PROCEEDINGS B

royalsocietypublishing.org/journal/rspb

Research

**Cite this article:** Sigeman H, Ponnikas S, Chauhan P, Dierickx E, Brooke M de L, Hansson B. 2019 Repeated sex chromosome evolution in vertebrates supported by expanded avian sex chromosomes. *Proc. R. Soc. B* **286**: 20192051. http://dx.doi.org/10.1098/rspb.2019.2051

Received: 5 September 2019
Accepted: 1 November 2019

**Subject Category:**
Evolution

**Subject Areas:**
evolution, genomics, bioinformatics

**Keywords:**
sex chromosome, neo-sex chromosome, recombination, degeneration, vertebrate, bird

**Authors for correspondence:**
Hanna Sigeman
e-mail: hanna.sigeman@biol.lu.se
Bengt Hansson
e-mail: bengt.hansson@biol.lu.se

Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.4730015.

THE ROYAL SOCIETY PUBLISHING

# Repeated sex chromosome evolution in vertebrates supported by expanded avian sex chromosomes

Hanna Sigeman[1], Suvi Ponnikas[1], Pallavi Chauhan[1], Elisa Dierickx[2], M. de L. Brooke[2] and Bengt Hansson[1]

[1]Department of Biology, Lund University, Ecology Building, 223 62 Lund, Sweden
[2]Department of Zoology, University of Cambridge, Downing Street, Cambridge CB23EJ, UK

HS, 0000-0002-1457-4174; SP, 0000-0003-3526-2118; ED, 0000-0002-4124-586X; BH, 0000-0001-6694-8169

Sex chromosomes have evolved from the same autosomes multiple times across vertebrates, suggesting that selection for recombination suppression has acted repeatedly and independently on certain genetic backgrounds. Here, we perform comparative genomics of a bird clade (larks and their sister lineage; Alaudidae and Panuridae) where multiple autosome–sex chromosome fusions appear to have formed expanded sex chromosomes. We detected the largest known avian sex chromosome (195.3 Mbp) and show that it originates from fusions between parts of four avian chromosomes: Z, 3, 4A and 5. Within these four chromosomes, we found evidence of five evolutionary strata where recombination had been suppressed at different time points, and show that stratum age explained the divergence rate of Z–W gametologs. Next, we analysed chromosome content and found that chromosome 3 was significantly enriched for genes with predicted sex-related functions. Finally, we demonstrate extensive homology to sex chromosomes in other vertebrate lineages: chromosomes Z, 3, 4A and 5 have independently evolved into sex chromosomes in fish (Z), turtles (Z, 5), lizards (Z, 4A), mammals (Z, 4A) and frogs (Z, 3, 4A, 5). Our results provide insights into and support for repeated evolution of sex chromosomes in vertebrates.

## 1. Introduction

Sex chromosomes contain sex-determining genes and have evolved from autosomes many times independently across the tree of life. A typical feature of emerging sex chromosomes is the evolution of progressive recombination suppression, which leads to diverging and eventually heteromorphic sex chromosomes, such as XY in mammals and ZW in birds [1,2]. The leading hypothesis for the evolution of recombination suppression on sex chromosomes stresses the importance of the genetic background of the sex-determining genes, and invokes a selective advantage of linkage between sex-determining and sexually antagonistic genes [3–5]. This hypothesis suggests that chromosomes harbouring such genes should frequently be involved in the formation, transition and turnover of sex chromosomes [6–9] and in the fusions between sex chromosomes and autosomes, forming neo-sex chromosomes [7,9–12]. A limited number of genes have taken on the sex-determining role in vertebrates, and evidence is accumulating that the chromosomes on which these reside have evolved into sex chromosomes several times independently in different lineages (reviewed in refs [9,13]). Empirical evidence for the importance of chromosome content for the evolutionary dynamics of sex chromosomes (in terms of recombination suppression, transitions and translocations) depends on well-studied independent origins of sex-linkage on a genomic level, which until recently have been very few (but see e.g. refs [14–17]). Moreover, the old age of many sex chromosome systems and the advanced stage of degeneration (gene loss) of the sex-limited chromosome (Y or W) means that material to study has

been lost and constitutes a major challenge in empirically assessing the evolutionary history of these chromosomes. Studying evolutionary young sex-linked regions, such as neo-sex chromosomes, offers an opportunity to test the putative selective forces driving these dynamics.

Avian sex chromosomes are highly stable as all birds studied so far share a homologous Z–W chromosome pair [18,19]. A few exceptions to this stability have been observed, such as a translocation event creating a multiple sex chromosome system in a species of penguin [20] and two cases of autosome–sex chromosome fusions [12,21]. In the passerine superfamily Sylvioidea, a neo-sex chromosome formed through a fusion between the first half (0–9.6 Mbp) of chromosome 4A (zebra finch *Taeniopygia guttata* nomenclature) and the ancestral sex chromosome [12,22,23]. Furthermore, two independent findings suggest that at least some species of larks (Alaudidae), a family within Sylvioidea, have acquired additional autosome–sex chromosome fusions. Firstly, heavily enlarged sex chromosomes were found in the karyotypes of the bimaculated lark (*Melanocorypha bimaculata*) and the horned lark (*Eremophila alpestris* [24]), and, secondly, genetic markers located on chromosome 3 and 5 were found to have sex-specific inheritance in the Raso lark (*Alauda razae* [25]).

Here, we use comparative genomics to characterize this expanded neo-sex chromosome system. First, we use whole-genome sequence data of three Alaudidae species—two *Alauda* species (Raso lark and Eurasian skylark *A. arvensis*) and one *Eremophila* species (horned lark)—and their sister species in the family Panuridae (the bearded reedling *Panurus biarmicus*) to identify which genomic regions have become fused to the sex chromosomes and stopped recombining. Next, we use dated phylogenies of Alaudidae, Panuridae, other Sylvioidea species and more distantly related outgroups, to determine and age several evolutionary strata, i.e. sex chromosome regions where recombination has been suppressed at different points in time [26], and test whether the age of the strata explains the degree of Z–W divergence of gametologous genes as predicted by sex chromosome evolution theory [26,27]. Then, we test the hypothesized importance of chromosome content for the formation of sex chromosomes by analysing whether the fused chromosomal regions are enriched for genes involved in differentiation of female and male reproductive systems, sex hormones and other sexual characteristics. As genes coding for sex-specific traits or sex-related functions are particularly likely to be involved in either past or present sexual antagonism [28], they offer a way to test if neo-sex chromosome formation has been driven by selection on specific genetic backgrounds [10]. Lastly, we evaluate signs of repeated sex chromosome evolution by searching for homologies between the fused sex chromosomes in larks and the sex chromosomes of other vertebrate lineages.

## 2. Results and discussion

### (a) Sex-linked genomic regions and age of evolutionary strata

We mapped whole-genome sequence data of one male and one female of each of the four species to de novo assembled reference genomes and anchored these data to the chromosome-level assembly of the zebra finch genome (taeGut3.4.2.; see Methods). We then scanned the genomes of each species (in 1 Mbp genome windows) for sex-specific signatures that signify sex chromosomes in different stages of degeneration: increased number of female-specific single nucleotide variants (SNVs) for slightly to moderately degenerated W chromosome regions, and decreased female read coverage depth for moderately and highly degenerated W chromosome regions. We found sex-specific signatures over the entire chromosome Z and on parts of chromosomes 3, 4A and 5 (figure 1; electronic supplementary material, table S5). The Z chromosome showed extremely low female coverage in all four species as expected for old, degenerated sex chromosomes. Chromosome 4A, the previously identified neo-sex chromosome in Sylvioidea [12], showed a high number of female-specific SNVs and moderately low female coverage over the first half of its length in all species. Finally, high amounts of female-specific SNVs, but no or moderate sex-specific coverage, were found over varying extents of chromosome 3 and 5 in the four species: small parts of chromosome 3 in the bearded reedling, slightly larger parts of chromosome 3 in the horned lark, and the greater part of chromosome 3 and 5 in the Raso lark and the Eurasian skylark (figure 1). These results support the existence of multiple autosome–sex chromosome fusions in varying stages of degeneration across several evolutionary strata, where recombination has been suppressed at different points in time across the Alaudidae/Panuridae phylogeny.

Next, we defined these non-recombining regions more precisely (at the scale of 0.1 Mbp; electronic supplementary material, figure S1 and table S6) and assessed the most parsimonious order of emergence of evolutionary strata based on dated phylogenies (figure 2*a*,*b*; [29–31]). We defined the entire ancestral sex chromosome (Z; 72.9 Mbp) as stratum 1, the oldest stratum, as this genomic region became sex-linked in an ancestor to birds *ca* 140 Myr ago [30]. The second oldest stratum, stratum 2, covers the first 9.6 Mbp of chromosome 4A [12], and was formed *ca* 21–19 Myr ago when Sylvioidea split from other passerines [31]. As expected, all four study species showed clear signatures of sex-linkage in these genomic regions. Stratum 3 is an 8.0 Mbp genomic region on chromosome 3 (between 8.4–10.4 and 18.1–24.1 Mbp) and is the earliest stratum being unique to the Alaudidae/Panuridae clade. This genomic region showed sex-specific genetic variation in all four species but is not known to appear in other Sylvioidea species. Its age is thus defined by the split of Alaudidae/Panuridae from other Sylvioidea families *ca* 19–17 Myr ago [31]. Stratum 4 constitutes a 3.6 Mbp region on chromosome 3 (between 10.4–14.0 Mbp) and occurs in all three lark species but not in the bearded reedling. This gives an estimated age of *ca* 17–14 Myr, defined by the split between Alaudidae and Panuridae [29]. The youngest evolutionary strata (5a and 5b) reside on parts of chromosome 3 (stratum 5a: 64.9 Mbp spanning the regions between 5.8–8.4, 14.0–18.1 and 29.8–88.0 Mbp) and chromosome 5 (stratum 5b: 36.3 Mbp, the region between 9.1–45.4 Mbp), which were sex-linked in the Raso lark and the Eurasian skylark in the genus *Alauda* but not in the other species. These strata are estimated to be *ca* 14–6 Myr based on the split between the horned lark and the *Alauda* larks [29]. Note that the more distinct sex-specific pattern in the Raso lark compared to the Eurasian skylark (figure 1; electronic supplementary material, figure S1) is likely caused by the former being a rare island endemic with extremely low genome-wide heterozygosity so that most genomic variation involves fixed differences between Z and W regions [25,32]. It should further be noted that the five

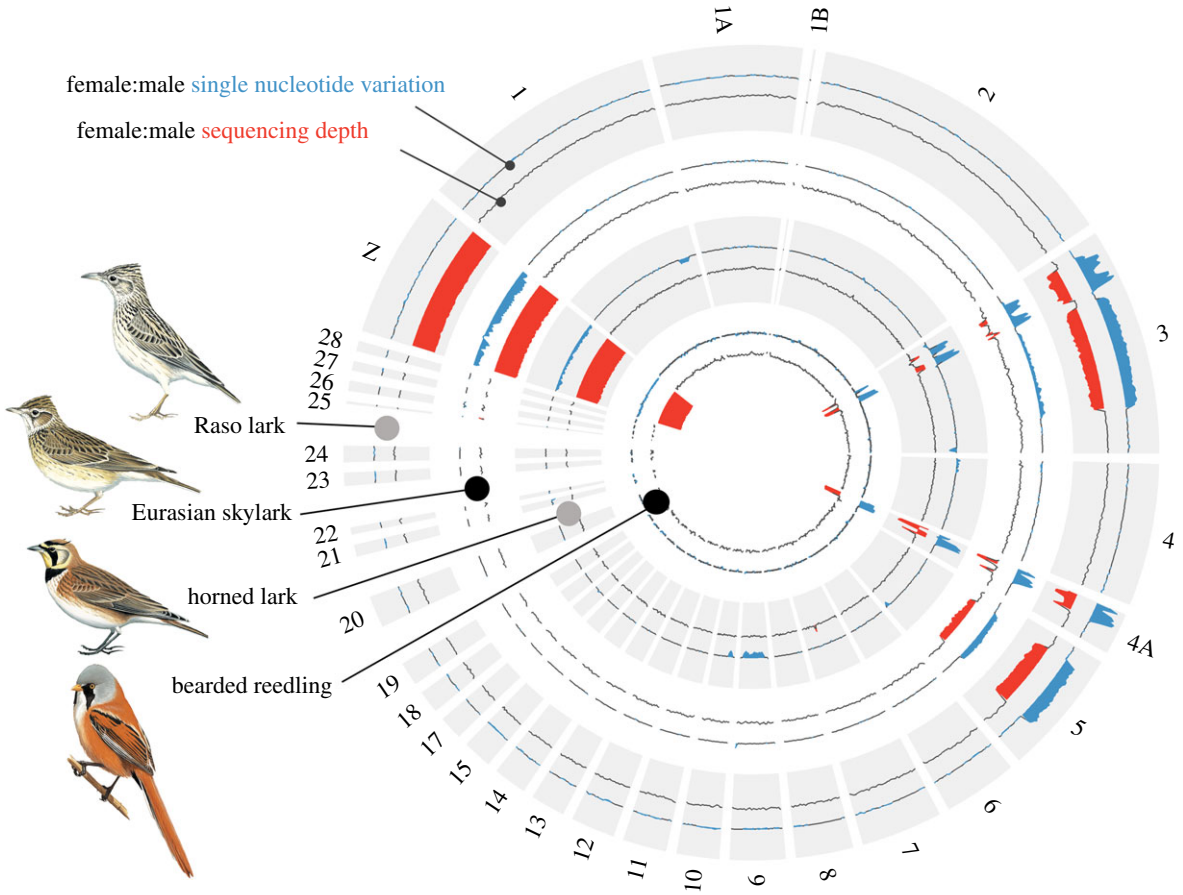


**Figure 1.** Genome-wide distribution of female-to-male difference in number of private single nucleotide variants (SNVs), and female-to-male coverage ratio in the four study species. The background colours grey and white separate the data for each of the species which are shown in the following order starting from the outer ring: (i) Raso lark, (ii) Eurasian skylark, (iii) horned lark, and (iv) bearded reedling. Within each ring, the outer line shows the average difference in number of private SNVs between females and males across 1 Mbp windows (blue: values greater than 500 or less than −500), and the inner line shows the average female-to-male coverage ratio across 1 Mbp windows (red: values greater than 1.1 or less than 0.9). Chromosome-wide averages are provided in electronic supplementary material, table S5. Bird drawings from HBW. (Online version in colour.)

evolutionary strata we have distinguished in *Alauda* larks (figure 2*a*,*b*) is an underestimation of the total number of strata in these species, as it is known from comparative genomics of deeper avian phylogenies that the ancestral sex chromosome (i.e. stratum 1 in our study) consists of several strata formed before the radiation of passerines [33,34].

Together, the sex-specific regions on chromosome Z, 4A, 3 and 5 in the Raso lark and the Eurasian skylark form the largest avian sex chromosome known to date: 195.3 Mbp constituting 16.3% of their genomes based on a genome size estimation of 1.2 Gbp (figures 1 and 2; electronic supplementary material, figure S1 and table S6). The size of the sex-linked region in the horned lark and in the bearded reedling was 94.1 Mbp (7.8%) and 90.5 Mbp (7.5%), respectively (electronic supplementary material, table S6). The 195.3 Mbp (16.3%) sex-linked region in *Alauda* corresponds well to the genomic proportion of the sex chromosomes in the karyotypes of the bimaculated lark (i.e. *ca* 15–20% [24]). The bimaculated lark karyotype further shows that the Z and W chromosomes are of similar size and drastically enlarged compared to the usual bird karyotype with heteromorphic sex chromosomes [24]. This suggests that the additional chromosome regions that are sex-linked in larks are fused to both Z and W. Moreover, the karyotype of a male horned lark (no female was karyotyped) indicates a Z chromosome of similar size to that of the bimaculated lark [24]. Therefore, it is likely that large parts of chromosome 3 and 5 are fused to the Z chromosome (and possibly W) in the horned lark, but as our results show are still recombining in that species (with the exception of strata 3 and 4 on chromosome 3 that are non-recombining). This reasoning is complemented by the results in Dierickx *et al*. [32] showing that different species and subspecies of *Alauda* (the oriental skylark *A. gulgula*, Eurasian skylark sspp. [possibly *A. a. arvensis*/*dulcivox*/*intermedia*], and Raso lark), and a species of the genus *Gallerida* (the crested lark *G. cristata*)—the sister genus of *Alauda*—have suppressed recombination on chromosome 5 (stratum 5b) but show recombination suppression to different degrees on chromosome 3 (stratum 5a).

A possible scenario is that the multiple autosome–sex chromosome fusions in larks involved the pseudo-autosomal region (PAR [1]), which would facilitate the integration of the fused elements from one sex chromosome (e.g. Z) to the other (e.g. W), followed by loss of recombination to various degrees in different lineages (figures 1 and 2). However, recombination-based genetic maps in one Sylvioidea species (the great reed warbler, *Acrocephalus arundinaceus*, in the family Acrocephalidae [12]; S. Ponnikas *et al*. in preparation) suggest that the fusion point between Z and 4A is not located in the region identified as the PAR in other passerines (e.g. collared flycatcher, *Ficedula albicollis* [35]). Unfortunately, neither genetic maps nor cytogenetic data are available in Alaudidae and Panuridae to help understand the seemingly complex patterns of splits and fusions, and consecutive phases of recombination suppression, that we have observed.

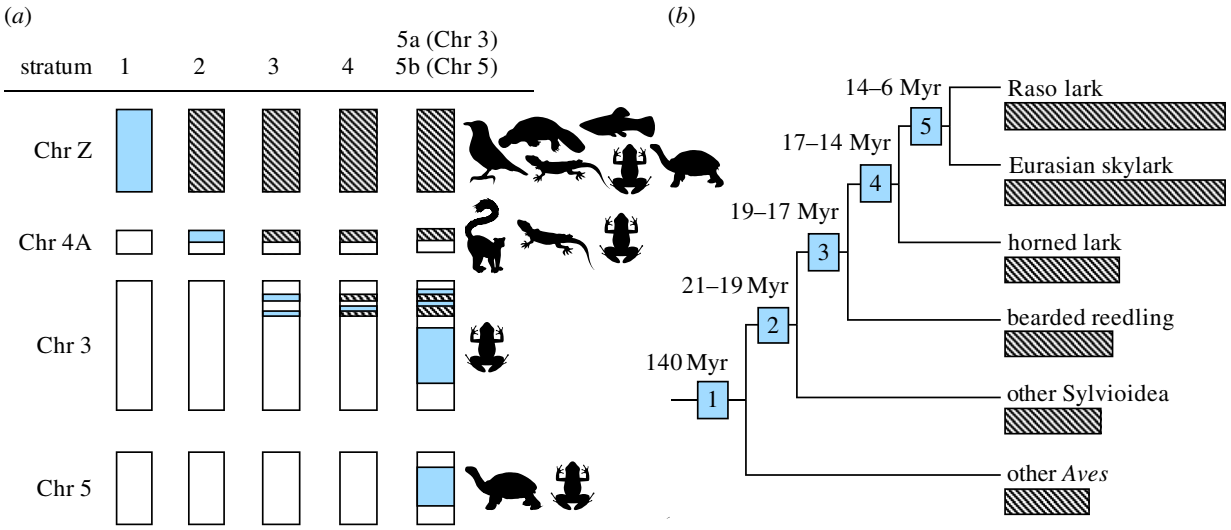


**Figure 2.** (*a*) Cartoon representation of the evolutionary strata in the most parsimonious order of appearance (starting with stratum 1 and ending with strata 5a and 5b). White represents autosomal regions and blue marks the stratum being added. Striped areas represent sex-linked regions that already exist at the timing of the new stratum. Animal silhouettes (from phylopic.org; credits in electronic supplementary material) represent vertebrate groups where homologues to these chromosomes act as sex chromosomes. (*b*) A cladogram showing the approximate time (Myr) when the evolutionary strata were formed. The bars under the tree labels represent relative sizes of sex chromosomes in the different groups (see electronic supplementary material, table S6 for details). (Online version in colour.)

## (b) Evolutionary rates of gametologous genes

Next, we analysed the degree of synonymous and non-synonymous divergence between gametologous gene pairs ($dS_{Z\text{-}W}$ and $dN_{Z\text{-}W}$) located on the different evolutionary strata in the Raso lark and the Eurasian skylark, the species with all five evolutionary strata. Our analysis of gametolog divergence rate is independent from the classification of strata, since we have inferred strata solely with phylogenetic approaches (figure 2). Also, since we are studying a relatively young neo-sex chromosome system with a moderately degenerated neo-W chromosome there are many gametologs to include in this analysis (e.g. 430 and 406 gametologs on the neo-sex regions in the Eurasian skylark and Raso lark, respectively, compared to 10 and 9 gametologs for the ancestral sex chromosome). We treated the two different parts of stratum 5 on chromosomes 3 and 5 separately in these analyses.

As expected from the different ages of the strata, $dS_{Z\text{-}W}$ varied substantially between them: median $dS_{Z\text{-}W}$ ranged from 0.224 at stratum 1 to 0.018 at stratum 5a in the Raso lark, and from 0.213 at stratum 1 to 0.018 at stratum 5a in the Eurasian skylark (figure 3). All pairs of strata differed significantly in $dS_{Z\text{-}W}$, except for strata 2 and 3 in the Raso lark, and strata 5a and 5b in both species. The $dN_{Z\text{-}W}$ values showed similar patterns, but less pronounced (median $dN_{Z\text{-}W}$ ranged from 0.030 at stratum 1 to 0.002 at stratum 5a in both species; figure 3). The $dN_{Z\text{-}W}$ values were significantly different between stratum 5a and all other strata, and between 5b and all other strata, in both species. The $dN_{Z\text{-}W}/dS_{Z\text{-}W}$ values were lowest at stratum 1 in both species (0.082 in the Eurasian skylark and 0.097 in the Raso lark) and highest at stratum 4 (0.196 in the Eurasian skylark and 0.294 in the Raso lark; figure 3). The $dN_{Z\text{-}W}/dS_{Z\text{-}W}$ values in stratum 4 differed significantly from stratum 5a in the Raso lark, and stratum 5a differed significantly from stratum 5b, in both species. For details, see electronic supplementary material, tables S7 and S8.

The level of synonymous substitutions ($dS_{Z\text{-}W}$), which is expected to have a relatively constant evolutionary rate [26,36], correlated strongly with relative age of the evolutionary strata in the *Alauda* larks (strata 5a and 5b being youngest and stratum 1 oldest; ordered-heterogeneity test: $r_s = 0.99$, $r_sP_c = 0.99$, $k = 6$, $p < 0.001$ for both species). This pattern was also noted for the non-synonymous divergence ($dN_{Z\text{-}W}$; $r_s = 0.99$, $r_sP_c = 0.99$, $k = 6$, $p < 0.001$ for both species). These results support the relative age of the evolutionary strata as inferred from the phylogeny as well as a key prediction from sex chromosome theory: continuous differentiation of non-recombining chromosomes [26,27]. The ratio between synonymous and non-synonymous substitutions ($dN_{Z\text{-}W}/dS_{Z\text{-}W}$) did not correlate with the timing of formation (Raso lark: $r_s = -0.41$, $r_sP_c = -0.41$, $k = 6$, $p \approx 0.11$; Eurasian skylark: $r_s = -0.46$, $r_sP_c = -0.46$, $k = 6$, $p \approx 0.11$). This is, however, not expected as $dN/dS$ values are heavily influenced by the strength and type of selection acting on specific genes. The lowest $dN_{Z\text{-}W}/dS_{Z\text{-}W}$ values are found on stratum 1 (figure 3*c*), the ancestral sex chromosome, where previous studies have shown strong purifying selection on the remaining gametologous gene pairs [37].

In general, Z–W divergence is expected to be driven by a higher rate of evolution at the W gametologs than at their Z counterparts due to their lack of recombination, low population size (Z: ¾; W: ¼) and loss of function. To study this in the *Alauda* larks, we compared the $dS$ and $dN$ values between the W gametologs and their homologous zebra finch genes ($dS_{W\text{-}zf}$ and $dN_{W\text{-}zf}$) to the corresponding values for the Z gametologs ($dS_{Z\text{-}zf}$ and $dN_{Z\text{-}zf}$), for all evolutionary strata except the ancestral sex chromosome (chromosome Z) (electronic supplementary material, table S9). The latter chromosome was excluded because it shares a more recent evolutionary history with the lark Z than with the lark W chromosome. We found that $dN_{W\text{-}zf}$ was significantly higher than $dN_{Z\text{-}zf}$ in both species for strata 2, 3 and 4 (paired samples Wilcoxon test; adjusted $p$ ranged between <0.001 and 0.005), while there was no difference between $dS_{W\text{-}zf}$ and $dS_{Z\text{-}zf}$ for these strata. For strata 5a and 5b, both the $dS_{W\text{-}zf}$ and $dN_{W\text{-}zf}$ values were significantly higher than the $dS_{Z\text{-}zf}$ and $dN_{Z\text{-}zf}$, except that $dS_{Z\text{-}zf}$ at stratum 5b in Raso lark was significantly higher than $dS_{W\text{-}zf}$ (adjusted $p$ ranged between <0.001 and 0.024). This shows that the W chromosome has diverged

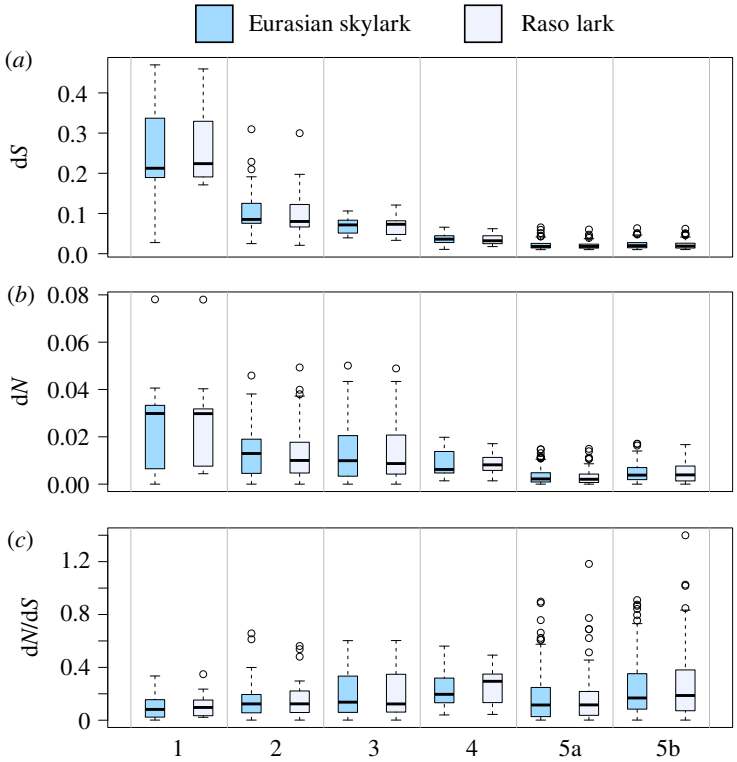


**Figure 3.** Z-to-W substitution rates for gametologous genes positioned within the different evolutionary strata in the Eurasian skylark (blue) and the Raso lark (light blue). Median (*a*) d*S*, (*b*) d*N*, and (*c*) d*N*/d*S* values are marked by the black line in each box, and the upper and lower hinges correspond to the first and third quartiles. The whiskers extend to no more than 1.5× the interquartile range from each hinge. (Online version in colour.)

more than the Z chromosome in terms of gene functionality (i.e. non-synonymous substitutions) for all strata. For strata 5a and 5b, but not for the other strata, the dS values also differed significantly. However, the power to detect small differences is higher for strata 5a and 5b due to a much larger number of genes (≈200 genes) compared to strata 2, 3 and 4 (≈20 genes; electronic supplementary material, table S9).

## (c) Gene enrichment and sex chromosome homology

A long-standing hypothesis posits that the formation and continuous evolutionary dynamics of sex chromosomes are governed by selection acting on linked genes, in particular on sex-determining and sexually antagonistic genes, as sex-linkage may resolve sexual conflicts by moving the sexes closer towards their respective fitness optimum [3–5]. This hypothesis implies that chromosomes containing such genes should be over-represented in sex chromosome formations, transitions and turnovers [6–9]. We test this hypothesis by looking for enrichment of genes with sex-related functions (using Gene Ontogeny (GO) annotations [38]) at the sex-linked regions and evolutionary strata. Genes coding for sex-related functions are particularly likely to be involved in either past or present sexual antagonism [28] and therefore an enrichment analysis of such genes offers a possibility to test if neo-sex chromosome formation has been driven by sexually antagonistic selection [10]. This does not, however, exclude the possibility that genes with other types of annotated functions could be sexually antagonistic or that many or even most sexually antagonistic genes are autosomal [39]. For example, in guppies (*Poecilia reticulata*) genes controlling bright coloration seem to be sexually antagonistic [40], in migratory warblers (*Acrocephalus arundinaceus*) wing length, a highly heritable trait, has sexually antagonistic fitness effects [41], and in *Drosophila* genes with sex-biased expression are located both on autosomes and the X chromosome although proportionally more frequently on the X chromosome than on autosomes [42].

In total, we found 323 genes with sex-related GO terms. Of these, 16 genes were located on the ancestral sex chromosome Z (stratum 1), 5 within the sex-linked part of chromosome 4A (stratum 2), 33 within the sex-linked region of chromosome 3 (strata 3, 4 and 5a) and 5 within the sex-linked part of chromosome 5 (stratum 5b) (figure 4*a*; electronic supplementary material, table S10; document S1). The different strata on chromosome 3 hold 5 (stratum 3), 0 (stratum 4) and 28 (stratum 5a) genes, respectively (figure 4*b*). This means that 59 genes with sex-related functions are sex-linked in the Raso lark and the Eurasian skylark, i.e. the species with all strata, compared to 16 in species that have only the ancestral avian sex chromosome, i.e. the majority of birds [33]. Binomial tests showed that the sex-linked parts of chromosome 3 (strata 3, 4 and 5a) were significantly enriched for sex-related genes (33 observed genes compared to 19.7 expected genes; binomial test: adjusted $p = 0.019$; figure 4*a*). The sex-linked regions on chromosomes Z, 4A and 5 showed no statistical difference between observed and expected number of genes with sex-specific functions. When analysing each stratum separately, stratum 5a was also significantly enriched for sex-related genes (28 observed genes compared to 16.7 expected genes; adjusted $p = 0.047$; figure 4*b*), while the other strata were not significantly enriched (electronic supplementary material, table S10; document S1).

Thus, we found statistical support for enrichment of sex-related genes for one of the chromosomes (chromosome 3) involved in the extensive neo-sex chromosome formation in larks. Moreover, our literature survey for homologies between the fused sex chromosomes in larks and

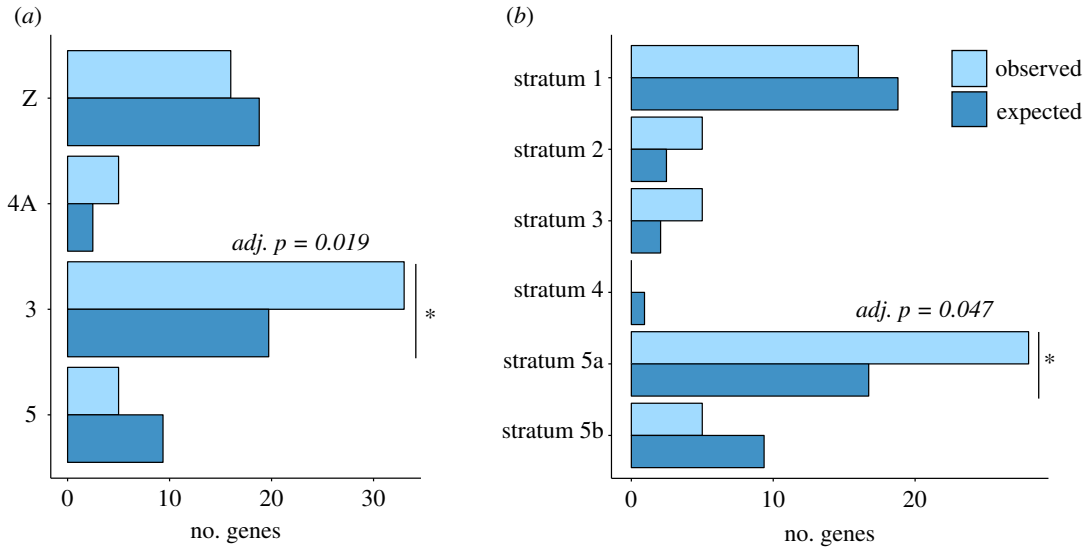


**Figure 4.** Observed and expected number of sex-related genes for (*a*) the sex-linked region of each chromosome and (*b*) each stratum. Binomial tests showed that the sex-linked region on chromosome 3 had significantly more sex-related genes than would be expected if the genes were randomly distributed throughout the genome, and that this enrichment on chromosome 3 was mainly due to a high number of sex-related genes on stratum 5a. (Online version in colour.)

sex chromosomes in other vertebrates showed that all four chromosomes have independently been recruited as sex chromosomes in several other vertebrate lineages. The Z chromosome—the sex chromosome in all birds, containing the putative sex determining gene *DMRT1* (*Doublesex and mab-3 related transcription factor 1* [43])—has independently been recruited as a sex chromosome in a flatfish (*Cynoglossus semilaevis* [14]), a turtle (*Staurotypus triporcatus* [44]), a gecko (*Gekko hokouensis* [45]), and in the platypus (*Ornithorhynchus anatinus* [46]). Chromosome 4A is homologous to the sex chromosome in lacertid lizards [47], the gecko genus *Paroedura* [15] and in all eutherian mammals [48], and contains several interesting genes, including *AR* (*androgen receptor*) and *SOX3* (*SRY-related HMG-box3*) (note, however, that *SOX3* is not sex-linked in Sylvioidea [12]). The homologue to chromosome 5 acts as a sex chromosome in two turtle groups (*Glyptemys* wood turtles and *Siebenrockiella* marsh turtles [44]). Finally, homologues of all four chromosomes act as sex chromosomes across different species of Ranidae frogs, as shown in a recent study [49]. This means that all four chromosomes that have become fused and stopped recombining in *Alauda* larks show repeated homology to sex chromosomes in other vertebrate lineages and that one is enriched for genes with predicted sex-specific functions (chromosome 3). Note, however, that there exist cases where chromosomes that are autosomal in larks act as sex chromosomes in other lineages, e.g. chromosomes 2 and 27 in caenophidian snakes [50,51], and chromosome 15 in soft-shell turtles [44]. To formally test the hypothesis of non-random recruitment of certain chromosomes to become sex-linked in vertebrates, current evidence of turnovers and autosome–sex chromosome fusions needs to be collected and analysed. Moreover, in many independently originated sex chromosomes the full gene content is not yet characterized, and their homology to other vertebrates are inferred through data of a few selected chromosomal markers that does not discover smaller translocations or gene movements between chromosomes. We hope that future studies will make use of whole-genome sequencing technologies to fill in this gap in our knowledge.

## 3. Conclusion

This study uncovers the largest known avian sex chromosome (195.3 Mbp) and shows that it originates from fusions between parts of four avian chromosomes (Z, 3, 4A and 5), illustrating an exception to the usually highly stable avian sex chromosomes [18,19]. Multiple autosome–sex chromosome fusions have also occurred in other clades on a family or genus level, including howler monkeys (genus *Alouatta* [52,53]), spiny rats (family Echimyidae [54]), bovids (family Bovidae [55,56]), iguanas (family Iguanidae [57,58]) and sea snakes (subfamily Hydrophiinae [57]). These patterns, now including those found in the families Alaudidae and Panuridae, hint at a phylogenetic component in neo-sex chromosome formation; once an autosome–sex chromosome fusion has occurred, additional ones may be expected [57]. If fusions are being selected for to resolve sexual conflicts [3–5], then previously fused regions (with already suppressed recombination) provide more opportunities for beneficial linkage to be formed between sex-linked genes and any additional genomic regions containing sexually antagonistic genes.

This reasoning is further supported by the significant enrichment of genes with sex-related functions on one of the chromosomes involved in the fusion (chromosome 3), and the independent recruitment of homologues to all these chromosomes as sex chromosomes in other vertebrate lineages (chromosomes Z, 3, 4A and 5). However, it should be noted that autosome–sex chromosome fusions can also reach fixation through meiotic drive [59] or heterozygote advantage [60], but these hypotheses are difficult to test with enrichment analyses as genes involved in these processes are not functionally identified (reviewed in ref. [2]). An alternative hypothesis is that fusions may become fixed as a consequence of non-selective processes [61]. However, we have presented several strands of evidence—the repeated evolution of recombination suppression, the enrichment of genes with sex-specific function and the homology to other vertebrate sex chromosomes—to support the hypothesis that the multiple fusion events that have formed these extraordinary neo-sex chromosomes in larks and in the bearded reedling have been driven by selective

processes acting on their gene content rather than by genetic drift. This adds to the accumulating evidences that specific chromosomes are non-randomly recruited as sex chromosomes in vertebrates [9,13].

## 4. Methods

DNA was extracted from blood samples of one female and one male for each of four study species: Raso lark (*Alauda razae*, from Cape Verde), Eurasian skylark (*A. arvensis cantarella*, from Italy), horned lark (*Eremophila alpestris flava*, from Sweden) and bearded reedling (*Panurus b. biarmicus*, from Sweden). DNA was sequenced with Illumina HiSeqX (150 bp, paired-end, 22x-51x).

Sequence reads were processed in several steps including trimming, building a de novo genome assembly of each species and remapping reads of each male and female to the most suitable genome assembly (electronic supplementary material, methods S1b). We identified sex-linked regions using two different genomic signatures indicative of W chromosomes in different stages of degeneration: (i) differential mapping success in males and females, and (ii) an excess or deficit of female-specific genetic variation. These measurements were calculated across 5 kbp genomic windows which were then anchored to chromosome positions using the genome assembly of the zebra finch (*Taeniopygia guttata*; taeGut.3.2.4 [62]; electronic supplementary material, methods S1c). Analyses of mean values were conducted on two scales, using window sizes of 1 Mbp and 0.1 Mbp, respectively (electronic supplementary material, methods S1d).

We extracted Z–W sequences of gametologous genes in the Raso lark and the Eurasian skylark by (i) conducting a lift-over of the zebra finch annotations to the Raso lark assembly (which was used as a mapping reference for both these species; electronic supplementary material, methods S1b), (ii) calling variants for every base pair within exons based on the genome coordinates from the lift-over, and (iii) *in silico* extracting gametologous (Z and W) gene sequences from sex-linked regions based on the genotypes of the female and male samples using in-house scripts (code S1; general methodology described in ref. [22]). Next, the extracted sequences were codon-aware aligned, and pairwise substitution rates (d$S$, d$N$ and d$N$/d$S$) between Z–W gametologs, and between the Z and W gametologs and the zebra finch homologues, were calculated using codeml [63] (electronic supplementary material, methods S1e).

We tested whether the divergence rates of the gametolog Z–W sequences differed between each pair of evolutionary strata using Kruskal–Wallis tests with Benjamini and Hochberg adjusted $p$-values. Values were $\log_2$-transformed prior to these tests to fulfil the criteria of similar shape distributions between test groups, and a low number (0.00001) was added to all non-synonymous substitution rate (d$N$) values as some contained zeros. Furthermore, we used ordered-heterogeneity (OH) tests [64] to evaluate if the gametolog divergence rates of the evolutionary strata correlated with their relative age. The OH test statistic value, $r_sP_c$, was calculated by multiplying (i) $P_c$, the complement of the $p$-value ($1 - p$-value) from a Kruskal–Wallis test of the difference in divergence rates of all strata, with (ii) $r_s$, the Spearman Rank rho value based on the median divergence rate of each stratum correlated with the rank order of their inferred age. As the relative timing of sex-linkage of strata 5a and 5b could not be distinguished using our data, these strata were given the same rank. The $p$-values of the OH test were extracted from the supplied figures in Rice & Gaines [64], and multiplied by two for two-tailed hypothesis testing. We tested the substitution rates between the Z or the W gametologs and the zebra finch homologues using paired samples Wilcoxon test, and corrected for multiple testing with Benjamini & Hochberg adjusted $p$-values.

We conducted gene enrichment analyses by downloading Gene Ontogeny (GO) annotations for all the genes in the zebra finch genome from Ensembl BioMart (taeGut3.2.4; accessed on 12 September 2018) and parsed the file for GO term names with the following pattern matches: 'sperm', 'ovarian', 'gonad', 'estrogen', 'testosterone', 'sex differentiation', 'sex determination', 'sexual characteristics', 'sexual reproduction' and 'oogenesis'. The zebra finch gene annotation had in total 323 genes matching these patterns. We then counted the number of genes matching any of these terms for (i) the sex-linked region for each chromosome separately, and (ii) within each of the identified evolutionary strata. We performed two-tailed binomial tests to see if these genomic regions contained either more or fewer sex-related genes than would be expected if the genes were randomly distributed across the genome. We calculated the proportion of the genome that made up each sex-linked region (1.253 Gbp is the full genome size of the zebra finch genome assembly used) and tested if the region contained a higher or lower proportion of sex-related genes than would be expected based on this probability. The $p$-values for the two categories of tests (i, ii) were adjusted using the Benjamini & Hochberg method.

Ethics. Samples were collected non-destructively and with permission from the relevant authorities (Direcção Geral do Ambiente, Cape Verde, and Malmö/Lund Ethical Committee for scientific work on animals, Sweden, no. 17277-18).

Data accessibility. Illumina sequence reads have been deposited in the NCBI Sequence Read Archive (Bioproject PRJNA578893) and genome assemblies in Dryad: https://doi.org/10.5061/dryad.95x69p8f9 [65].

Authors' contributions. H.S., S.P. and B.H. conceived the study. H.S. and P.C. performed analyses. E.D. and M.d.L.B. provided samples. H.S. and B.H. wrote the paper with input from all co-authors.

Competing interest. We declare we have no competing interests.

Funding. Sequencing was performed by the SNP&SEQ Technology Platform in Uppsala, which is part of the NGI Sweden and SciLifeLab, supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation. Bioinformatics analyses were performed on resources provided by SNIC at UPPMAX. The research was funded by grants from the Swedish Research Council (to B.H.: nos 621-2014-5222 and 621-2016-689), the Royal Physiological Society in Lund, the Erik Philip-Sörensen's Foundation, Stiftelsen Olle Engkvist and the Wenner-Gren Foundations (to S.P.).

Acknowledgements. We wish to thank O. Berglund, P. Zehtindjiev and S. Bensch for DNA samples.

## References

1. Bergero R, Charlesworth D. 2009 The evolution of restricted recombination in sex chromosomes. *Trends Ecol. Evol.* **24**, 94–102. (doi:10.1016/j.tree.2008.09.010)
2. Ponnikas S, Sigeman H, Abbott JK, Hansson B. 2018 Why do sex chromosomes stop recombining? *Trends Genet.* **34**, 492–503. (doi:10.1016/j.tig.2018.04.001)
3. Fisher RA. 1931 The evolution of dominance. *Biol. Rev.* **6**, 345–368. (doi:10.1111/j.1469-185X.1931.tb01030.x)
4. Bull JJ. 1983 *Evolution of sex determining mechanisms*. Menlo Park, CA: Benjamin Cummings.
5. Rice WR. 1987 The accumulation of sexually antagonistic genes as a selective agent promoting the evolution of reduced recombination between primitive sex chromosomes. *Evolution* **41**, 911–914. (doi:10.1111/j.1558-5646.1987.tb05864.x)

6. Van Doorn GS, Kirkpatrick M. 2007 Turnover of sex chromosomes induced by sexual conflict. *Nature* **449**, 909–912. (doi:10.1038/nature06178)
7. Ross JA, Urton JR, Boland J, Shapiro MD, Peichel CL. 2009 Turnover of sex chromosomes in the stickleback fishes (Gasterosteidae). *PLoS Genet.* **5**, e1000391. (doi:10.1371/journal.pgen.1000391)
8. Bachtrog D, Kirkpatrick M, Mank JE, McDaniel SF, Pires JC, Rice W *et al*. 2011 Are all sex chromosomes created equal? *Trends Genet.* **27**, 350–357. (doi:10.1016/j.tig.2011.05.005)
9. O'Meally D, Ezaz T, Georges A, Sarre SD, Graves JAM. 2012 Are some chromosomes particularly good at sex? Insights from amniotes. *Chromosome Res.* **20**, 7–19. (doi:10.1007/s10577-011-9266-8)
10. Charlesworth D, Charlesworth B. 1980 Sex differences in fitness and selection for centric fusions between sex-chromosomes and autosomes. *Genet. Res.* **35**, 205–214. (doi:10.1017/S0016672300014051)
11. Kitano J, Ross JA, Mori S, Kume M, Jones FC, Chan YF *et al*. 2009 A role for a neo-sex chromosome in stickleback speciation. *Nature* **461**, 1079–1083. (doi:10.1038/nature08441)
12. Pala I, Naurin S, Stervander M, Hasselquist D, Bensch S, Hansson B. 2012 Evidence of a neo-sex chromosome in birds. *Heredity* **108**, 264–272. (doi:10.1038/hdy.2011.70)
13. Ezaz T, Srikulnath K, Graves JAM. 2016 Origin of amniote sex chromosomes: an ancestral super-sex chromosome, or common requirements? *J. Hered.* **108**, 94–105. (doi:10.1093/jhered/esw053)
14. Chen S, Zhang G, Shao C, Huang Q, Liu G, Zhang P *et al*. 2014 Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nat. Genet.* **46**, 253–260. (doi:10.1038/ng.2890)
15. Rovatsos M, Farkačová K, Altmanová M, Pokorná MJ, Kratochvíl L. 2019 The rise and fall of differentiated sex chromosomes in geckos. *Mol. Ecol.* **28**, 3042–3052. (doi:10.1111/mec.15126)
16. Nielsen SV, Daza JD, Pinto BJ, Gamble T. 2019 ZZ/ZW sex chromosomes in the endemic Puerto Rican leaf-toed gecko (*Phyllodactylus wirshingi*). *Cytogenet. Genome Res.* **157**, 89–97. (doi:10.1159/000496379)
17. Darolti I, Wright AE, Sandkam BA, Morris J, Bloch NI, Farré M *et al*. 2019 Extreme heterogeneity in sex chromosome differentiation and dosage compensation in livebearers. *Proc. Natl Acad. Sci. USA* **3**, 201905298. (doi:10.1073/pnas.1905298116)
18. Shetty S, Griffin DK, Graves JAM. 1999 Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. *Chromosome Res.* **7**, 289–295. (doi:10.1023/A:1009278914829)
19. Ellegren H. 2010 Evolutionary stasis: the stable chromosomes of birds. *Trends Ecol. Evol.* **25**, 283–291. (doi:10.1016/j.tree.2009.12.004)
20. Gunski, RJ, Cañedo AD, del Valle Garnero A, Ledesma MA, Coria N, Montalti D *et al.* 2017 Multiple sex chromosome system in penguins (Pygoscelis, Spheniscidae). *Comp. Cytogenet.* **11**, 541–552. (doi:10.3897/CompCytogen.v11i3.13795)
21. Gan HM, Falk S, Morales HE, Austin CM. 2019 Genomic evidence of neo-sex chromosomes in the eastern yellow robin. *Gigascience* **8**, giz111. (doi:10.1093/gigascience/giz111)
22. Sigeman H, Ponnikas S, Videvall E, Zhang H, Chauhan P, Naurin S, Hansson B. 2018 Insights into avian incomplete dosage compensation: sex-biased gene expression coevolves with sex chromosome degeneration in the Common Whitethroat. *Genes* **9**, 373. (doi:10.3390/genes9080373)
23. Leroy T, Anselmetti Y, Tilak M, Bérard S, Csukonyi L, Gabrielli M *et al*. 2018 A bird's white-eye shot: looking down on a new avian sex chromosome evolution. *bioRxiv*, 505610. (doi:10.1101/505610)
24. Bulatova, NS. 1981 A comparative karyological study of passerine bird. *Acta. Sci. Nat. Brno.* **15**, 1–44.
25. Brooke ML, Welbergen JA, Mainwaring MC, Van Der Velde M, Harts AMF, Komdeur J *et al.* 2010 Widespread translocation from autosomes to sex chromosomes preserves genetic variability in an endangered lark. *J. Mol. Evol.* **70**, 242–246. (doi:10.1007/s00239-010-9333-3)
26. Lahn BT, Page DC. 1999 Four evolutionary strata on the human X chromosome. *Science* **286**, 964–967. (doi:10.1126/science.286.5441.964)
27. Rice WR. 1994 Degeneration of a nonrecombining chromosome. *Science* **263**, 230–232. (doi:10.1126/science.8284674)
28. Rice WR. 1984 Sex chromosomes and the evolution of sexual dimorphism. *Evolution* **38**, 735–742. (doi:10.2307/2408385)
29. Alström P, Barnes KN, Olsson U, Barker FK, Bloomer P, Khan AA *et al*. 2013 Multilocus phylogeny of the avian family Alaudidae (larks) reveals complex morphological evolution, non-monophyletic genera and hidden species diversity. *Mol. Phylogenet. Evol.* **69**, 1043–1056. (doi:10.1016/j.ympev.2013.06.005)
30. Cortez D, Marin R, Toledo-Flores D, Froidevaux L, Liechti A, Waters PD *et al*. 2014 Origins and functional evolution of Y chromosomes across mammals. *Nature* **508**, 488–493. (doi:10.1038/nature13151)
31. Moyle RG, Oliveros CH, Andersen MJ, Hosner PA, Benz BW, Manthey, JD *et al*. 2016 Tectonic collision and uplift of Wallacea triggered the global songbird radiation. *Nat. Commun.* **7**, 12709. (doi:10.1038/ncomms12709)
32. Dierickx E, Sin S, van Veelen P, Brooke ML, Liu Y, Edwards S. 2019 Neo-sex chromosomes and demography shape genetic diversity in the Critically Endangered Raso lark. *bioRxiv* **132**, 617563. (doi:10.1101/617563)
33. Zhou Q, Zhang J, Bachtrog D, An N, Huang Q, Jarvis ED *et al*. 2014 Complex evolutionary trajectories of sex chromosomes across bird taxa. *Science* **346**, 1246338. (doi:10.1126/science.1246338)
34. Xu L, Auer G, Peona V, Suh A, Deng Y, Feng S *et al*. 2019 Dynamic evolutionary history and gene content of sex chromosomes across diverse songbirds. *Nat. Ecol. Evol.* **3**, 834–844. (doi:10.1038/s41559-019-0850-1)
35. Smeds L, Kawakami T, Burri R, Bolivar P, Husby A, Qvarnström A *et al*. 2014 Genomic identification and characterization of the pseudoautosomal region in highly differentiated avian sex chromosomes. *Nat. Commun.* **5**, 5448. (doi:10.1038/ncomms6448)
36. Nam K, Ellegren H. 2008 The chicken (*Gallus gallus*) Z chromosome contains at least three nonlinear evolutionary strata. *Genetics* **180**, 1131–1136. (doi:10.1534/genetics.108.090324)
37. Bellott DW, Skaletsky H, Cho T, Brown L, Locke D, Chen N *et al*. 2017 Avian W and mammalian Y chromosomes convergently retained dosage-sensitive regulators. *Nat. Genet.* **49**, 387–394. (doi:10.1038/ng.3778)
38. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM *et al*. 2000 Gene Ontology: tool for the unification of biology. *Nat. Genet.* **25**, 25–29. (doi:10.1038/75556)
39. Fry JD. 2010 The genomic location of sexually antagonistic variation: some cautionary comments. *Evolution* **64**, 1510–1516. (doi:10.1111/j.1558-5646.2009.00898.x)
40. Wright AE, Darolti I, Bloch NI, Oostra V, Sandkam B, Buechel SD *et al*. 2017 Convergent recombination suppression suggests role of sexual selection in guppy sex chromosome formation. *Nat. Commun.* **8**, 14251. (doi:10.1038/ncomms14251)
41. Tarka M, Akesson M, Hasselquist D, Hansson B. 2014 Intralocus sexual conflict over wing length in a wild migratory bird. *Am. Nat.* **183**, 62–73. (doi:10.1086/674072)
42. Meisel RP, Malone JH, Clark AG. 2012 Disentangling the relationship between sex-biased gene expression and X-linkage. *Genome Res.* **22**, 1255–1265. (doi:10.1101/gr.132100.111)
43. Smith CA, Roeszler KN, Ohnesorg T, Cummins DM, Farlie PG, Doran TJ *et al*. 2009 The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature* **461**, 267–271. (doi:10.1038/nature08298)
44. Montiel EE, Badenhorst D, Lee LS, Literman R, Trifonov V, Valenzuela N. 2016 Cytogenetic insights into the evolution of chromosomes and sex determination reveal striking homology of turtle sex chromosomes to amphibian autosomes. *Cytogenet. Genome Res.* **148**, 292–304. (doi:10.1159/000447478)
45. Kawai A, Ishijima J, Nishida C, Kosaka A, Ota H, Kohno S *et al*. 2008 The ZW sex chromosomes of *Gekko hokouensis* (Gekkonidae, Squamata) represent highly conserved homology with those of avian species. *Chromosoma* **118**, 43–51. (doi:10.1007/s00412-008-0176-2)
46. Grützner F, Rens W, Tsend-Ayush E, El-Mogharbel N, O'Brien PCM, Jones RC *et al*. 2004 In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes. *Nature* **432**, 913–917. (doi:10.1038/nature03021)
47. Rovatsos M, Vukić J, Altmanová M, Pokorná MJ, Moravec J, Kratochvíl L. 2016 Conservation of sex chromosomes in lacertid lizards. *Mol. Ecol.* **25**, 3120–3126. (doi:10.1111/mec.13635)
48. Ross, MT, Grafham DV, Coffey AJ, Scherer S, McLay K, Muzny D *et al*. 2005 The DNA sequence of

the human X chromosome. *Nature* **434**, 325–337. (doi:10.1038/nature03440)

49. Jeffries DL, Lavanchy G, Sermier R, Sredl MJ, Miura I, Borzée A *et al*. 2018 A rapid rate of sex-chromosome turnover and non-random transitions in true frogs. *Nat. Commun.* **9**, 11. (doi:10.1038/s41467-018-06517-2)
50. Matsubara K, Tarui H, Toriba M, Yamada K, Nishida-Umehara C, Agata K *et al*. 2006 Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. *Proc. Natl Acad. Sci. USA* **103**, 18 190–18 195. (doi:10.1073/pnas.0605274103)
51. Rovatsos M, Vukić J, Lymberakis P, Kratochvíl L. 2015 Evolutionary stability of sex chromosomes in snakes. *Proc. R. Soc. B* **282**, 20151992. (doi:10.1098/rspb.2015.1992)
52. de Oliveira EHC, Neusser M, Figueiredo WB, Nagamachi C, Pieczarka JC, Sbalqueiro IJ *et al*. 2002 The phylogeny of howler monkeys (Alouatta, Platyrrhini): reconstruction by multicolor cross-species chromosome painting. *Chromosome Res.* **10**, 669–683. (doi:10.1023/A:1021520529952)
53. Steinberg ER, Nieves M, Mudry MD. 2014 Multiple sex chromosome systems in howler monkeys (Platyrrhini, Alouatta). *Comp. Cytogenet.* **8**, 43–69. (doi:10.3897/CompCytogen.v8i1.6716)
54. da Silva WO, da Costa MJR, Pieczarka JC, Rissino J, Pereira JC, Ferguson-Smith MA *et al*. 2019 Identification of two independent X-autosome translocations in closely related mammalian (Proechimys) species. *Sci. Rep.* **9**, 4047. (doi:10.1038/s41598-019-40593-8)
55. Vassart M, Séguéla A, Hayes H. 1995 Chromosomal evolution in gazelles. *J. Hered.* **86**, 216–227. (doi:10.1093/oxfordjournals.jhered.a111565)
56. Vozdova M, Ruiz-Herrera A, Fernandez J, Cernohorska H, Frohlich J, Sebestova H *et al*. 2016 Meiotic behaviour of evolutionary sex-autosome translocations in Bovidae. *Chromosome Res.* **24**, 325–338. (doi:10.1007/s10577-016-9524-x)
57. Pokorná M, Altmanová M, Kratochvíl L. 2014 Multiple sex chromosomes in the light of female meiotic drive in amniote vertebrates. *Chromosome Res.* **22**, 35–44. (doi:10.1007/s10577-014-9403-2)
58. Giovannotti M, Trifonov VA, Paoletti A, Kichigin IG, O'Brien PCM, Kasai F *et al*. 2016 New insights into sex chromosome evolution in anole lizards (Reptilia, Dactyloidae). *Chromosoma* **126**, 245–260. (doi:10.1007/s00412-016-0585-6)
59. Yoshida K, Kitano J. 2012 The contribution of female meiotic drive to the evolution of neo-sex chromosomes. *Evolution* **66**, 3198–3208. (doi:10.1111/j.1558-5646.2012.01681.x)
60. Charlesworth B, Wall JD. 1999 Inbreeding, heterozygote advantage and the evolution of neo-X and neo-Y sex chromosomes. *Proc. R. Soc. Lond. B* **266**, 51–56. (doi:10.1098/rspb.1999.0603)
61. Pennell MW, Kirkpatrick M, Otto SP, Vamosi JC, Peichel CL, Valenzuela N *et al*. 2015 Y fuse? Sex chromosome fusions in fishes and reptiles. *PLoS Genet.* **11**, e1005237. (doi:10.1371/journal.pgen.1005237)
62. Warren WC, Clayton DF, Ellegren H, Arnold AP, Hillier LW, Künstner A *et al*. 2010 The genome of a songbird. *Nature* **464**, 757–762. (doi:10.1038/nature08819)
63. Yang Z. 2007 PAML 4: Phylogenetic Analysis by Maximum Likelihood. *Mol. Biol. Evol.* **24**, 1586–1591. (doi:10.1093/molbev/msm088)
64. Rice WR, Gaines SD. 1994 Extending nondirectional heterogeneity tests to evaluate simply ordered alternative hypotheses. *Proc. Natl Acad. Sci. USA* **91**, 225–226. (doi:10.1073/pnas.91.1.225)
65. Sigeman H, Ponnikas S, Chauhan P, Dierickx E, Brooke MdeL, Hansson B. 2019 Data from: repeated sex chromosome evolution in vertebrates supported by expanded avian sex chromosomes. Dryad Digital Repository. (https://doi.org/10.5061/dryad.95x69p8f9)