sex-linked transcripts, and we showed that sex-biased regulation evolved independently in association with sex linkage in each of these species as well. These findings are thus concordant across these three frog species, each with independently evolved sex chromosomes, and each with distinctive sex-

linked genomic regions. These results point to independent evolution of *cis*-regulation in the sex-linked regions of each species that occurred contemporaneously with the onset of sex linkage, as opposed to having been present ancestrally in an autosomal precursor of each sex chromosome. When analysis was performed at the gene level on the transcriptome data from all three species, the signal of sex-biased expression by sex-linked genes was less substantial, which suggests that this effect is largely driven by allele-specific and splice variant-specific expression. Technical artefacts undoubtedly also affect our quantification of sex-biased expression, but we have no reason to suspect that this would result in an enrichment of sex-biased expression of transcripts encoded by genes in three different sex-linked regions of three species.

In the laboratory strain of *X. borealis* previously studied by Furman & Evans [26,29], sex chromosome differentiation was detected that coincided with recombination suppression [29]. Our study suggests that this strain originates from central Kenya (figures 2 and 3), shows that the extent of sex chromosome differentiation varies among populations of *X. borealis* (figure 4), and links recombination suppression and sex chromosome differentiation to the sex-biased expression of transcripts encoded by sex-linked genes in *X. borealis* from central and eastern Kenya. In a small sample of *X. borealis* from eastern Kenya, two previously identified loci on chr8L had female-specific heterozygous sites and a higher abundance of sex-linked markers in females than males (electronic supplementary material, figure S13), which is consistent with female heterogamy. By contrast, a lack of substantial differentiation between male and female *X. borealis* from western Kenya (figure 4) suggests that the sex chromosomes in these specimens are mostly pseudoautosomal regions that (by definition) recombine in both sexes. We were unable to identify sex-linked variation on chr8L in the western population of *X. borealis* (electronic supplementary material, figure S13), so it is conceivable that this population has a system for sex determination that is distinctive from *X. borealis* from central and eastern Kenya in terms of where in the genome the trigger for sex-determination resides, and/or whether males or females are the heterogametic sex.

At this time, we can only speculate about the mechanisms behind recombination suppression in the sex-linked portions of the sex chromosomes of *X. borealis* from central and eastern Kenya (figure 4). One possibility is that specimens from central and eastern Kenya (including our laboratory strain) have an inversion on the W-chromosome but not on the Z-chromosome and that suppresses recombination between this portion of these sex chromosomes. This inversion could be absent on both sex chromosomes of *X. borealis* from western Kenya—either because it was lost or because it was never present. If recombination suppression in the central and eastern populations is newly evolved compared to sex chromosomes in the western populations, this would suggest that sex-biased expression of sex-linked transcripts evolved quickly because divergence between these populations is relatively recent. Alternatively and more plausibly, *X. borealis* from west Kenya may have recently lost recombination suppression on chr8L, for example, if new sex-determining locus evolved on another chromosome in an ancestor of *X. borealis* from west Kenya. This scenario would be consistent with a more protracted period for sex-biased expression of sex-linked transcripts to arise in the

central and eastern population, but it would still be more recently than the age of the most recent common ancestor of *X. laevis* and *X. borealis* (approximately 40 Ma [32]) because the sex chromosomes of *X. borealis* arose after this ancestor diversified [26]. Further study of sex linkage in *X. borealis* from west Kenya, and in other closely related species in the *muelleri* species group (figure 2) may help distinguish between these alternatives.

(b) Effects of sex chromosome evolution on the transcriptome

In three frog species, we observed a higher density of sex-biased transcripts encoded by genes on the sex-linked portion of the sex chromosomes as compared to those encoded by genes located in the rest of the genome. In *X. borealis*, which has female heterogamy, this higher density of sex-biased transcripts was significantly skewed towards female-biased transcripts compared to the rest of the genome (table 1 and figure 5), but in *X. tropicalis*, a higher proportion of transcripts with male-biased expression are encoded in the sex-linked region. In *X. laevis*, there is also a high density of sex-biased transcripts in the sex-linked region, but no substantial skew towards these transcripts being more female- or male-biased as compared to sex-biased transcripts encoded by genes elsewhere. Several factors discussed below could account for strong sex-biased expression skews of transcripts encoded by sex-linked genes, including sex chromosome degeneration, and adaptive evolution of the sex-specific (W- or Y-) or sex-shared (X- or Z-) sex chromosome—including faster-X/Z effects.

(i) Sex chromosome degeneration

In *X. tropicalis*, there is polymorphism in sex chromosomes, with W-, Z- and Y-chromosomes being present in different laboratory strains [23] and the Y-chromosome evolved from ancestral Z-chromosomes [28]. Male-biased expression of sex-linked genes is also observed in *X. tropicalis* tadpole gonad/mesonephros tissue, and patterns of nucleotide polymorphism in expressed transcripts *X. tropicalis* are consistent with degeneration of the W-chromosome as a primary mechanism driving male-biased expression [28]. The similarity in expression profiles reported here for adult liver tissue suggests that degeneration of the small sex-linked portion of the W-chromosome may account for the male-biased expression of sex-linked transcripts in many or all tissues throughout development.

In *X. borealis*, however, this explanation does not immediately explain the pattern of sex-biased expression that we observed because a strong skew towards female-biased expression is not predicted from W-chromosome degeneration. One hypothetical scenario that could explain these observations is that this biased expression is a consequence of degeneration of an ancestral Y-chromosome that eventually evolved into the contemporary Z-chromosome in association with an ancestral X-chromosome evolving to become the contemporary W-chromosome (table 1C in [42]). A transition from an XY to a ZW system is theoretically disfavoured by natural selection if it involves fixation of a degenerate Y-chromosome [20], but is still possible if the deleterious load of the ancestral Y-chromosome is modest. Amphibians are not known to have dosage compensation

[43] and tolerance of dosage differences between the sexes is evidenced here by the sex-biased expression of transcripts encoded by genes in the sex-linked region (figure 5). However, molecular polymorphism of expressed sex-linked transcripts in male *X. borealis* is not substantially lower than that of expressed non-sex-linked transcripts (electronic supplementary material). This suggests that if a sex chromosome transition in *X. borealis* did occur, it did not occur recently because non-recombining portions of Y-chromosomes are expected to have much lower polymorphism than autosomes [1].

(ii) Adaptive evolution on sex-specific sex chromosome

Another factor that potentially could contribute to higher expression in the heterogametic sex is adaptive evolution on the sex-specific sex chromosome, such as the W-chromosome of *X. borealis* or the Y-chromosome of *X. tropicalis*. While we do not have evidence for or against this possibility, adaptive evolution is generally expected to be faster on the X or Z-chromosome than on the Y- or W-chromosome [44,45].

(iii) Adaptive evolution on sex-shared sex chromosome

In general, theory predicts adaptive evolution is generally faster on the autosomes than on the X-chromosome, but under certain conditions, this will be reversed with faster evolution on the X- (or Z-) chromosome [4]. Theoretical conditions for faster-X evolution include that (i) beneficial mutations are partially or completely recessive, (ii) fitness effects of mutations are similar in each sex, (iii) mutation rates and distributions of fitness effects of mutations are similar in each sex, (iv) the substitution rate is the product of the number of mutations per generation and their fixation probabilities (which are determined by selection coefficients in each sex), and (v) relative effective population size (N_e) on the X-chromosome is three-quarters that of the autosomes [4,46,47]. Departures from these assumptions, such as increased relative N_e of the X- as a consequence of sexual selection on males, can increase faster-X evolution, though this effect is diminished by high levels of random offspring mortality [48]. For faster Z-effects, expectations are similar, though faster-Z effects may be amplified by a higher male than female mutation rate, which is common in many species [49].

By way of comparison, in *Drosophila*, expression divergence of male-biased genes on the X-chromosome evolves faster than their counterparts in the autosome [50], and signatures of adaptive evolution are prominent in some male-biased genes on the X-chromosome [51]. It is also possible that faster-X (or faster-Z) effects benefit the homogametic sex by favouring the fixation of recessive alleles that are expressed in the heterogametic sex that benefit the homogametic sex. In fruit flies with male heterogamy, for example, there is evidence of faster-X effects in female-biased transcripts [52–54]. In fact, in species with male heterogamy, there is often a higher proportion of female- than male-biased expression in sex-linked transcripts [55–59], and in some species with female heterogamy, there can be an enrichment in sex-linked genes of transcripts with male-biased expression [60,61].

Could faster-Z effects account for male-biased expression in *X. borealis*? Relevant to this possibility is that adaptation which involves allele frequency changes at many sites throughout the genome (polygenic adaptation) or beneficial

mutations that appear recurrently or at a high rate are not necessarily expected to have faster-X evolution [46]. This is because these conditions render the connection between the rate of substitution and the rate of beneficial mutation more complex (and idiosyncratic owing to effects of epistatic interactions for polygenic adaptation) as compared to the rate of adaptive evolution that occurs via a series of successive selective sweeps at individual sites [46]. Additionally, faster-X and faster-Z effects are expected to primarily exploit genetic variation that has been consistently beneficial since the mutations arose, as opposed to exploiting standing genetic variation where fitness effects of a mutation change as a consequence of changed environmental conditions [4,62]. Thus, if the sex-biased expression of transcriptomes encoded by sex-linked genes is owing to faster-X-like and faster-Z effects, this suggests that it should primarily be owing to new mutations, as opposed to standing variation that was previously at mutation-selection equilibrium. These factors collectively limit explanatory power of faster-Z effects as a major driver of widespread skew in the sex-biased expression of sex-linked transcripts in *Xenopus* or at least argue that it would take considerable time to manifest. Because we have no reason to suspect that mutation rates in *Xenopus* are particularly unusual, we prefer the hypothesis of degeneration of an ancestral Y-chromosome followed by a transition from a male (XY) to female (ZW) heterogamy.

It would be fascinating for future efforts to evaluate the functional significance of sex-biased expression patterns by comparing populations with distinctive sex chromosomes (e.g. the western versus eastern populations of *X. borealis*, including zones of intergradation if they exist). The observation of population variation in sex chromosomes of *X. borealis* (figure 4) opens the possibility that a sex chromosome turnover may have taken place in *X. borealis* from western Kenya. This raises the questions of what the sex-determination system of the western population of *X. borealis* is, where it is located in the genome, whether there is a strong skew of transcripts with female-biased expression encoded by sex-linked genes, and whether this population still has the Z-chromosome that is present in the central/eastern population and that potentially could be derived from a degenerate Y-chromosome.

5. Concluding remarks

In the Marsabit clawed frog, *X. borealis*, there exists population variation in the extent of differentiation between the W- and Z-chromosomes on a large sex-linked region of these sex chromosomes. This sex-linked region has a far

higher density of transcripts with sex-biased expression—especially female-biased expression—as compared to orthologous and homeologous genomic regions, and to other recombining pseudoautosomal and autosomal genomic regions. This demonstrates that sex-biased expression of sex-linked genes occurred in concert with the origin of the new sex chromosomes of *X. borealis*. Similar findings were also recovered from transcriptomes of *X. laevis* and *X. tropicalis*, each of which has independently evolved sex chromosomes. Additionally, we found that transcripts encoded by sex-linked genes in *X. borealis* and *X. tropicalis* have a strong sex bias (female- or male-biased, respectively). A plausible explanation for the intense, sex-bias of transcripts that emerge from the sex-linked region is that it is owing to degeneration of a sex-specific sex chromosome (the W- in *X. tropicalis* and an ancestral Y- in *X. borealis*). For *X. borealis*, this scenario would involve a recent homologous sex chromosome turnover, wherein an ancestral sex-determination system on chromosome 8L with male (XY) heterogamy evolved into the current system on the same chromosome with female (ZW) heterogamy, with the ancestral X-chromosome becoming the contemporary W-chromosome and the ancestral Y-chromosome becoming the contemporary Z-chromosome. This hypothesis can be tested with comparative data from other populations and species. Still unknown are the functional consequences of extensive sex-biased expression of transcripts that are tethered to various sex-determining loci in different *Xenopus* species. This raises the question of whether and how this variation might clash or synergize in individuals in nature.

Ethics. This work was approved by the Animal Care Committee at McMaster University (AUP no. 17-12-43).

Data accessibility. The RRGs and RNAseq data from this study have been deposited in the Short Read Archive of NCBI (BioProject PRJNA616217). All new Sanger sequences were submitted to GenBank (accession nos MT998323–MT998434).

Authors' contributions. B.L.S.F., D.V.W. and B.J.E. performed the field-work. X.-Y.S., B.L.S.F., T.P., M.K., C.M.S.C., I.D. and B.J.E. collected and analysed data. X.-Y.S. and B.J.E. wrote the paper, and all authors edited the paper.

Competing interests. We declare have no competing interests.

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