

1 **Genomics of expanded avian sex chromosomes**
2 **shows that certain chromosomes are predisposed**
3 **towards sex-linkage in vertebrates**

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5 Running head: Genomics of lark sex chromosomes

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16 ABSTRACT

17 Sex chromosomes have evolved from the same autosomes multiple times across vertebrates,
18 suggesting that certain genomic regions are predisposed towards sex-linkage. However, to test this
19 hypothesis detailed studies of independently originated sex-linked regions and their gene content
20 are needed. Here we address this problem through comparative genomics of birds where multiple
21 chromosomes appear to have formed neo-sex chromosomes: larks (Alaudidae; Sylvioidea). We
22 detected the largest known avian sex chromosome (195.3 Mbp) and show that it originates from
23 fusions between (parts of) four avian chromosomes (Z, 3, 4A and 5). We found evidence of five
24 evolutionary strata where recombination has been suppressed at different time points, and that
25 these time points correlate with the level of Z-W gametolog differentiation. We show that there is
26 extensive homology to sex chromosomes in other vertebrate lineages: three of the fused
27 chromosomes (Z, 4A, 5) have independently evolved into sex chromosomes in fish (Z), turtles (Z,
28 5), lizards (Z, 4A) and mammals (Z, 4A). Moreover, we found that the fourth chromosome,
29 chromosome 3, was significantly enriched for genes with predicted sex-specific functions. These
30 results support a key role of chromosome content in the evolution of sex chromosomes in
31 vertebrates.

32

33 Sex chromosome | fusion | resequencing | Sylvioidea | larks | birds

34 1. INTRODUCTION

35 Sex chromosomes have evolved from autosomes many times independently across the tree of life. The most
36 generic hypothesis for the evolution of sex chromosomes invokes a selective advantage of linkage between
37 sex-determining and sexually antagonistic genes (Haldane 1922; Fisher 1931; Lewis & John 1968; D.
38 Charlesworth & B. Charlesworth 1980). Accordingly, this hypothesis suggests that the chromosomes
39 harbouring these genes should frequently be involved in the formation, transition and turnover of sex

40 chromosomes (Bachtrog et al. 2011; Ross et al. 2009; O'Meally et al. 2012). Indeed, there are a limited
41 number of genes across vertebrates that have taken on the sex-determining role, and evidence is
42 accumulating that the chromosomes on which these reside have evolved into sex chromosomes several
43 times independently in different lineages (reviewed in O'Meally et al. 2012; Ezaz et al. 2006; Ezaz et al.
44 2016). Furthermore, it has been suggested that some autosomes fuse with existing sex chromosomes into
45 neo-sex chromosomes more easily than others due to their gene content (Kitano et al. 2009; Ross et al.
46 2009; Pala, Hasselquist, et al. 2012; Kitano & Peichel 2011; Yoshida et al. 2014; O'Meally et al. 2012). The
47 importance of chromosome content for the evolutionary dynamics of sex chromosomes is, however, still
48 poorly supported due to a limited number of well-studied origins of sex-linkage.

49
50 The sex chromosomes of birds are highly stable with a Z chromosome size of approximately 73 Mbp
51 (Ellegren 2010). One of the few known exceptions to this stability is the neo-sex chromosome of passerines
52 in the superfamily Sylvioidea, which has been formed by a fusion between half of chromosome 4A
53 (according to the zebra finch *Taeniopygia guttata* nomenclature) and the ancestral sex chromosome (Pala,
54 Naurin, et al. 2012; Pala, Hasselquist, et al. 2012; Sigeman et al. 2018). Moreover, two independent findings
55 suggest that at least some species of larks (Alaudidae), a family within Sylvioidea, have acquired autosome–
56 sex chromosome fusions in addition to the one including parts of 4A. Firstly, heavily enlarged sex
57 chromosome karyotypes were found in the bimaculated lark (*Melanocorypha bimaculata*) and the horned lark
58 (*Eremophila alpestris*; Bulatova 1981), and, secondly, genetic markers located on chromosome 3 and 5 were
59 found to have sex-specific inheritance in the Raso lark (*Alauda razae*; Brooke et al. 2010).

60
61 Here, we use comparative genomics to study this neo-sex chromosome system across three Alaudidae
62 species – two *Alauda* species (Raso lark and Eurasian skylark *A. arvensis*) and one *Eremophila* species (horned
63 lark) – and their sister species in the family Panuridae (the bearded reedling *Panurus biarmicus*). We use whole-
64 genome sequence data of these species to characterise in detail which genomic regions have become fused
65 to the sex chromosomes, and use phylogenetic information to determine the appearance and age of several
66 evolutionary strata where recombination has been suppressed at different points in time (*sensu* Lahn & Page

67 1999). Then, we test whether the age of the strata explains the degree of Z-W divergence of gametologous
68 genes, as predicted by sex chromosome theory (Rice 1994; Lahn & Page 1999). Next, we test the
69 hypothesised importance of chromosome content for the formation of sex chromosomes by analysing
70 whether the fused chromosomal regions are enriched for genes with sex-specific functions. Lastly, we
71 evaluate signs of repeated sex chromosome evolution by searching for homologies between the fused sex
72 chromosomes in larks and the sex chromosomes of other vertebrate lineages. Our study supports a key role
73 of chromosome content in the evolution of sex chromosomes and highlights the importance of studying
74 independently originated sex chromosomes for understanding how and why vertebrate sex chromosomes
75 evolve.

76 2. MATERIALS & METHODS

77 2.1 Sequence data

78 We extracted DNA from blood samples using a phenol–chloroform protocol (Sambrook & W Russel, 2001)
79 of one female and one male individual from each of our four study species: Raso lark (*Alauda razae*, from
80 Cape Verde), Eurasian skylark (*A. arvensis cantarella*, from Italy), horned lark (*Eremophila alpestris flava*, from
81 Sweden) and bearded reedling (*Panurus b. biarmicus*, from Sweden). According to *cytochrome b* sequence data,
82 the two *Alauda* species diverged c. 6 million years ago (Mya), *Alauda* and *Eremophila* c. 14 Mya (Alström et
83 al. 2013), and Alaudidae and Panuridae c. 17 Mya (Moyle et al. 2016). All samples were collected non-
84 destructively and with permission from the relevant authorities (Direcção Geral do Ambiente, Cape Verde,
85 and Malmö/Lund Ethical Committee for scientific work on animals, Sweden, no. 17277-18). DNA was
86 sequenced with Illumina HiSeqX (150 bp, paired-end) by SciLifeLab Sweden.

87 2.2 *De novo* assembly and mapping

88 To obtain reference genomes, we created *de novo* genome assemblies from the male sequence data for each
89 of the study species. The resequencing data was trimmed using nelson clip ([https://github.com/Victorian-](https://github.com/Victorian-Bioinformatics-Consortium/nesoni)
90 [Bioinformatics-Consortium/nesoni](https://github.com/Victorian-Bioinformatics-Consortium/nesoni)) with a minimum read quality of 20 and minimum read length of 20 bp.
91 The trimmed reads were then assembled with Spades v3.5.0 (Bankevich et al. 2012) using 5 different kmer
92 lengths (21, 33, 55, 77 and 127) and the setting “careful”. Scaffolds shorter than 1 kbp were discarded.
93 Quality statistics from the assemblies were calculated using Quast v4.5.4 (Gurevich et al. 2013). Assemblies
94 with N50 over 50 kbp were kept for further analysis. This threshold meant keeping the assemblies of the
95 Raso lark (N50=103 kbp, 28304 scaffolds) and the bearded reedling (N50=68 kbp, 36455 scaffolds), while
96 the assemblies for the Eurasian skylark (N50=8 kbp, 256822 scaffolds) and horned lark (N50=22 kbp,
97 109088 scaffolds) were discarded.

98
99 Reads from all of the samples (n = 8) were cleaned for adaptor sequences with Trimmomatic v.0.3.6 (Bolger
100 et al. 2014) using the adaptor file TruSeq3-PE and options seedMismatches: 2, palindromeClipThreshold:
101 30 and simpleClipThreshold: 10. Trimming of low-quality bases was done using a quality threshold of 15
102 from the leading end and 30 from the trailing end. The reads were further trimmed for a minimum quality
103 of 20 over sliding windows of 4 bp. Lastly, any reads shorter than 90 bp were excluded from further analyses.
104 The number of remaining reads in our samples ranged from 176 to 404 million. The samples were quality
105 checked using fastqc v0.11.7 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

106
107 The male and female samples of Raso lark and bearded reedling were aligned to their respective genome
108 assemblies, while the Eurasian skylark and horned lark individuals were aligned to the genome assembly of
109 their closest relative, the Raso lark. The alignment was done with bwa mem v0.7.17 (Li & Durbin 2009),
110 marking shorter split hits as secondary (option -M) for downstream compatibility. The aligned reads were
111 sorted with samtools v1.7 (Li et al. 2009), and duplicated reads were removed using picardtools v2.18.0
112 (<http://broadinstitute.github.io/picard>). Assembly and alignment statistics are provided in Suppl. Tables
113 S1,2.

114 2.3 Chromosome anchoring

115 The scaffolds in the genome assemblies were grouped into different chromosomes and ordered into
116 chromosome-level using the genome assembly of the zebra finch (*Taeniopygia guttata*; (Warren et al. 2010).
117 The zebra finch genome assembly tacGut.3.2.4 was downloaded from Ensembl (Cunningham et al. 2015)
118 and transformed into a database using the last v876 (Kielbasa et al. 2011) program lastdb. The two genome
119 assemblies, of the Raso lark and bearded reedling, were aligned to the zebra finch genome using the program
120 lastal and converted to psl format using the script maf-convert, both from the same software suite last v876.
121 From there, we extracted chromosome anchoring coordinates based on the longest match to the zebra finch
122 genome for each 5 kbp window in the Raso lark and bearded reedling assemblies, with a minimum
123 requirement of 500 matching base pairs per 5 kbp window. The assembly positions from the output files of
124 the coverage and single nucleotide variant (SNV) analyses were then translated to the starting positions of
125 the match to the zebra finch genome assembly (see section below).

126

127 2.4 Identification of sex-linked genomic regions

128 We identified sex-linked regions using two different kinds of genomic signatures: (i) differential mapping
129 success in males and females (i.e. sex-specific genome coverage), and (ii) an excess or deficit of female-
130 specific genetic variation. Sex chromosomes almost invariably evolve recombination suppression in the
131 heterogametic sex so that regions that have been sex-linked for a long time (such as the sex chromosomes
132 that formed in the ancestor of all birds) will show pronounced sequence divergence and degeneration in the
133 non-recombining chromosome (the W in birds; Zhou et al. 2014). The regions belonging to the ancestral
134 sex chromosomes can thus be identified by lower female coverage, and fewer female-specific genetic
135 variants compared to males. This is because reads from the female-specific W-chromosome will either not
136 map to the male reference genome due to substantial differentiation between the Z and W or because of
137 deletions on the W chromosome. More-recently formed sex-linked regions may be identified by lower
138 mapping success in females, although we expect a subtler difference as the W-linked genomic region may

139 not have yet developed substantial differentiation from the Z-linked homologous region. Here, we also
140 expect a higher amount of female-specific mutations compared to males, due to differentiation between the
141 Z and W sex chromosome copies.

142

143 To uncover differential mapping success in males and females for each species, we parsed the alignment
144 files for reads with more than two mismatches and calculated genome-wide coverage for 5 kbp windows
145 with bedtools v2.71.1 (Quinlan & Hall 2010). All genome coverage values were normalised between the
146 female and the male sample, based on the number of reads in the trimmed and adaptor-free fastq files. To
147 identify sex-linked regions, we binned the female-to-male coverage values for every 1 Mbp genomic region
148 and extracted the mean value from each bin. Following discovery of sex-linked genomic regions, we
149 performed another normalisation step by dividing the median female-to-male coverage ratio for each 5 kbp
150 window by the genome-wide median female-to-male coverage ratio counting only chromosomes without
151 sex-linked regions. We then binned the data into 0.1 Mbp windows and calculated the mean female-to-male
152 coverage ratio within each bin.

153

154 To analyse female-specific variation, we called variants in the alignment files (with all mismatches allowed)
155 with freebayes v1.1.0 for each species separately ($n = 2$ in each analysis) using freebayes-parallel (Garrison
156 & Marth 2012) and parallel v20180322 (Tange 2018). The output was then parsed for any SNP that had
157 been marked with a flag other than PASS (--remove-filtered-all), a minimum quality of 20 and minimum
158 depth of 3x using vcftools v0.1.15 (Danecek et al. 2011). Private alleles (minor alleles occurring only in one
159 sample in a heterozygous state) were extracted with vcftools using option --singletons. We calculated the
160 difference between the number of female-specific private alleles and male-specific private alleles for each 5
161 kbp window and extracted the average difference across 1 Mbp and 0.1 Mbp windows.

162 2.5 Analysis of divergence between gametologous genes

163 We used the whole-genome synteny aligner program SatsumaSynteny v. 2.0 (Grabherr et al. 2010) to align
164 the Raso lark assembly to the zebra finch assembly (taeGut.3.2.4), and then used kraken (Zamani et al. 2014,

165 downloaded 18 June 2018) to make a lift-over of the zebra finch annotations to the Raso lark assembly. Of
166 the 18204 transcripts and 17488 genes in the zebra finch annotation, 14466 transcripts (79 %) from 13764
167 genes (79 %) were annotated in the Raso lark. We used Freebayes v.1.1.0 (Garrison & Marth 2012) (--report-
168 monomorphic) to call variants for every base pair within all exons in the Raso lark based on the genome
169 coordinates from the lift-over.

170

171 We *in silico* extracted gametologous gene sequences from sex-linked regions using an in-house script (code
172 provided as Supplementary Code S1; general methodology described in (Sigeman et al. 2018) based on the
173 genotypes of the female and male samples. The script uses sex-specific genotype information to phase the
174 data into a Z and W sequence. We replaced a site by “N” if it had a quality score or sequence depth below
175 20 or if the genotype in either sample was not called. Any site without variants in either sample was extracted
176 as such, and remaining variants between the male and female were phased based on sex-specific allele
177 compositions provided in Suppl. Table S3.

178

179 To confirm that both Z and W gametologs were present in the data, we calculated genome coverage values
180 for every exon using bedtools v.2.27.1 multicov (Quinlan & Hall 2010) and normalised the values between
181 the two samples of each species. Exons with female coverage less than 75 % of the male sample were
182 masked with N:s, as this suggests absence of a W gametolog.

183

184 We used TransDecoder v3.0.1 (<https://github.com/TransDecoder/TransDecoder/>) to find the longest
185 open reading frames for each of the sequences (--retain_pfam_hits, --retain_blastp_hits, --single-best-orf).
186 Orthologous genes from the zebra finch, collared flycatcher (*Ficedula albicollis*) and chicken (*Gallus gallus*)
187 were downloaded through BioMart (database: Ensembl Genes 93), and the longest transcript from the
188 flycatcher and chicken corresponding to the zebra finch transcripts in the annotation was added as additional
189 sequences. These gene sequences were not used in further analyses but aided in evaluating the quality of the
190 alignments. The sequences were codon-aware aligned using *Prank* v.150803 (Loytynoja 2014). Each N in
191 the sequences was transformed into “-”, and all sites including this character in any sequence were then

192 removed using gblocks v0.91b (Castresana 2000) . We calculated pairwise substitution rates between Z–W
193 gametologs using codeml (estimated kappa and codon frequency F3X4) from the PAML v4.9 package (Yang
194 2007). Gene sequences longer than 500 bp, and where the Z and W sequences within a species had a
195 synonymous substitution rate (dS) value above 0.01, were kept for further analysis. We used Kruskal-Wallis
196 tests to investigate potential differences in sequence divergence between the identified sex-linked genomic
197 regions (or strata, see below) as the data were not normally distributed. All values were \log_2 -transformed
198 prior to these tests to fulfil the criteria of similar distributions between groups and a low number (0.00001)
199 was added to all synonymous substitution rate (dN) values as some contained zeros. Significance levels of
200 differences between groups were calculated using pairwise Wilcoxon tests with Benjamini & Hochberg
201 adjusted p values. Furthermore, we used ordered heterogeneity tests (OH; Rice & Gaines 1994) to evaluate
202 if the difference between Z–W nucleotide divergences between groups correlated with relative timing of
203 sex-linkage between the evolutionary strata. The OH value r_{spc} was calculated by multiplying the
204 complement of the p value from the Kruskal-Wallis tests ($1 - p$ value) with the Spearman Rank rho value
205 from the median nucleotide divergence estimates for each stratum correlated with the order in which they
206 appear based on the phylogeny of the studied species. The relative timing of sex-linkage of stratum 5a and
207 5b (see below) could not be distinguished using our data and were thus given the same rank. P values were
208 extracted from the supplied figures in Rice & Gaines (1994), and multiplied by two for two-tailed hypothesis
209 testing.

210 2.6 Gene enrichment analysis

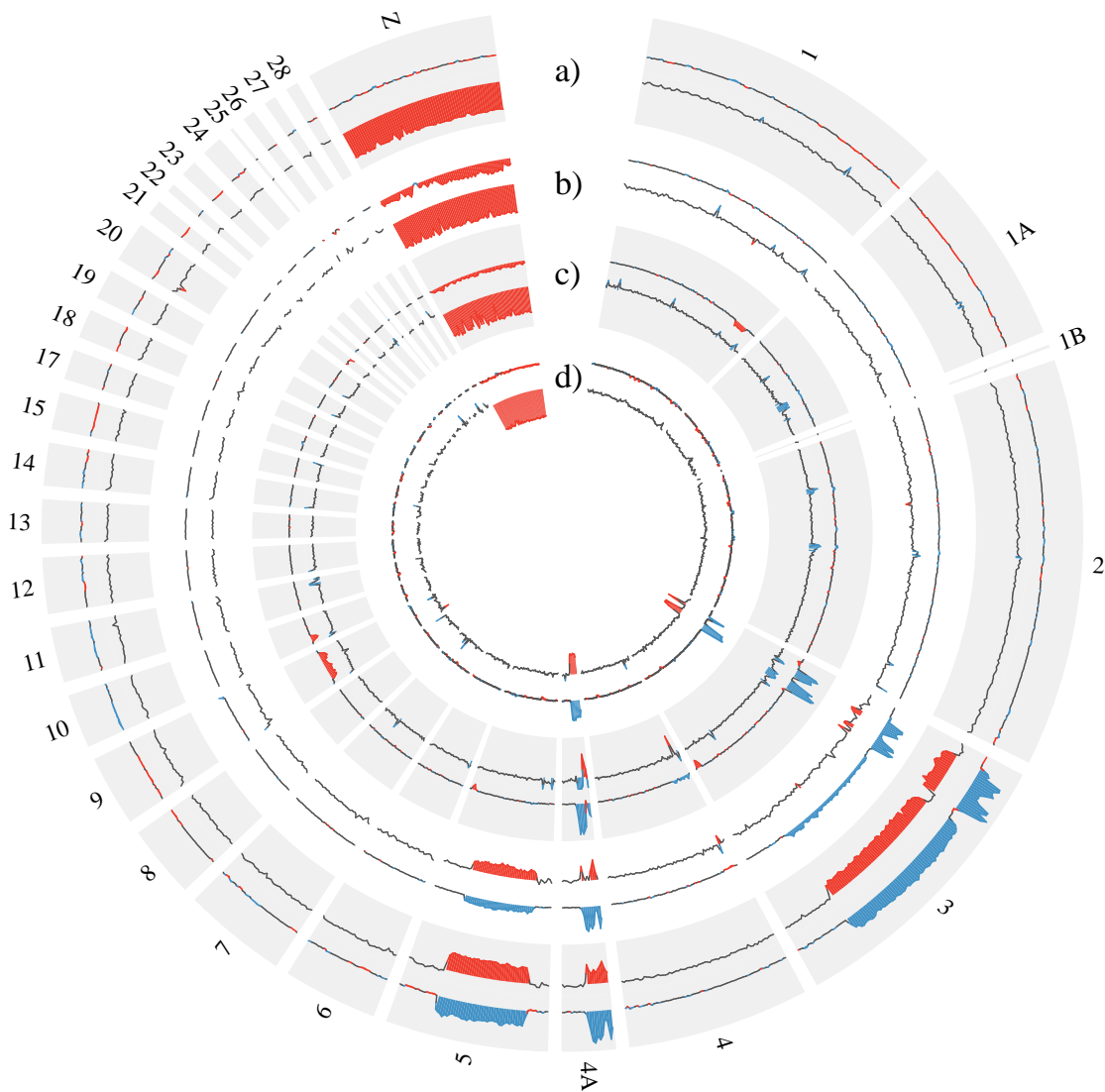
211 We downloaded Gene Ontology (GO) annotations for all the genes in the zebra finch genome from
212 Ensembl BioMart (taeGut3.2.4; accessed on 12 September 2018) and parsed the file for GO term names
213 with the following pattern matches: “sperm”, “ovarian”, “gonad”, “estrogen”, “testosterone”, “sex
214 differentiation”, “sex determination”, “sexual characteristics”, “sexual reproduction” and “oogenesis”. The
215 zebra finch gene annotation had in total 323 genes matching to any of these patterns. We then counted the
216 number of genes matching any of these terms for i) the sex-linked region for each chromosome separately,
217 and ii) within each of the identified evolutionary strata (see below). We performed two-tailed binomial tests

218 to see if these genomic regions contained either more or fewer sex-related genes than would be expected if
219 the genes were randomly distributed across the genome. We calculated the proportion of the genome that
220 made up each studied sex-linked region (1.253 Gbp is the full genome size of the zebra finch genome
221 assembly used) and tested if the region contained a higher or lower proportion of sex-related genes than
222 would be expected based on this probability. The p values for the two categories of tests (i, ii) were adjusted
223 using the Benjamini & Hochberg method.

224 3. RESULTS

225 3.1 Identification of sex-linked genomic regions

226 A genome-wide scan for sex-specific genetic variation (measured as the average difference in number of
227 female and male private SNVs per Mbp), and sex-specific genome coverage (measured as the average
228 female-to-male read coverage ratio per Mbp), revealed that four chromosomes stood out from the
229 autosomal pattern across the species (Figure 1; Suppl. Table S4). First, the ancestral Z chromosome showed
230 substantially lower female coverage, and no or a moderately lower amount of female-specific genetic
231 variation, in all four species (Figure 1). Secondly, the first half of chromosome 4A showed substantially
232 higher number of female-specific SNVs and moderately lower female coverage in all species. Thirdly, a
233 substantially higher amount of female-specific SNVs and no or moderate sex-specific coverage were found
234 over varying extents of chromosome 3 and 5 in the four species – small parts of chromosome 3 in the
235 bearded reedling, slightly larger parts of chromosome 3 in the horned lark, and the greater part of
236 chromosome 3 and 5 in the Raso lark and the Eurasian skylark (Figure 1). These results support the existence
237 of multiple autosome–sex chromosome fusions and several evolutionary strata where recombination has
238 been suppressed at different points in time across the avian and the lark phylogeny.
239



240

241 Figure 1. Genome-wide distribution of female-to-male difference in number of private single nucleotide variants
242 (SNVs), and female-to-male coverage ratio, in the four study species. The background colours grey and white separate
243 the data for each of the four species which are shown in the following order starting from the outer ring: a) Raso lark,
244 b) Eurasian skylark, c) horned lark and d) bearded reedling. Within each ring, the outer line shows the average
245 difference in number of private SNVs between females and males across 1 Mbp windows (with values > 500 in blue
246 and values < -500 in red), and the inner line shows the average female-to-male coverage ratio across 1 Mbp windows
247 (with values > 1.1 in blue and values < 0.9 in red). Chromosome-wide averages are provided in Suppl. Table S4.

248 To define the evolutionary strata more precisely, and to estimate their age, we assessed (i) the genomic
249 signatures of sex-linkage (i.e., sex-specific genetic variation and genome coverage as above) at the scale of

250 0.1 Mbp regions in each species (Table 1; Suppl. Figure S1), and (ii) the most parsimonious order of
251 emergence based on dated phylogenies (Figure 2; Alström et al. 2013, Cortez et al. 2014, Moyle et al. 2016).
252 Stratum 1 corresponds to the entire ancestral Z chromosome (72.9 Mbp), which was formed c. 140 Myr
253 ago (Cortez et al. 2014), and stratum 2 corresponds to the previously known Sylvioidea neo-sex
254 chromosome, i.e. the first 9.6 Mbp of chromosome 4A (Pala et al. 2012), which was formed c. 21-19 Myr
255 ago when Sylvioidea split from other passerines (Moyle et al. 2016). For both these strata, all four study
256 species showed clear signatures of sex-linkage (Table 1; Suppl. Figure S1). Likewise, all four study species
257 showed sex-specific genetic variation and coverage in the regions between 8.4–10.4 and 18.1–24.1 Mbp on
258 chromosome 3 (with a total size of 8.0 Mbp): this constitutes stratum 3, which is the earliest stratum being
259 unique to the Alaudidae/Panuridae clade. The age of stratum 3 is defined by the split of
260 Alaudidae/Panuridae from other Sylvioidea families c. 19-17 Myr ago (Moyle et al. 2016). The next stratum,
261 stratum 4, has a size of 3.6 Mbp and spans the region between 10.4–14.0 Mbp on chromosome 3. It occurs
262 in all three lark species, but not in the bearded reedling, which gives an estimated age of c. 17-14 Myr as
263 defined by the split between Alaudidae and Panuridae (Alström et al. 2013). Finally, the Raso lark and the
264 Eurasian skylark had two additional strata on chromosome 3 (stratum 5a: 64.9 Mbp spanning the regions
265 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
266 between 9.1-45.4 Mbp), respectively, that were not shared with the other two species. These strata form the
267 youngest sex chromosome layer, estimated to be c. 14-6 Myr based on the split between the horned lark
268 and the *Alauda* larks (Alström et al. 2013). Together, these sex-specific regions on chromosome Z, 4A, 3
269 and 5 amounts to 195.3 Mbp of the genome of the Raso lark and Eurasian skylark (16.3% of the genome
270 based on a genome size estimation of 1.2 Gbp) (Table 1; Suppl. Figure S1). The horned lark had 94.1 Mbp
271 (7.8%), and the bearded reedling 90.5 Mbp (7.5%), sex-linked genomic material (Table 1; Suppl. Figure S1).
272 Figure 2a shows a summary of the chromosomal position of the evolutionary strata and their most
273 parsimonious order of appearance based on presence and absence in the studied species (also shown are
274 silhouettes indicating vertebrate groups where homologs to these chromosomes act as sex chromosomes;
275 see Discussion). Figure 2b summarises the timings of sex-linkage based on dated phylogenies (Cortez et al.
276 2014, Alström et al. 2013, Moyle et al. 2016).

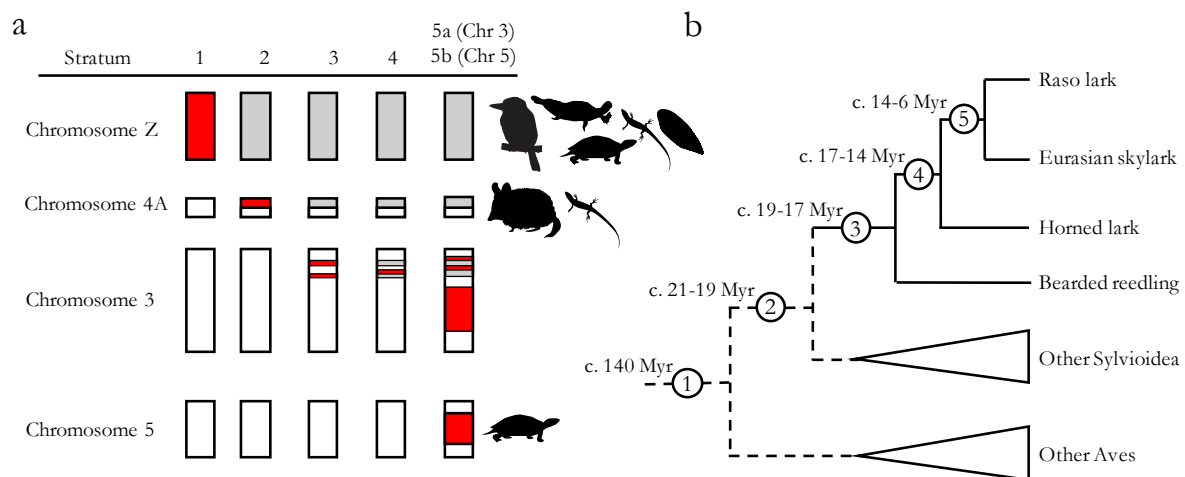
277

278 Table 1. Sex-linked regions in each of the four species. The strata are numbered according to the most parsimonious
 279 order of emergence based on phylogenetic analyses. Stratum 1 (homologous to chromosome Z in zebra finch) acts as
 280 the sex chromosome in all birds, and sex-linkage of stratum 2 (chromosome 4A in zebra finch) is common for all birds
 281 belonging to the superfamily Sylvioidea, which includes larks and the bearded reedling. Stratum 3 was seen to be sex-
 282 linked in all four studied species and is therefore older than stratum 4, which appears as sex-linked in all larks but not
 283 the bearded reedling. Stratum 5a and 5b appear as sex-linked in the Raso lark and Eurasian skylark, but not in horned
 284 lark or bearded reedling, and is therefore the youngest stratum but here divided into two substrata as they originated
 285 from different autosomes. The table provides mean values for the male-to-female coverage ratio (coverage) and female-
 286 to-male difference in number of private SNVs across 0.1 Mbp windows for each stratum. Bearded reedling and horned
 287 lark have no sex-linkage for stratum 5a and 5b, and bearded reedling not for stratum 4, and are therefore marked with
 288 NA in addition to the corresponding values.

Stratum	Chromosome	Genomic region (Mbp)	Stratum Size (Mbp)		Raso lark	Eurasian skylark	Horned lark	Bearded reedling
1	Z	0-72.9	72.9	Coverage	0.52	0.55	0.54	0.52
				SNV	-17.77	-992.10	-375.85	-160.48
2	4A	0-9.6	9.6	Coverage	0.73	0.89	1.06	0.66
				SNV	2957.35	2227.36	2518.46	2413.16
3	3	8.4-10.4, 18.1-24.1	8.0	Coverage	0.74	0.95	1.20	0.68
				SNV	3386.68	2435.65	3404.79	2787.17
4	3	10.4-14.0	3.6	Coverage	0.69	0.84	0.97	NA (1.00)
				SNV	3019.06	2161.91	2512.71	NA (-10.54)
5a	3	5.8-8.4, 14.0-18.1, 29.8-88.0	64.9	Coverage	0.74	0.98	NA (1.00)	NA (0.99)
				SNV	1785.39	451.48	NA (1.73)	NA (1.77)
5b	5	9.1-45.4	36.3	Coverage	0.69	0.85	NA (1.00)	NA (0.99)
				SNV	1952.04	879.14	NA (20.19)	NA (-37.92)

289

290

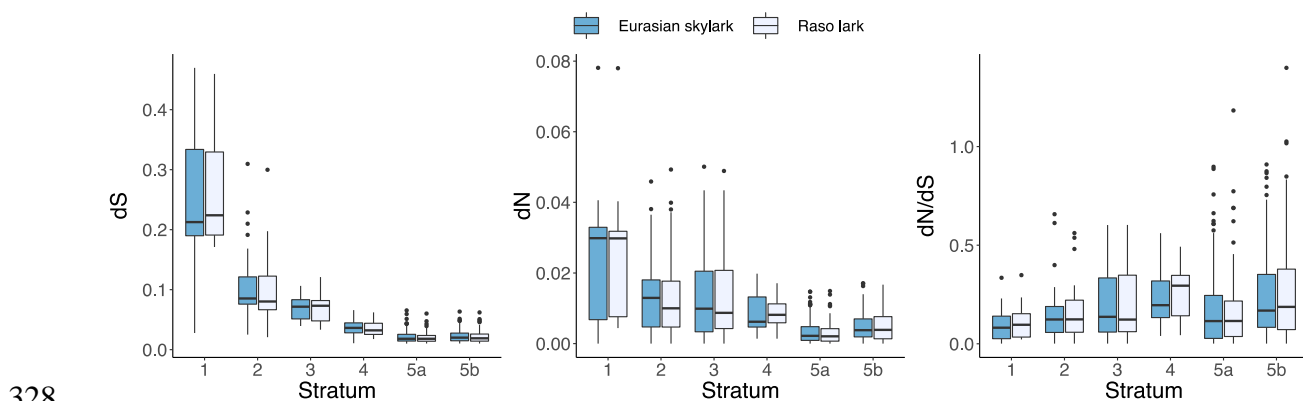


291
 292 Figure 2. a) Cartoon representation of the evolutionary strata in the most parsimonious order of appearance (starting
 293 with stratum 1 and ending with stratum 5a and 5b). White colour represents autosomal regions and red colour marks
 294 the stratum being added. Grey colour represents sex-linked regions that already exist at the timing of the new stratum.
 295 Silhouettes indicate vertebrate groups where homologs to these chromosomes act as sex chromosomes (see
 296 Discussion): chromosome Z acts as the sex chromosome in all birds (Smith et al. 2009) and its homolog acts as sex
 297 chromosome in a flatfish (*Cynoglossus semilaevis*; Chen et al. 2014), a turtle (*Staurotypus triporcatus*; Montiel et al. 2016), a
 298 gekko (*Gekko bokouensis*; Kawai et al. 2008) and in the platypus (*Ornithorhynchus anatinus*; Grützner et al. 2004); the
 299 homolog of chromosome 4A is the sex chromosome in a lizard (*Takydromus sexlineatus*; Rovatsos et al. 2016) and in all
 300 eutherian mammals (Ross et al. 2005); and the homolog of chromosome 5 acts as sex chromosome in two turtle groups
 301 (*Glyptemys* wood turtles and *Siebenrockiella* marsh turtles; Montiel et al. 2016). (b) A cladogram showing when in time
 302 (Myr) the evolutionary strata were formed. The dating of stratum 1 is based on analyses of gametologous gene pairs in
 303 Cortez et al. (2014), stratum 2 and 3 on speciation estimates in Moyle et al. (2016), and stratum 4 and 5 (5a and 5b) on
 304 speciation estimates in Alström et al. (2013).

305 3.2 Analysis of divergence between gametologous genes

306 We analysed the Z–W divergence from the gametologous genes located on the different strata across the
 307 sex chromosome in the Raso lark and the Eurasian skylark, i.e. the species with all five evolutionary strata.
 308 We analysed the two different parts of stratum 5 on chromosome 3 and 5 separately in these analyses (i.e.,
 309 stratum 5a and 5b, respectively). The degree of synonymous substitutions (median dS) between the Z and

310 W gametologs ranged from 0.22 at stratum 1 to 0.02 at stratum 5a and 5b in the Raso lark, and from 0.21
 311 at stratum 1 to 0.02 at stratum 5a and 5b in the Eurasian skylark (Figure 3, Suppl. Table S5). In the Raso
 312 lark, the log₂-transformed dS values differed significantly between all strata except between stratum 2 and
 313 stratum 3, and between stratum 5a and 5b (Kruskal-Wallis rank sum test: chi-squared = 154.0, df = 4, p <
 314 0.001; for pairwise comparison values see Suppl. Table S6). In the Eurasian skylark, the log₂-transformed
 315 dS differed significantly between all strata, except between stratum 5a and 5b (Kruskal-Wallis rank sum test:
 316 chi-squared = 143.0, df = 4, p < 0.001; pairwise comparison values in Suppl. Table S6). The degree of non-
 317 synonymous substitutions (dN) between the Z and W gametologs showed similar patterns, but less
 318 pronounced (median dN ranged from 0.03 at stratum 1 to 0.002 at stratum 5a in the Raso lark, and from
 319 0.03 at stratum 1 to 0.002 at stratum 5a in the Eurasian skylark; Figure 3, Suppl. Table S5). The dN values
 320 were significantly different between stratum 5a and all other strata, and between 5b and all other strata in
 321 both species, and between stratum 1 and stratum 4 in the Raso lark (Raso lark: chi-squared = 88.7, df = 4,
 322 p < 0.001; Eurasian skylark: chi-squared = 143.0, df = 4, p < 0.001; for pairwise comparison values see
 323 Suppl. Table S6). The dN/dS values were lowest at stratum 1 in both species (0.082 in the Eurasian skylark
 324 and 0.097 in the Raso lark) and highest at stratum 4 (0.196 in the Eurasian skylark and 0.294 in the Raso
 325 lark). The dN/dS values in stratum 4 differed significantly from strata 1, 2 and 5a, and stratum 5a differed
 326 significantly from stratum 5b, in both species (Raso lark: chi-squared = 17.7, df = 4, p = 0.001; Eurasian
 327 skylark: chi-squared = 14.0, df = 4, p = 0.007; pairwise comparisons in Suppl. Table S6).



329 Figure 3. Z-to-W substitution rates for gametologous genes positioned within the different evolutionary strata in the
 330 Eurasian skylark (dark blue) and the Raso lark (light blue). (a) Median dS values, (b) median dN values, and (c) median

331 dN/dS values are marked by the black line in each box, and the upper and lower hinges correspond to the first and
332 third quartiles (the 25 and 75 percentiles). The whiskers extend to no more than $1.5\times$ the interquartile range from
333 each hinge. See Suppl. Table S5 for details and main text for results from analyses of variances between strata based
334 on \log_2 -transformed values.

335

336 In both Raso larks and Eurasian skylarks, the dS values correlated significantly with the relative age of the
337 strata (stratum 1 being oldest and strata 5a and 5b being youngest) (Ordered-heterogeneity test: $r_sP_c = 0.99$,
338 $k = 6$, $p < 0.001$ for both species). This was also true for the dN values ($r_{spc} = 0.99$, $k = 6$, $p < 0.001$ for
339 both species). However, the dN/dS values were not significantly associated with the age of the strata (Raso
340 lark: $r_{spc} = 0.41$, $k = 6$, $p \sim 0.11$; Eurasian skylark: $r_{spc} = 0.46$, $k = 6$, $p \sim 0.11$).

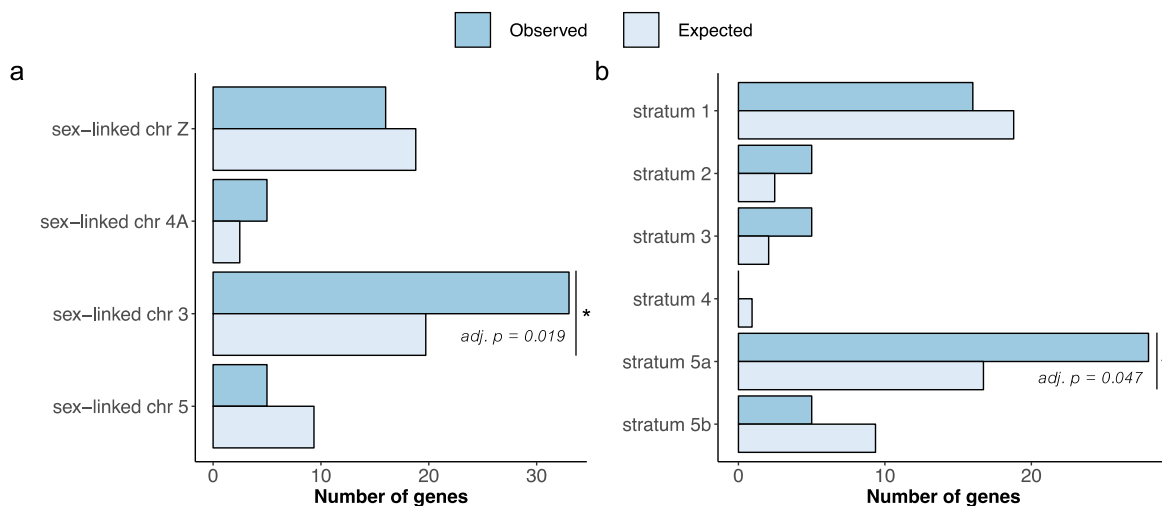
341 3.3 Gene enrichment analysis

342 Of the 323 genes with sex-related GO term names (see Methods section), 16 genes were located on the
343 ancestral avian sex chromosome Z (stratum 1), 5 within the sex-linked part of chromosome 4A (stratum 2),
344 33 within the sex-linked region of chromosome 3 (strata 3, 4 and 5a) and 5 within the sex-linked part of
345 chromosome 5 (stratum 5b) (Figure 4a; Suppl. Table S7). The different strata on chromosome 3 hold 5
346 (stratum 3), 0 (stratum 4) and 28 (stratum 5a) genes, respectively (Figure 4b; Suppl. Table S7). This means
347 that 59 genes with these hypothesised sex-related functions are sex-linked in the Raso lark and in the
348 Eurasian skylark, i.e. the species with all strata, compared to 16 in species only having the ancestral avian
349 sex chromosome.

350

351 Binomial tests showed that the sex-linked part of chromosome 3 (strata 3, 4 and 5a) had significantly more
352 sex-related genes than would be expected if the genes were randomly distributed over the genome (33
353 observed genes compared to 19.7 expected genes; Binomial test: adjusted $p = 0.019$; Figure 4a; Suppl. Table
354 S7). The sex-linked regions on chromosomes Z, 4A and 5 showed no statistical difference between observed
355 and expected number of genes (NS; Suppl. Table S7). When analysing each stratum separately, stratum 5a

356 was also significantly enriched for sex-related genes (28 observed genes compared to 16.7 expected genes;
357 adjusted $p = 0.047$; Figure 4b; Suppl. Table S7), while the other strata were not significantly enriched (NS;
358 Suppl. Table S7). Information about the analysed genes (chromosomal positions, GO terms and gene ID)
359 are provided in Supplementary Document S1.
360



361
362 Figure 4. Observed and expected number of sex-related genes for (a) the sex-linked region of each chromosome and
363 (b) each stratum. Binomial tests (Suppl. Table S7) showed that the sex-linked region on chromosome 3 had significantly
364 more sex-related genes than would be expected if the genes were randomly distributed throughout the genome
365 (adjusted $p = 0.019$), and that this enrichment on chromosome 3 was mainly due to a high number of sex-related genes
366 on stratum 5a (adjusted $p = 0.047$).

367 4. DISCUSSION

368 By using comparative genomics in larks and their closest relative, the bearded reedling, we have identified
369 the precise genomic regions that have evolved sex-linkage across a group of birds with multiple autosome–
370 sex chromosome fusions. The extent of sex-linkage varied between species, and for two of them (Raso lark
371 and Eurasian skylark in the genus *Alauda*), we detected the largest known avian sex chromosome (195.3
372 Mbp) and show that it originates from fusions between (parts of) four avian chromosomes (Z, 3, 4A and
373 5). We found evidence of five evolutionary strata where recombination has been suppressed at different

374 time points; from c. 140 Myr ago (stratum 1, the sex chromosome common to all birds) to c. 14-6 Myr ago
375 (stratum 5a and 5b, the layer unique to *Alauda*; the strata and their age estimates are summarised in Figure
376 2). We further show that the substitution rates between Z and W gametologs increase with increasing age
377 of the strata. This gives support for a key prediction from sex chromosome theory: continuous
378 differentiation of non-recombining chromosomes (Rice 1994; Lahn & Page 1999).

379
380 With the available genomic data, we are not able to determine the chromosomal structure of this neo-sex
381 chromosome. However, the 195.3 Mbp sex-linked region in the two *Alauda* study species (Raso lark and
382 Eurasian skylark) constitutes c. 16.3% of their expected genome size (1.2 Gbp), which in turn corresponds
383 well to the genomic proportion of the sex chromosomes in the karyotypes of the bimaculated lark (i.e., c.
384 15-20%; Bulatova 1981). The bimaculated lark karyotypes further show that the Z and W chromosomes are
385 of similar size and drastically enlarged compared to the usual bird karyotype (Bulatova 1981). Therefore, we
386 believe that the additional chromosomes that are sex-linked in larks have fused to the Z as well as the W
387 chromosome. Moreover, the karyotype of a male horned lark (no female was karyotyped) indicates a Z
388 chromosome of similar size to that of the bimaculated lark (Bulatova 1981). Therefore, it is possible that
389 large parts of chromosome 3 and 5 are fused to the Z chromosome also in the horned lark, but as our results
390 show are still recombining in that species (with the exception of strata 3 and 4 on chromosome 3 that are
391 non-recombining). This reasoning is complemented by the results in E. Dierickx et al. (unpublished
392 manuscript) showing that different species and subspecies of *Alauda* (the oriental skylark *A. gulgula*, Eurasian
393 skylark ssp. [possibly *A. a. arvensis/dulcivox/intermedia*], and Raso lark), and a species of the genus *Gallerida*
394 (the crested lark *G. cristata*) – the sister genus of *Alauda* – have suppressed recombination on chromosome
395 5 (stratum 5b) but show recombination suppression to different degrees on chromosome 3 (stratum 5a).

396
397 A long-standing hypothesis in sex chromosome research posits that the evolutionary dynamics of sex
398 chromosomes are governed by selection acting on linked genes, in particular on sex-determining and
399 sexually antagonistic genes (Haldane 1922; Fisher 1931; Lewis & John 1968; D. Charlesworth & B.
400 Charlesworth 1980). This hypothesis states that chromosomes that harbour such genes should be over-

401 represented in sex chromosome formations, transitions and turnovers (Bachtrog et al. 2011; Ross et al. 2009;
402 O'Meally et al. 2012). In support of this hypothesis, we found that the sex-linked region of chromosome 3,
403 and one of the strata on that chromosome (stratum 5a), were significantly enriched for genes with sex-
404 related functions (adjusted $p = 0.019$ and 0.047 , respectively). Among these genes is *ESR1* (*Estrogen receptor*
405 *1*), which has been suggested to be involved in sex reversal in the American alligator (*Alligator mississippiensis*;
406 Kohno et al. 2015). The other chromosomes (and strata) involved in the extensive neo-sex chromosome
407 formation in larks were not significant enriched for sex-related genes. However, all of them have
408 independently been recruited as sex chromosomes in other vertebrate lineages. The Z chromosome – the
409 sex chromosome in all birds, containing the putative sex determining gene *DMRT1* (*Doublesex and mab-3*
410 *related transcription factor 1*; Smith et al. 2009) – has independently been recruited as a sex chromosome in a
411 flatfish (the half-smooth tongue sole *Cynoglossus semilaevis*; Chen et al. 2014), a turtle (the Mexican musk turtle
412 *Staurotypus triporcatus*; Montiel et al. 2016), a gecko (the Kwangsi gecko *Gekko bokouensis*; Kawai et al. 2008),
413 and in the platypus (*Ornithorhynchus anatinus*; Grützner et al. 2004). Chromosome 4A is homologous to the
414 sex chromosome in a lizard (the Asian grass lizard *Takydromus sexlineatus*; Rovatsos et al. 2016) and in all
415 eutherian mammals (Ross et al. 2005), and contains several interesting genes, including *AR* (*Androgen receptor*)
416 and *SOX3* (*SRY-related HMG-box 3*) (note, however, that *SOX3* is not sex-linked in Sylvioidea; Pala, Naurin,
417 et al. 2012; this study). Finally, the homolog to chromosome 5 acts as sex chromosome in two turtle groups
418 (*Glyptemys insculpta* and *Siebenrockiella crassicolis*; Montiel et al. 2016). An alternative hypothesis is that fusions
419 may become fixed as a consequence of non-selective processes (see e.g. Pennell et al. 2015). However, we
420 have presented several strands of evidence – the enrichment of genes with sex-specific function and the
421 repeated homology to other vertebrate sex chromosomes – to support the hypothesis that the multiple
422 fusion events between chromosome Z, 4A, 3 and 5, that have formed these extraordinary neo-sex
423 chromosomes in larks and in the bearded reedling, have been driven by selective processes acting on their
424 gene content. This adds to the accumulating evidences that specific chromosomes are non-randomly
425 recruited as sex chromosomes in vertebrates (O'Meally et al. 2012; Ezaz et al. 2006; Ezaz et al. 2016).

426

427 Multiple autosome–sex chromosome fusions have also occurred in other clades, including gazelles and
428 snakes (Vassart et al. 1995; Pokorná et al. 2014). In snakes, all six neo-sex chromosome systems identified
429 so far are from a single family, Elapidae (Pokorná et al. 2014). These patterns, now also including those
430 found in the larks, hint at a phylogenetic component in neo-sex chromosome formation; that once an
431 autosome–sex chromosome fusion has occurred, additional ones may be expected (Pokorná et al. 2014). If
432 the fusions are being selected for because of any advantageous linkage that they may create, then previously
433 fused regions (with already suppressed recombination) provide more opportunities for beneficial linkage to
434 be formed between the sex-linked genes and new additional genomic regions.

435

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446

447 Supplementary Material and Data Availability

448 Suppl. Figure S1 and Suppl. Tables S1-7 are provided together as a separate file. Suppl. Document S1 is
449 provided as a separate file. In-house scripts are given in Suppl. Code S1 (available upon request). Illumina

450 HiSeqX raw reads (150 bp, paired-end) will be deposited at NCBI sequence read archives upon
451 acceptance.

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