

1 **Diversity of reptile sex chromosome evolution revealed by cytogenetic and linked-read**
2 **sequencing**

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30

31 **Abstract**

32 Reptile sex determination is attracting much attention because the great diversity of sex-
33 determination and dosage compensation mechanisms permits us to approach fundamental
34 questions about sex chromosome turnover and evolution. However, reptile sex chromosome
35 variation remains largely uncharacterized and no reptile master sex determination genes have yet
36 been identified. Here we describe a powerful and cost-effective “chromosomics” approach,
37 combining probes generated from the microdissected sex chromosomes with transcriptome
38 sequencing to explore this diversity in non-model Australian reptiles with heteromorphic or
39 cryptic sex chromosomes. We tested the pipeline on a turtle, a gecko, and a worm-lizard, and we
40 also identified sequences located on sex chromosomes in a monitor lizard using linked-read
41 sequencing. Genes identified on sex chromosomes were compared to the chicken genome to
42 identify homologous regions among the four species. We identified candidate sex determining
43 genes within these regions, including conserved vertebrate sex-determining genes *pdgfa*, *pdgfra*,
44 *amh* and *wt1*, and demonstrated their testis or ovary-specific expression. All four species showed
45 gene-by-gene rather than chromosome-wide dosage compensation. Our results imply that reptile
46 sex chromosomes originated by independent acquisition of sex-determining genes on different
47 autosomes, as well as translocations between different ancestral macro- and micro-chromosomes.
48 We discuss the evolutionary drivers of the slow differentiation, but rapid turnover, of reptile sex
49 chromosomes.

50 **Introduction**

51 Sex can be determined either by genes on specialized chromosomes (genetic sex determination,
52 GSD) or by environmental factors (environmental sex determination, ESD). Much of our
53 knowledge on sex chromosome evolution has come from studies of model organisms such as
54 *Drosophila*, chicken and mammals (principally humans and mice), in which species master sex
55 determining genes have been identified ¹. Their heteromorphic sex chromosomes can be easily
56 identified by cytogenetic observations because the male-specific Y chromosome, or the female-
57 specific W chromosome is morphologically different from the X or Z chromosome. Sex
58 chromosome differentiation occurs as the result of suppression of recombination, and is
59 manifested by massive accumulation of massive transposable elements and inactivation or loss of
60 genes ². The sex chromosomes of many model vertebrate species have been evolutionarily stable
61 for more than 100 million years, judging from the homology of the pair of sex chromosomes
62 within their clade ^{3,4}.

63 However, in many reptiles, amphibians and fish, there are frequent transitions between
64 different sex determination mechanisms ^{5, 6, 7}. Reptiles represent an extraordinary variety of sex
65 determining mechanisms, including GSD and TSD, XY and ZW systems with varying degrees of
66 sex chromosome differentiation ⁸. However, we know little about reptile sex chromosomes and
67 sex determining genes.

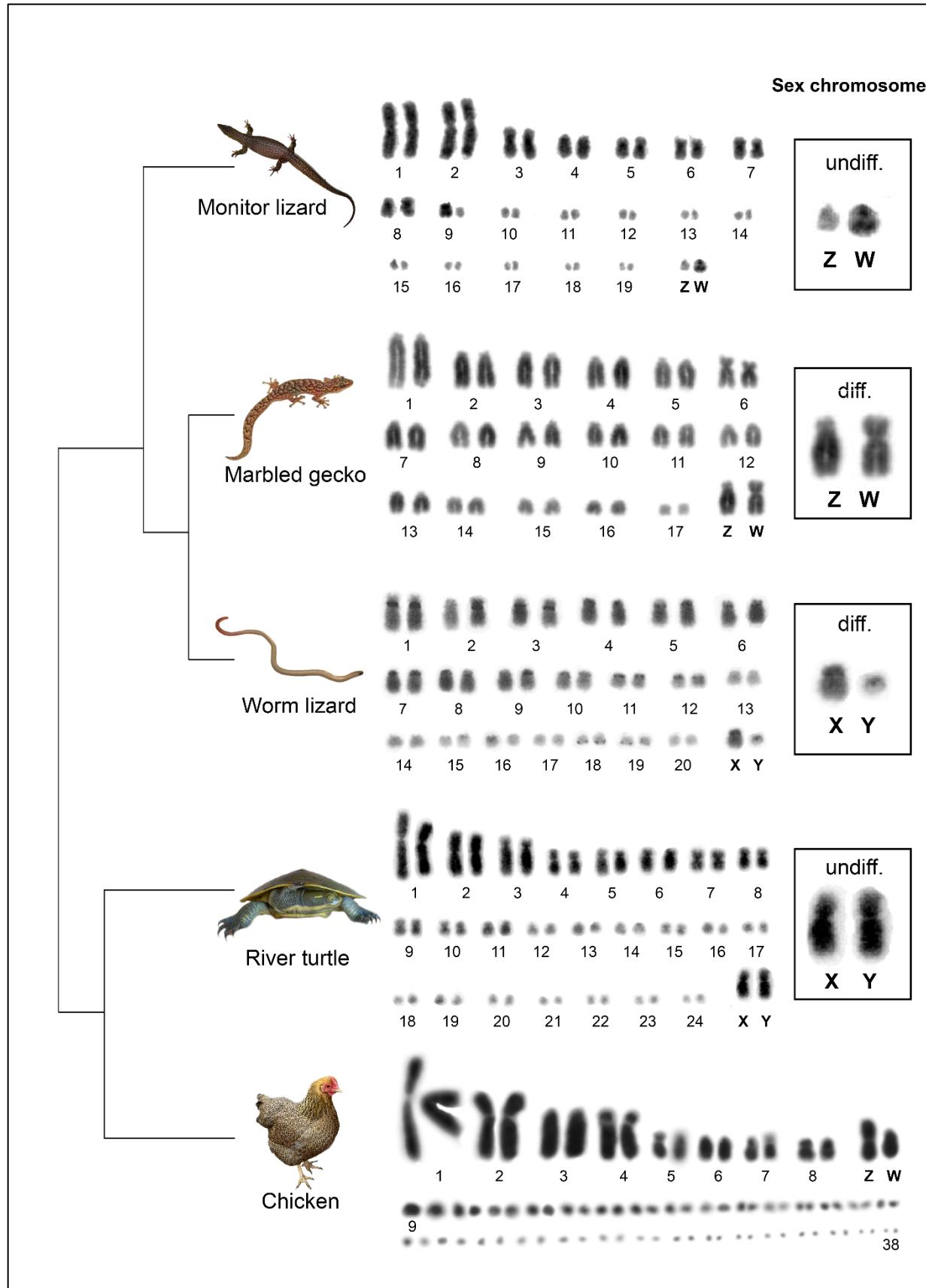
68 The evolutionary variety of vertebrate sex determining systems has long been recognized.
69 Cytological observations and limited gene mapping data reveal that multiple transitions between
70 ESD and GSD, and between XY and ZW sex chromosome systems, have occurred in reptiles ⁵,
71 teleost fish ⁶ and anurans ⁷. However, despite this variety, extensive cytogenetic mapping of the
72 reptile orthologues of genes that are located on sex chromosomes of model organisms (e.g.,
73 human and chicken) revealed a surprisingly frequent over-representation of particular ancestral
74 autosomes or genomic regions ^{9, 10, 11}.

75 With the development of long-read sequencing and Hi-C technologies, many genomic
76 consortia (e.g., Vertebrate Genome Project ¹² and the Earth Biogenome Project ¹³ aim to finish
77 the complete genomes of most vertebrate species on earth in the next few years. However
78 sequencing projects usually represent sex chromosomes poorly; either the homogametic sex (XX
79 female or ZZ male) is sequenced, and the male-specific Y or female-specific W is ignored; or the
80 heterogametic sex only is sequenced with poor representation of the X or Z, and there is great

81 difficulty in assembling the repeat-rich Y or W^{14, 15}.

82 Here, we develop a cost-effective method to identify genes borne on sex chromosomes,
83 combining microdissection of sex chromosomes and high-throughput sequencing, followed by
84 PCR validation and assessment as candidate sex determining genes. A similar method was
85 pioneered to identify novel genes on the Y chromosome of marsupials¹⁶. We applied the method
86 to four reptile species, revealing the great diversity of sex chromosomes, and their independent
87 evolutionary origins.

88 We chose four Australian reptile species, a turtle and three lizard species to represent the
89 variety of reptile sex determining systems (**Figure 1**). The Murray River turtle *Emydura*
90 *macquarii*¹⁷ (referred as ‘river turtle’ hereafter) has a cryptic XX/XY sex chromosome system in
91 which minimally differentiated X and Y are macrochromosomes, whereas the pink-tailed worm
92 lizard *Aprasia parapulchella*¹⁸ (referred as ‘worm lizard’ hereafter) has a highly differentiated
93 XX/XY sex chromosome system in which the X and Y are microchromosomes. The marbled
94 gecko *Christinus marmoratus*¹⁹ (referred as ‘marbled gecko’ hereafter) has a pair of ZZ/ZW sex
95 chromosomes, in which the Z and W heteromorphy involves pericentric inversion, whereas the
96 spiny-tailed monitor lizard *Varanus acanthurus*²⁰ (referred as ‘monitor lizard’ hereafter) has
97 ZZ/ZW heteromorphic sex chromosomes in which Z and W chromosomes are minimally
98 differentiated microchromosomes (**Figure 1**).



100 **Figure 1 The diversity of reptile sex chromosomes.**

101 Cladogram and karyotypes of the studied reptile species river turtle (*Emydura macquarii*), worm
102 lizard (*Aprasia parapulchella*), Marbled gecko (*Christinus marmoratus* and monitor lizard
103 (*Varanus acanthurus*) with two types of sex chromosome systems. Species with differentiated
104 sex chromosomes are labelled with “diff.”, otherwise with “undiff.”. Photo credit: see
105 acknowledgements.

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107 **Results**

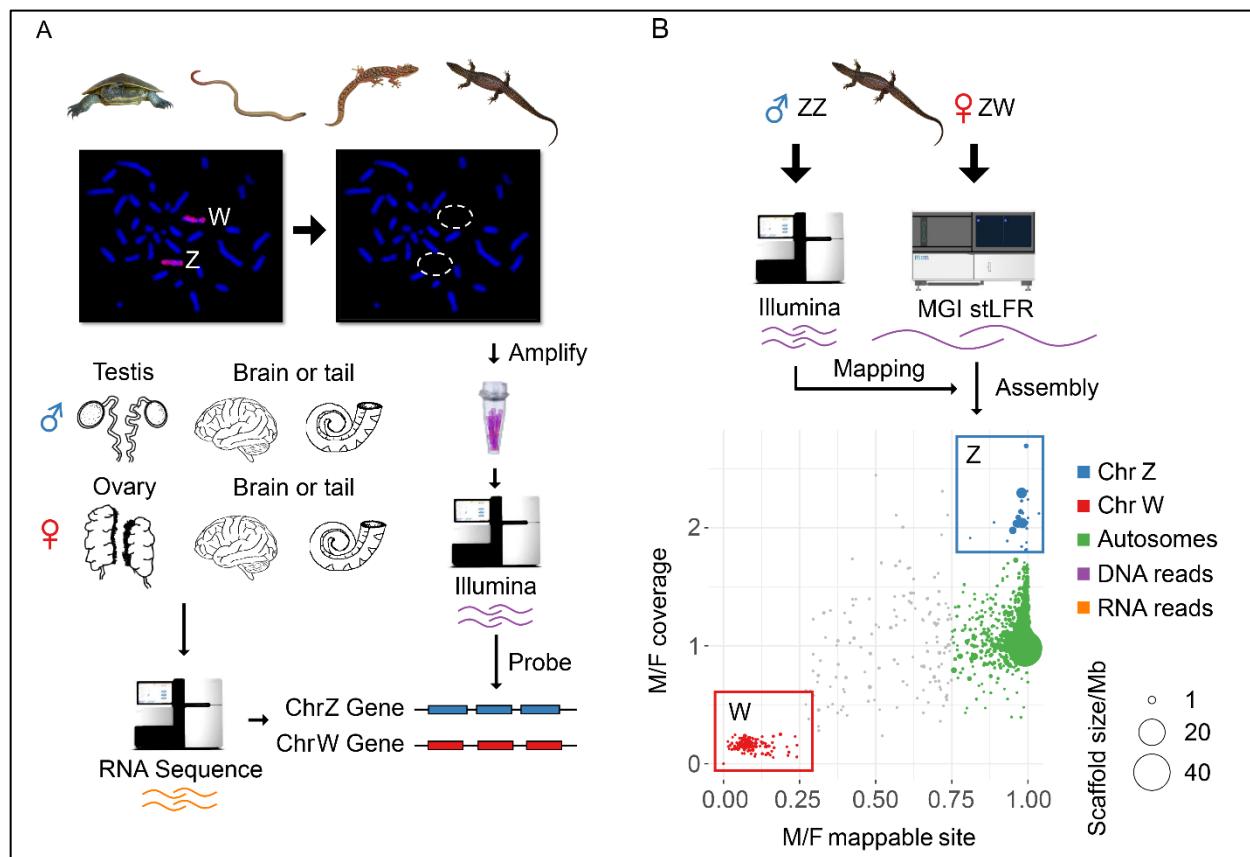
108 **Transcriptome and genome assemblies of sex chromosomes of the four reptile species**

109 The four reptile species have cytologically distinguishable sex chromosome pairs (**Figure 1**,
110 **Supplementary Fig. S1**); these were morphologically differentiated in the worm lizard and the
111 monitor lizard, but subtle for the river turtle and the marbled gecko^{18, 19, 20}. For each species, we
112 microdissected each of their sex chromosomes, performed linear genome amplification and
113 validated the sex chromosome specificity of the DNA products by chromosome painting (**Figure**
114 **2A, Supplementary Fig. S1**).

115 For each sex chromosome, we generated up to 2Gb clean paired-end (PE) Illumina reads
116 from the microdissected sex chromosome DNA (**Supplementary Table S1**). To identify genes
117 borne on sex chromosomes, we also produced 2Gb transcriptomes from the gonads and brain
118 tissues for males and females of monitor lizard, river turtle and marbled gecko and somatic
119 transcriptomes (tail tissue) from a male and female worm lizard (**Figure 2B**). Genomic reads
120 derived from each sex chromosome were then used to identify sex-linked genes from *de novo*
121 assembled transcript sequences of each species. We annotated a total of 11299, 15202 and 10507
122 non-redundant transcripts, respectively for the worm lizard, the marbled gecko, and the river
123 turtle, using chicken genes as a reference for each.

124 For the monitor lizard inferences based on transcripts and microdissected sex
125 chromosome sequences were uncertain because its sex chromosomes were reported to have
126 originated from translocations between fragments of multiple ancestral autosomes (also see
127 below)²¹, as well as the poor quality of sequences obtained from microdissected monitor lizard
128 sex chromosomes. Therefore, we generated 200 Gb (135x genomic coverage) single-tube long
129 fragment linked reads (stLFR)²² from a female individual, and 30 Gb Illumina PE reads from a
130 male individual. We performed *de novo* genome assembly and produced a female draft genome

131 with the total length of 1.46Gb and the scaffold N50 length of 12.8Mb (**Supplementary Table**
132 **S2**). The high continuity of the draft genome was evident from 94 very large scaffolds that
133 accounted for 80% of the entire genome. Using protein sequences of human and chicken as
134 reference, we annotated a total of 14521 genes for the monitor lizard and identified its sex-linked
135 sequences based on the comparisons of mapped read patterns between sexes. The putative W-
136 linked scaffolds showed female specificity in both their mapped read number and mapped sites,
137 whereas the Z-linked scaffolds showed a 2-fold increase of male mapped reads compared to that
138 of female mapped reads, but an equal number of mapped sites for putative autosomal scaffolds
139 between sexes (**Figure 2B**). Using this approach, we identified 10.81 Mb Z-linked scaffolds
140 with 337 genes, and 7.10 Mb W-linked scaffolds with 87 genes.



141
142 **Figure 2. Transcriptome and genome assemblies of four Australian reptile species.**
143 A. The pipeline of identifying sex-linked transcripts of river turtle, worm lizard and marbled
144 gecko using transcriptomes of sexed tissues, and amplified probes from the microdissected sex
145 chromosomes. The probes have been validated by chromosome painting, and Illumina reads
146 generated from the probes were used to identify the sex chromosome genes from the *de novo*

147 assembled transcripts. **B.** Identifying sex-linked sequences of monitor lizard based on the *de*
148 *novo* genome assembly generated from linked (stLFR) reads. For the assembled Z-linked
149 sequences (in blue), we found a 2-fold male vs. female (M/F) ratios of Illumina DNA sequencing
150 coverage, but an equal mappable site between sexes. While the W-linked sequences (in red)
151 exhibited a female-specific pattern of read coverage ratio and mappable sites. The size of each
152 dot is scaled to its scaffold length.

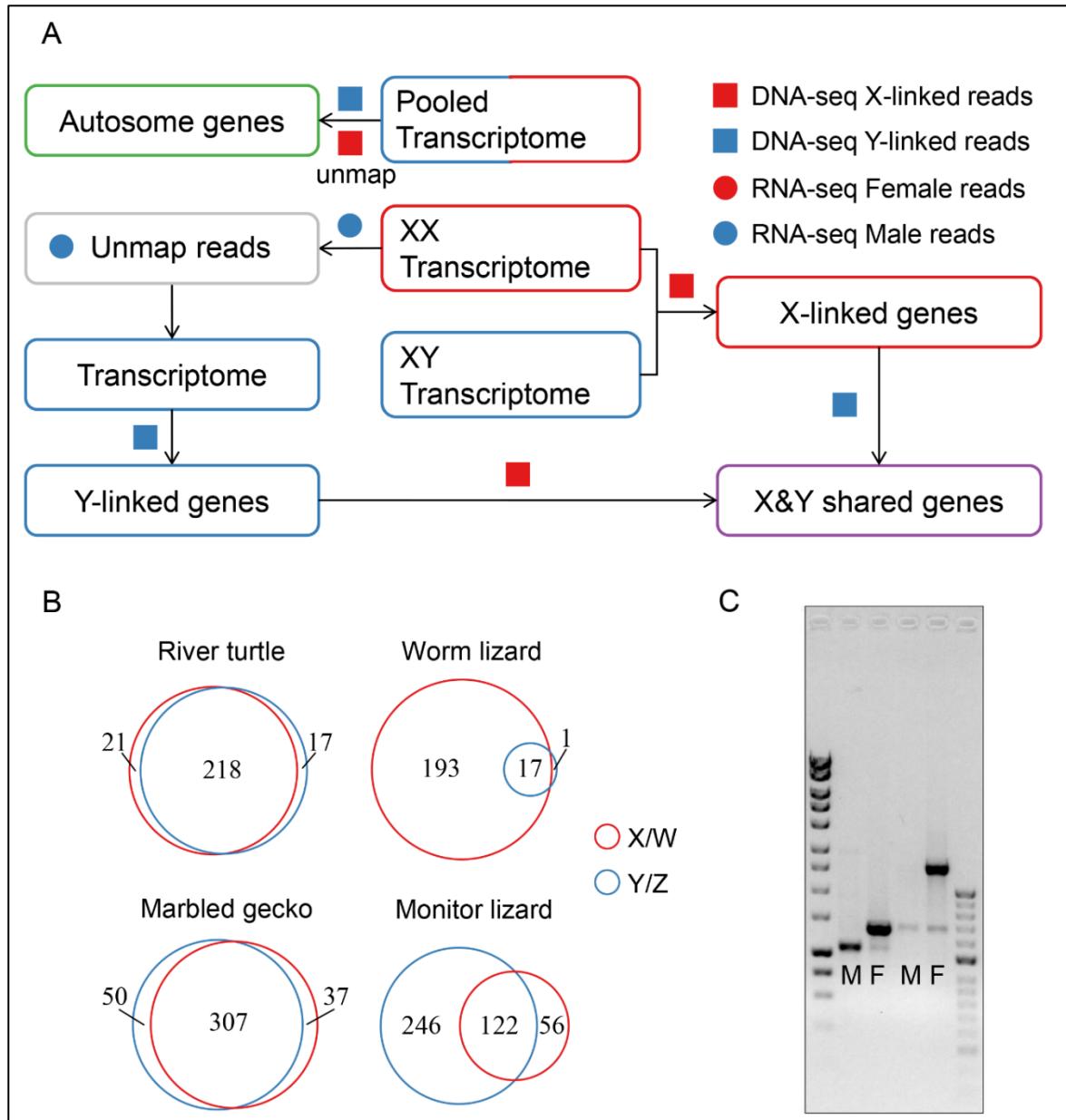
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154 **Identification of sex-linked genes**

155 To identify the sex chromosome-borne genes in three species other than the monitor lizard, we
156 developed a pipeline to separately assemble transcripts of genes that are X- or Y-borne (or Z-
157 and W-borne) using the sexed transcriptomes (**Figure 3A**). In brief, we considered that
158 transcripts that were assembled using pooled RNA-seq reads of both sexes and could not be
159 aligned using the sex chromosome DNA probes were autosomal genes. Conversely, male RNA-
160 seq reads in XY species that could not be aligned to the female transcripts were assembled into
161 candidate Y- borne transcript sequences. Then by comparing the mapped read numbers of Y- or
162 X-borne probes for each candidate Y-borne transcript or each transcript assembled from female
163 RNA-seq, we were able to categorize them into the genes that were specific to the X or to Y
164 chromosome, or were shared between X and Y. We also conducted the same process for the ZW
165 marbled gecko but in reverse.

166 Following our stringent filtering criteria (see Methods), we identified 193 X-borne genes,
167 1 Y-borne genes and 17 shared genes between X/Y chromosomes in the worm lizard, 21 X-borne
168 genes, 7 Y-borne genes and 218 shared genes in the river turtle and 50 Z-borne genes 37 W-
169 borne genes and 307 shared genes in the gecko (**Figure 3B, Supplementary Table S3**). We
170 considered these numbers to be conservative estimates of the sex chromosome-borne genes in
171 these species because genes with low expression levels may not be well assembled in our
172 transcriptome data and there could also be a sampling bias in the sex chromosome probes
173 captured by microdissection. We further designed primers spanning regions of insertions or
174 deletions between the sex chromosomes and confirmed their length variations between sexes by
175 PCR for the sex chromosome-borne genes of the monitor lizard (**Figure 3C**). We found no indel
176 sequences within the coding regions of sex chromosome borne genes of the other three species,
177 hence did not design primers for validation.

178 The proportion of genes that are specific for one or other sex chromosome, and the
179 proportion that are shared, provide a good indication of the degree of genetic differentiation of
180 the sex chromosome pair, and correlate well with our cytogenetic observations (**Supplementary**
181 **Fig. S1**). The high numbers of genes shared between the X and Y chromosomes of the river
182 turtle suggest that its sex chromosome pair is not highly differentiated, which is consistent with
183 the subtle difference in size and morphology of the X and Y (**Figure 1**). Among the three lizard
184 species, the numbers of shared versus sex chromosome-specific genes also implied different
185 degrees of sex chromosome differentiation. Consistent with the cytogenetic data (**Figure 1**,
186 **Supplementary Fig. S1**)¹⁸, the Z and W chromosomes of the marbled gecko also shared most
187 genes, whereas the monitor lizard showed an intermediate level of shared Z- and W-borne genes.
188 In contrast, the worm lizard had many X-specific but very few Y-specific genes, and only a few
189 X-Y shared genes, implying that the Y chromosome is highly degraded.



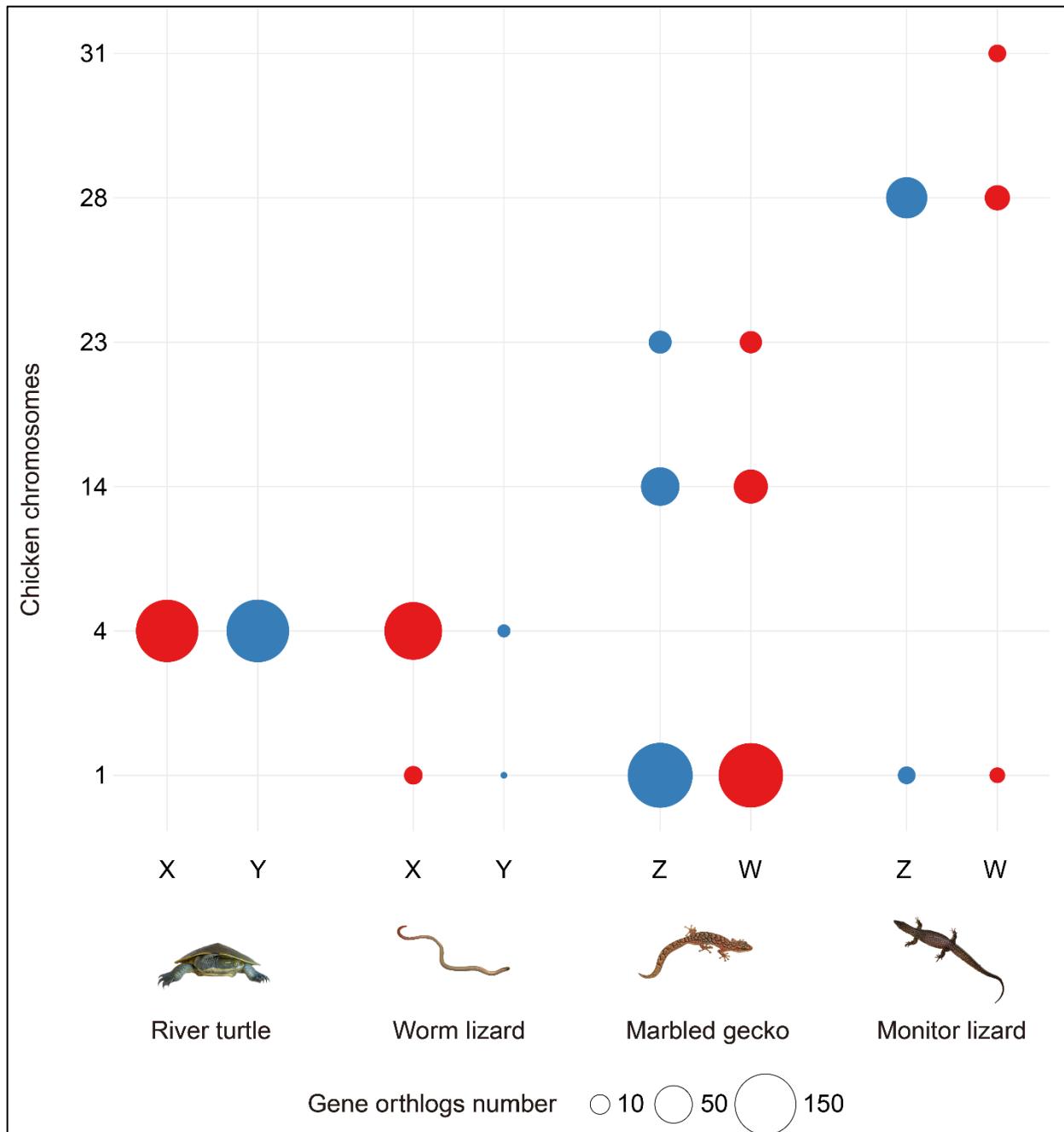
192 A. Pipeline to separately assemble the X-linked (red box), Y-linked (blue box) genes using the
193 sexed transcriptomes (red: female; blue: male). Squares (DNA) and circles (RNA) refer to the
194 reads from different resources. Shared genes, which can be aligned by probes from both sex
195 chromosomes are labelled in purple colour. The autosomal genes, which cannot be aligned by the
196 sex chromosome DNA probes are labelled in green; B. Numbers of sex-linked genes in the four
197 reptile species. X-linked and W-linked genes are labelled in red colour, while Y-linked and Z-
198 linked genes are labelled in blue colour. The overlapping areas refer to the genes shared between

199 the two sex chromosomes; **C.** An example of PCR validation of sex-linked sequences of the
200 monitor lizard. M refers male individual and F refers female individual; outside lane size
201 standard 1kb (left) and 50bp (right) ladder.

202

203 **Origins of sex chromosomes of the four reptile species**

204 By mapping the orthologues of sex-linked genes of the four reptiles to the chicken genome
205 (GGA), we found evidence for both independent origin and convergent evolution of sex
206 chromosomes (**Figure 4, Supplementary Fig. S2**).



215 on a single chicken chromosome, though in three of the species there were other minor clusters.
216 Sex chromosomes of the three lizards were homologous to quite different regions of the chicken
217 genome, on chromosomes GGA1, GGA4 and GGA28 respectively, implying independent
218 origins. However, the sex chromosomes of the river turtle largely overlapped with those of the
219 worm lizard on GGA4q, the long arm of chicken chr4. This is unlikely to represent sex
220 chromosome identity by descent, since the turtles are more closely related to birds (in which this
221 region is autosomal) than they are to squamates, with divergence times of ~250 million years ago
222 (MYA) and 285 MYA respectively²³.

223 Secondary sites of homology between our four reptile species and the chicken genome
224 represent fragments of sex chromosomes with a different evolutionary origin. For instance,
225 homologs of some river turtle sex chromosome-borne genes were found on chicken
226 microchromosome GGA32 (**Supplementary Fig. 2**). This supports the hypothesis that the sex
227 chromosomes of the river turtle originated by a recent translocation between an ancestral sex
228 chromosome pair (GGA4) and a microchromosome pair^{17, 24}.

229 The sex chromosomes of marbled gecko were mainly homologous to GGA1p, with
230 strong secondary signals on GGA14 and GGA23 that contained very similar numbers of Z- and
231 W-borne genes. Genes on the sex chromosomes of the monitor lizard were mainly homologous
232 to GGA28, with strong secondary sites at GGA31, GGA33 and Z that contained similar numbers
233 of sex chromosome-borne genes (**Figure 4, Supplementary Fig. 2**).
234

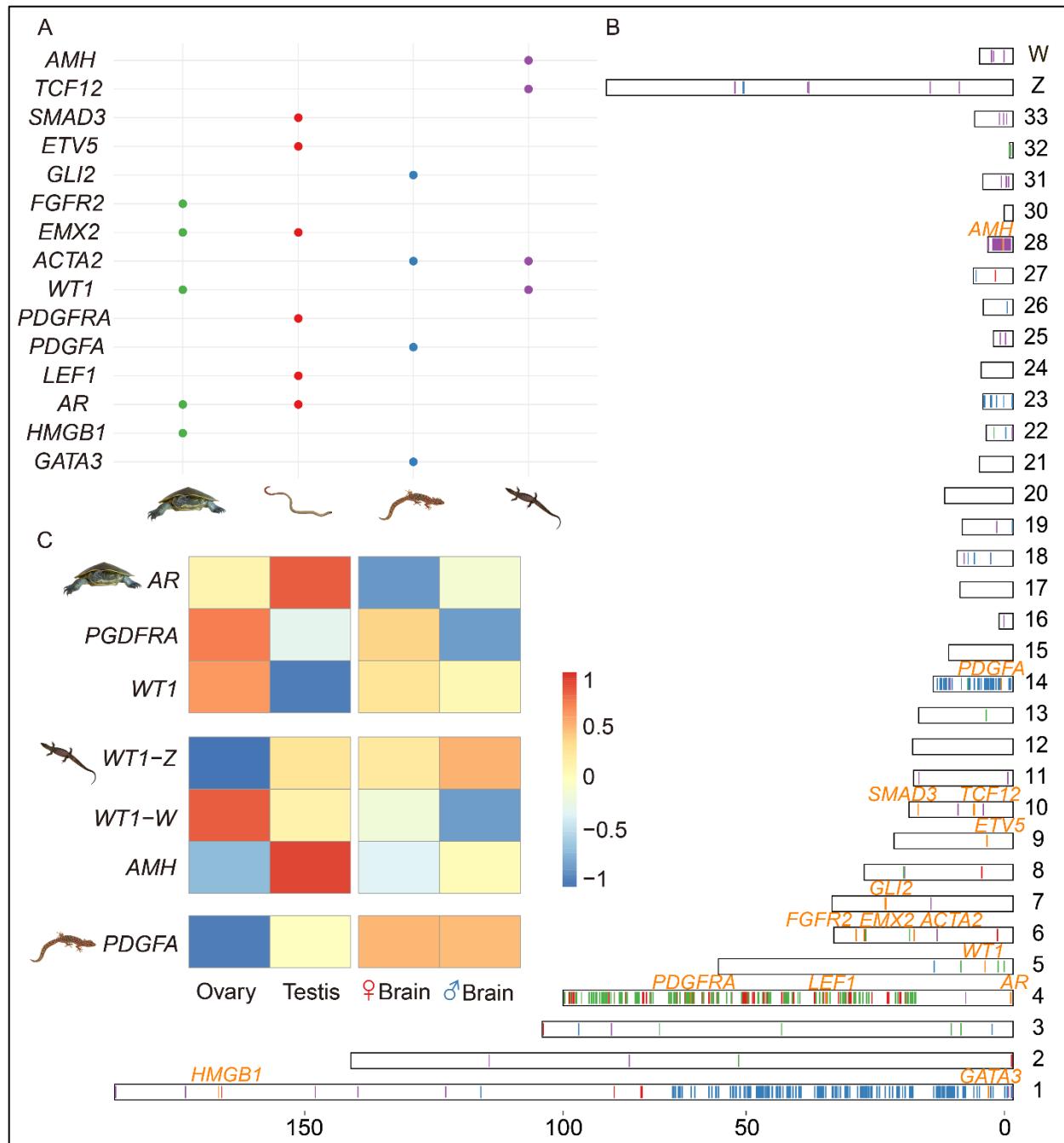
235 **Candidate sex-determining genes of the four reptile species**

236 Novel sex chromosomes may arise when an autosomal gene acquires a sex determining function.
237 Sex chromosome turnovers have occurred many times during reptile evolution^{25, 26, 27}, possibly
238 by a novel sex determining gene usurping the established gene²⁸ (e.g., *sdY* in rainbow trout²⁹, or
239 a change to environmental sex determination and the subsequent evolution of novel genetic
240 systems³⁰. It would not, therefore, be unexpected to find different candidate sex determining
241 genes within the genomic regions that we have identified in the four reptiles (**Figures 1 and 4**).

242 To test this, we compiled a list of genes reported to be involved in the sex-determining
243 pathways of other vertebrates (**Supplementary Table S4, Supplementary Figs. S3-4**) and
244 looked for their orthologs among genes that were either identified as sex-linked or fell within the
245 identified sex-linked region in each studied species (**Figure 5A-B, Supplementary Table S5-7**).

246 Included in the region on GGA4q that overlaps the homologous regions of the river turtle
247 and the worm lizard X chromosomes, was one candidate male-determining gene *pdgfra* (platelet-
248 derived growth factor receptor alpha). Another candidate sex determining gene *AR* (Androgen
249 receptor) located on GGA4p was also annotated as X-borne in these two species. This suggests
250 an independent acquisition of this gene because GGA4p is a microchromosome in all other birds
251 which was fused recently. The *pdgfa* (platelet-derived growth factor alpha polypeptide) gene and
252 its receptor *pdgfra* (platelet-derived growth factor receptor alpha) have been shown to be critical
253 for testis development, particularly Leydig (male steroidogenic) cell development in mammals
254 and turtles ^{31, 32}, whereas *AR* is more likely to be involved in the downstream sexual
255 differentiation process after the gonad sex is determined ^{33, 34}. We confirmed *pdgfra* to be X-
256 borne in the worm lizard using our transcriptome assembly and sex-linked probes from
257 microdissected sex chromosomes. In the river turtle, we could not annotate *pdgfra* as a sex
258 chromosome-borne gene because of a lack of mapped sex-chromosome borne probes, but it was
259 embedded among other sex chromosome-borne genes in, so that is likely to be sex chromosome-
260 borne also in the river turtle (**Figure 5B**).

261 For the marbled gecko, a promising candidate sex-determining gene is the Z-borne *pdgfa*
262 (with a chicken orthologue on GGA14). For the monitor lizard, the most promising candidate
263 sex-determining gene was *amh* (anti Mullerian hormone), which (or the duplicated copy of
264 which) is located on GGA28 and plays a conserved role in testis development in multiple teleost
265 species ²⁸, birds ³⁵, turtles ³⁶ and even the platypus ³⁷. Intriguingly, the ortholog of *wt1* (Wilms
266 Tumour 1), an important regulator of *amh* and master male-determining gene *Sry* in human ³⁸,
267 was determined to be X and Y-borne in the river turtle, and Z and W-borne in the monitor lizard,
268 and was located on a secondary chicken site of GGA5 (**Figure 5B, Supplementary Fig. 2**).



269

270 **Figure 5. Candidate sex-determining genes of the four reptile species.**

271 **A.** The distribution of orthologs of vertebrate sex-determining genes that were also identified as
272 on the sex chromosomes in this study. The coloured dots correspond to such genes within each
273 species, which were identified by blast search against the chicken genome. For figures B, C and
274 D, the river turtle is shown by green dots or bars, the monitor lizard by purple, the worm lizard
275 by red and the marbled gecko by blue. B. Shows the ortholog positions of the sex chromosome-

276 borne genes of these four reptile species on chicken chromosomes, with different colours of lines
277 for different species' orthologs. **C.** Gene expression patterns in the gonad and somatic tissues of
278 candidate sex-determining genes of the three reptile species. We did not show it for the worm
279 lizard due to unavailability of gonad tissues.

280
281 The expression patterns of these candidate sex-determining genes within the three reptiles
282 for which we collected the gonad transcriptomes in this study further supported a function in the
283 sex-determination pathway of each species (**Figure 5C, Supplementary Fig. S5**). The Z-borne
284 *pdgfa* was specifically expressed in the testis of the marbled gecko; whereas its downstream
285 receptor *pdgfra*, which is X-borne in the river turtle, was strongly expressed in the ovary. The X-
286 borne *wtl* of the river turtle, as well as the W-linked *wtl* of the monitor lizard, were both
287 expressed specifically in the ovary. The Z-borne *wtl* and *amh* of the monitor lizard were both
288 specifically expressed in the testis. In summary, turnover of sex determining genes between the
289 studied reptile species probably accounts for their sex chromosome turnovers.

290
291 **Evolution of dosage compensation and sex-linked gene expression in the four reptile species**
292 Having identified the sex chromosome-borne genes of the four distantly related reptiles, we set
293 out to examine their diversity of dosage compensation based on comparison of gene expression
294 levels between sexes, and between the autosomes and the sex chromosomes. Since sex
295 chromosomes may undergo meiotic sex inactivation in germ cells, and gonads are probably not
296 appropriate for direct comparison between sexes³⁹, we focused on comparing the expression
297 levels between sexes in their somatic (brain, tail or blood) tissues.

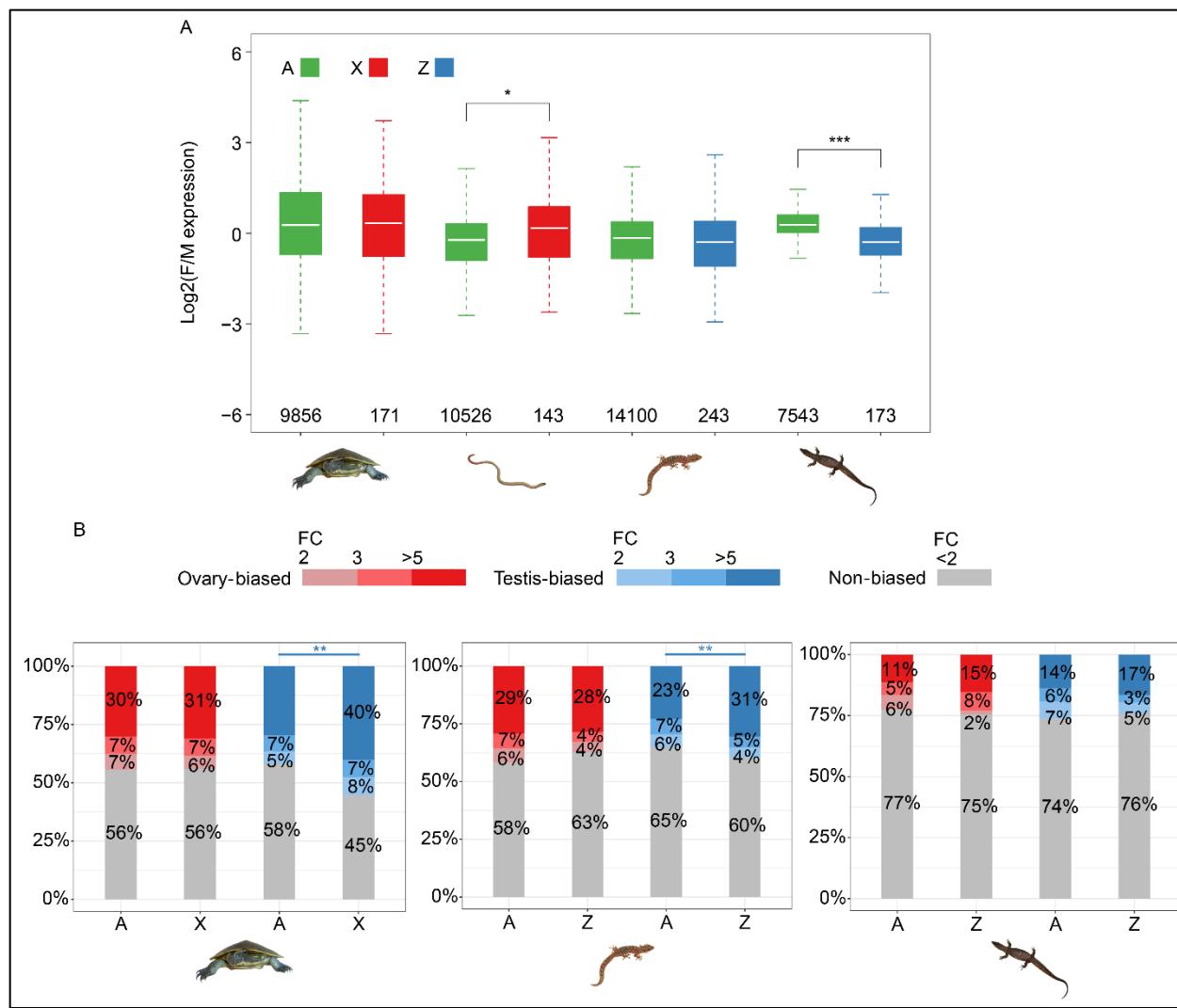
298 Among the four species, the worm lizard (with an XY sex system) and the monitor lizard
299 (with a ZW sex system) have highly or moderately differentiated sex chromosomes. These two
300 species exhibited a significantly ($P<0.05$, Wilcoxon test) different female vs. male expression
301 ratio between autosomes and sex chromosomes (**Figure 6A, Supplementary Fig. S6**). The X-
302 borne genes were more female-biased in the worm lizard, and Z-linked genes were more male-
303 biased in the monitor lizard, indicating incomplete dosage compensation in the two species. In
304 contrast, genes on the undifferentiated sex chromosomes, as well as autosomes, of marbled
305 gecko and river turtle showed no significant difference of their expression ratios between sexes.
306 This was because their Y- or W-linked genes have not degraded yet, so most genes on their X or

307 Z chromosomes still have active partners on the Y (W) and thus there is no dosage difference
308 between the sexes that selects for dosage compensation.

309 For the three species with gonad transcriptomes (river turtle, marbled gecko and monitor
310 lizard), we compared gene expression levels between sex chromosome vs. autosomes in the
311 gonad, with the expectation that gonad-specific genes may have been preferentially selected to
312 be located or not located on the sex chromosomes due to sex chromosomes' sex-biased selective
313 regimes. Previous studies in *Drosophila*⁴⁰ and other dipteran species⁴¹ found
314 underrepresentation of male-biased or testis-biased genes, and overrepresentation of female-
315 biased or ovary-biased genes on the X chromosome, supporting such sex-biased selective
316 regime. We found that testis-biased genes were overrepresented ($P<0.001$, chi-square test), while
317 ovary-biased genes were underrepresented ($P<0.005$, chi-square test) on the Z chromosome of
318 the marbled gecko (**Figure 6B**). However, a similar masculinization and defeminization pattern
319 was not found on the undifferentiated Z chromosome of the monitor lizard, probably because
320 few Z-borne genes were hemizygous (**Figure 4**).

321 The river turtle with undifferentiated XY sex chromosomes, unexpectedly showed a
322 significant enrichment of testis-biased genes on the X chromosome relative to autosomal genes.
323 This was probably because of cross-mapping of the reads of Y-borne genes that were not highly
324 differentiated from those of X-borne genes. When we examined only the hemizygous X-linked
325 genes (those without a Y-linked homolog) there was no such enrichment pattern. This suggests
326 some Y-borne genes of the river turtle have undergone a masculinization process even though
327 they were still retained by the Y chromosome.

328



329

330 **Figure 6. Dosage compensation and sex-linked gene expression in the four reptile species**

331 **A.** Comparisons of the expression levels between sexes in the somatic (brain or tail) tissues of
 332 the four species. Genes from autosomes and the sex chromosomes are labelled in different
 333 colours, (autosomes: green, chrX: red and chrZ:blue). Only genes that have orthologs in chicken
 334 are considered and the respective gene numbers are shown on the X-axis. **B.** The comparisons of
 335 gonad specific gene expression levels between the sex chromosome against autosomes in three
 336 reptile species. Stacked bars shows the proportions of biased genes with more red or blue, the
 337 higher gonad-biased. Only genes with the most gonad-biased (the bluest/reddest part) were used
 338 in the significance testing when evaluating the level of masculinization.

339

340

341 **Discussion**

342 Given the large genomes of many reptile species (up to 5.3Gb), fully sequencing sex
343 chromosomes remains costly, despite the development of long-read sequencing and Hi-C
344 technologies. So far, in depth studies of the gene content and dosage compensation of sex
345 chromosomes have been carried out in a handful of lizards and snake species ^{8, 42, 43, 44, 45, 46}
346 although ZW chromosome have been sequenced and a candidate sex determining gene identified
347 in the central bearded dragon ⁴⁷.

348 Here, we developed a cost-effective method to expand our knowledge of sex-linked
349 genes and sex chromosomes in a range of non-model reptiles and applied it to four distantly
350 related reptile species. We used it to map sex chromosome-borne genes from male and female
351 transcriptomes that were identified by screening with DNA probes from microdissected sex
352 chromosomes. We also applied the novel stLFR linked-read sequencing technology ⁴⁸ and
353 assembled the draft genome of monitor lizard, *V. acanthurus*, including the sex chromosome
354 sequences. The newly identified gene content of the sex chromosomes of these four distantly
355 related reptile species provided new insights into reptile sex chromosome evolution and dosage
356 compensation.

357 Mapping the chicken orthologues of sex chromosome-borne genes of the monitor lizard
358 (*V. acanthurus*), worm lizard (*A. parapulchella*) and marbled gecko (*C. marmoratus*) onto the
359 chicken genome revealed examples of recruitment of different ancestral autosomes. We found
360 that the sex chromosomes of the monitor lizard (*V. acanthurus*), worm lizard (*A. parapulchella*)
361 and marbled gecko (*C. marmoratus*) have homologues on different chicken autosomes. This
362 implies that they evolved from different autosomes in a common reptilian ancestor.

363

364 However, our finding that sex chromosomes of the distantly related pink-tailed worm
365 lizard and river turtle (*E. macquarii*) both have homology to GGA4q provides a striking example
366 of convergent recruitment of ancestral autosome regions. The long arm of the chicken
367 chromosome 4 (GGA4q) has also been previously reported to be recruited as sex chromosomes
368 of pygopodid gecko ⁴⁵. This homology may signify that the same gene (likely to be *pdgfra*) has
369 independently acquired a role in sex determination in all these species. Convergent recruitment
370 of ancestral chromosome is a region orthologous to GGA23, which we identified to be part of the
371 sex chromosomes of marbled gecko, and the central bearded dragon (*Pogona vitticeps*) ⁴⁶.

372 Several general patterns emerged from these comparative analyses of the location of the
373 chicken orthologues of genes on reptile sex chromosomes. Firstly, sex chromosomes seemed to
374 have frequently originated by fusion of ancestral micro- and macro-chromosomes, or between
375 micro-chromosomes ⁴⁹. In addition to homology to the chicken microchromosome GGA28 that
376 was reported, and also confirmed in this work as the ancestral sex chromosome of Anguimorpha
377 species including spiny tailed monitor lizard ^{50, 51}, we found that other chicken
378 microchromosomes GGA31, 33 contained fragments homologous to genes on the sex
379 chromosomes of spiny tailed monitor lizard. Microchromosomes also seemed to have
380 contributed to the sex chromosomes of three other reptiles (**Figures 3 and 4**), as well as in the
381 previously reported green anole lizard ⁵², bearded dragon lizard ⁴⁷, soft-shell turtles ^{53, 54}. The
382 short arm of chicken chromosome 4 (which is homologous to the conserved region of the X
383 chromosome of therian mammals, is a microchromosome in all species other than the
384 Galliformes. These observations of homologies with chicken microchromosomes are not
385 surprising given that half the chicken genes lie on microchromosomes.

386 A microchromosome origin might have contributed to the second feature of reptile sex
387 chromosomes, most of which are less differentiated than those of birds and mammals.
388 Homomorphic or partially differentiated sex chromosomes were found in three out of four
389 reptiles we examined and are also described also in the giant musk turtle ⁵⁵, eyelid geckos ^{55, 56}
390 and some other gecko species ⁵⁷, and skinks ⁵⁸. The preponderance of poorly differentiated sex
391 chromosomes in reptiles could be the result either of slow differentiation, or rapid turnover, or
392 both. A potential cause for the generally slower rate of sex chromosome differentiation in
393 reptiles could be the high recombination rate and gene density of the ancestral
394 microchromosomes ⁵⁹, which might prevent extensive recombination suppression and rapid
395 differentiation between sex chromosomes in these reptiles.

396 Alternatively, rapid turnover of reptile sex chromosomes could explain the “ever young”
397 partially differentiated sex chromosomes that are so common in reptiles. We have previously
398 demonstrated ⁵ rapid transitions between sex determination systems in agamid lizards, and our
399 present results expand the variety and independent origins of reptile sex chromosomes. In
400 addition, the ability to switch into an environmental sex determination mode, and then to evolve
401 novel genetic sex determination systems, may greatly facilitate turnovers. GSD and TSD have
402 been reported within and between closely related reptile species, e.g., in agamid lizards ⁶⁰, in

403 viviparous skink⁶¹, some turtles⁶² and eye-lid geckos⁶³. In the Australian bearded dragon, the
404 transition from GSD to TSD was observed both in the lab and in the field⁶⁴, despite its
405 possession of a pair of highly differentiated sex microchromosomes⁶⁵.

406 Our identification of genes on reptile sex chromosomes enabled us to assess their
407 transcription and assess dosage compensation. We found no evidence of global dosage
408 compensation, even in the worm lizard *A. parapulchella* with highly differentiated X and Y
409 chromosomes. This is similar to the absence of global dosage compensation in birds⁶⁶ and other
410 reptiles⁴⁵, but contrasts with the recently reported case of green anole lizard^{67, 68}, in which the
411 single copy of the X chromosome is upregulated in XY males through an epigenetic mechanism
412 similar to that in *Drosophila*. The absence of global dosage compensation in *A. parapulchella*
413 could reflect dosage mitigation or tolerance at post-transcriptional levels, or it may be a
414 consequence of its dosage-dependent sex-determination mechanism, similar to that in chicken, in
415 contrast to a male-dominant XY system of the green anole.

416 In this work we combined cytogenetics and high-throughput sequencing to characterize
417 the sex chromosomes of four reptile species. This greatly widened our knowledge of sex
418 chromosome birth, death and dosage compensation in a vertebrate class that shows particular
419 variety in modes and turnover of sex determining systems.

420 Thus, we used DNA from microdissected sex chromosomes to identify transcripts of
421 genes located on the XY or ZW chromosome pairs in each species, and located their chicken
422 orthologues on different chicken chromosomes. This revealed the diverse origins of sex
423 chromosomes, but detected convergent evolution between distantly related reptiles (turtle and
424 worm lizard). Our novel pipeline efficiently identified candidate sex determining genes, which
425 differed from those of birds and mammals. We found that none of the four species showed
426 transcription profiles expected of global chromosomal dosage compensation.

427 In summary, our molecular and cytogenetic characterisation of sex chromosomes in
428 diverse taxa greatly expands our knowledge of reptile sex determination. By identifying reptile
429 candidate sex genes and providing the means with which to identify more, we hope to realise the
430 value of this particularly variable, but understudied, vertebrate class, the only one for which no
431 master sex determining gene has yet been discovered.

432 The inexpensive and efficient method developed here can be applied to studying any
433 species of eukaryote with cytologically distinct sex chromosomes, providing the basis with

434 which to better understand the ecological and evolutionary drivers of sex chromosomes and sex
435 determination systems.

436

437 **Materials and Methods**

438 **Chromosome preparations, sex chromosome microdissection, probe preparations and** 439 **FISH analysis**

440 Animal collection, microdissection, preparation of sex chromosome specific probes and
441 validation of probes were described in our previous studies ^{18, 19, 20}. Briefly, we labelled sex
442 chromosome probes by nick translation incorporating SpectrumGreen-dUTP (Abbott, North
443 Chicago, Illinois, USA) or SpectrumOrange-dUTP (Abbott) and precipitated with 20 µg
444 glycogen. After decantation, labeled probe pellets were resuspended in a 15 µl hybridization
445 buffer. The resuspended probe mixture was hybridized with a drop of metaphase chromosome
446 suspension fixed on a glass slide, covered with coverslips, and sealed with rubber cement. The
447 slide was then denatured on a hot plate at 68.5°C for 5 min and was hybridized overnight in a
448 humid chamber at 37°C for two days. The slides were then washed first with 0.4×SSC, 0.3%
449 IGEPAL (Sigma-Aldrich) at 55°C for 2 min followed by 2×SSC, 0.1% IGEPAL for 1 min at
450 room temperature. The slides were dehydrated by ethanol series and air-dried and then mounted
451 with anti-fade medium Vectashield (Vector Laboratories, Burlingame, California, USA)
452 containing 20 µg/ml DAPI (4',6-diamidino-2-phenylindole.).

453

454 **Transcriptome assembly and annotation**

455 RNA-Seq data from gonads and brain tissues for males and females of monitor lizard (*V.*
456 *acanthurus*), river turtle (*E. macquarii*) and marbled gecko (*C. marmoratus*) and tail tissue from
457 a male and female worm lizard (*A. parapulchella*) were used to perform *de novo* assembly of
458 each species with Trinity v2.4.0 pipeline ⁶⁹. Then we used transcoder ⁶⁹ to do ORF prediction
459 and cd-hit (v4.7) ⁷⁰ to remove the redundant sequences with the parameters -c 1.00 -b 5 -T 8. For
460 evaluating the quality of the assembly, we examine the number of transcripts that appear to be
461 full-length or nearly full-length by BLAST+ (v2.6.0) ⁷¹ with the e-value 1e-3. For worm lizard
462 and marbled gecko, the reference species is *G. japonicus* while for river turtle, the reference
463 species is *P. sinensis*, and transcripts with a minimum 30% coverage of reference were selected.
464 We used the Trinotate ⁶⁹ pipeline to annotate the transcriptome. First, we aligned the transcripts

465 to the reference library consisting of human and chicken using blastx and the protein file using
466 blastp with the e-value 1e-3. Also, we used HMMER to do another annotation which aligned the
467 transcripts to the Pfam protein library according to the hidden Markov algorithm with the default
468 parameters. Later, the transcripts and the protein, along with the alignments from blast and
469 HMMER were fed to Trinotate to annotate the transcriptome. The transcriptomes were evaluated
470 by assessing the number of fully reconstructed coding transcripts with their reference species,
471 which are *G. japonicus* for worm lizard and marbled gecko, and *P.sinensis* for river turtle.
472

473 **Genome assembly and annotation**

474 We used SOAPdenovo2 (v2.0.4) pipeline ⁷² to assemble the Illumina DNA reads from
475 microdissected sex chromosomes. In brief, we first tried several times with default parameters, to
476 find the best K-mer with the longest N50. Then, we adjusted the average insertion size according
477 to the best result and re-run the scaffold step. Afterwards, we used kgf(v1.16) with the
478 parameters -m 5 -t 6 and Gapcloser(v1.12) to fill the gaps ⁷², which finally built a de novo draft
479 assembly for sex chromosomes of our species.

480 We constructed the genome assembly of monitor lizard (*V. acanthurus*) with the
481 Supernova v2.1.1 pipeline ⁷³ with the default parameters, which is a package for de novo
482 assembly based on 10X sequencing. Briefly, the approach is to first build an assembly using read
483 kmers (default is 48), then resolve this assembly using read pairs (to K = 200), then use barcodes
484 to effectively resolve this assembly to K ≈ 100,000. The final step pulls apart homologous
485 chromosomes into phase blocks, which create diploid assemblies of large genomes.

486 We annotated the genome of monitor lizard (*V. acanthurus*) with the Braker2 v2.1.5
487 pipeline ⁷⁴ which combined evidence of protein homology, transcriptome and *de novo* prediction.
488 First, we used RepeatMasker (v4.0.7) ⁷⁵ with parameters: -xsmall -species squamata -pa 40 -e
489 ncbi, and the Repbase(v21.01) to annotate the repeat sequences. Then we aligned all available
490 RNA-seq reads to the reference genome by STAR(v2.5) ⁷⁶ to construct transcriptome evidence.
491 Later we fed the masked genome, the alignment of RNA-seq, and the reference protein
492 sequences, which were human and chicken here, to Braker2 with parameter: --prg=exonerate,
493 setting exonerate for protein homology prediction. Finally, the package outputs the GFF file
494 containing the gene models, along with protein sequences and CDS sequences. Additionally, we
495 also separately annotated the sex chromosome of monitor lizard. First, we aligned our sequences

496 to the reference protein sequences using tblastn with parameters: -F F -p tblastn -e 1e-5. The
497 results were then refined by GeneWise (v2.4.1)⁷⁷, and for each candidate gene, we kept the one
498 with the best score. Within these genes, we filtered them if premature stop codons or frameshift
499 mutations reported by GeneWise or single-exon genes with a length shorter than 100bp, or multi-
500 exon genes with a length shorter than 150bp, or if the repeat content of the CDS sequence is
501 larger than 20% exists.

502

503 **Sex-linked sequences identification**

504 For worm lizard (*A. parapulchella*), river turtle (*E. macquarii*) and marbled gecko (*C.*
505 *marmoratus*), using an XY system as an example, we first assembled all the RNA-seq reads into
506 a pooled transcriptome, the female reads into a XX transcriptome, and the male reads into a XY
507 transcriptome. Then male RNA-seq reads were mapped to the XX transcriptome with bowtie2
508 (v2.2.9)⁷⁸ with default parameters. Read depth was then calculated using SAMtools (v1.6)⁷⁹,
509 and those reads unmapped were assembled into a transcriptome which was considered to be X
510 reads excluded.

511 The pooled transcriptomes were directly mapped by Illumina DNA reads from the X and
512 Y chromosomes, and those sequences not mapped by either X or Y reads were assigned as
513 autosomal genes. XX transcriptome and XY transcriptome were both mapped by DNA reads
514 from the X and Y chromosomes, the reads depth (coverage/mappable site) was calculated for
515 each genomic regions mapped, and sequences with a depth higher than 3 and a minimum
516 coverage of 10% with X reads, simultaneously with no alignments with Y reads were assigned as
517 X-linked. For the transcriptome with X reads excluded, the same steps were repeated and
518 sequences with a depth higher than 3 and a minimum coverage of 10% with Y reads and no
519 alignments with X reads were assigned as Y-linked. Afterwards, for sequences with both reads
520 depth (X reads and Y reads) higher than 3, along with a minimum 10% coverage with both X
521 and Y reads, we assigned them as shared genes.

522 To identify the sex-linked sequences in monitor lizard (*V. acanthurus*), Illumina reads
523 from both sexes were aligned to the scaffold sequences using bowtie2 with default parameters.
524 Read depth of each sex was then calculated using SAMtools in 10kb non-overlapping windows
525 and normalized against the median value of depths per single base pair throughout the entire
526 genome for the comparison between sexes. Those sequences with depth ratio of male-vs-female

527 (M/F) ranging from 1.75 to 3, along with a read coverage ratio of male-vs-female higher than 0.8
528 were assigned as Z-linked sequences. For the rest of the sequences, those with M/F ratio of depth
529 and coverage both ranging from 0.0 to 0.25 are assigned as W-linked sequences, and the
530 remaining are assigned as autosomes.

531

532 **Homology comparisons**

533 To find the orthologs of our genes with chicken, we compared the sex-linked transcripts of worm
534 lizard (*A. parapulchella*), river turtle (*E. macquarii*) and marbled gecko (*C. marmoratus*), and
535 the sex-linked genes annotated of the monitor lizard (*V. acanthurus*) 10X assembly to the
536 proteins of chicken (v6, Ensembl), respectively using blastx with the e-value 1e-5. The result
537 was filtered with the aligned AAs > 30% coverage of the reference chicken protein, along with a
538 minimum 50% identity, and returned the one-to-one best hits, with the duplications retained.
539 Then we merged the alignment sites from the four species and calculated the total number of
540 orthologs on the relative chicken chromosomes. With the same protocols, we found the orthologs
541 of our sex-linked genes with chicken sex-determining genes, except for the threshold of identity
542 which were adjusted to 40%.

543

544 **Gene expression analyses**

545 To quantify gene expression, RNA-seq reads were mapped to the transcripts of worm lizard (*A.*
546 *parapulchella*), river turtle (*E. macquarii*) and marbled gecko (*C. marmoratus*) and the CDS of
547 monitor lizard (*V. acanthurus*) by bowtie2. The raw read counts were estimated by RSEM
548 (v1.3.1)⁸⁰, with both TPM expressions calculated. Those genes which have orthologs with
549 chicken were filtered for dosage compensation analysis. Correlation within sexes for each
550 species was tested using a Wilcoxon rank-sum test, where a significant differentiation within
551 samples was found, with a p-value smaller than 0.05.

552

553 Gonadal biased genes were identified by calculating the fold change of gonadal to somatic
554 expressions of the four species. For both ovary and testis, bias genes were classified into 4
555 categories of TPM ratio, namely <2, 2 to 3, 3 to 5, >5. For those genes that have a ratio <2 are
556 assigned as negative, for those >=2, are assigned as gonadal biased, and only those genes with a
557 ratio higher than 5 were calculated in the correlation test for masculinization.

558

559 **INDEL calling and PCR validation of sex specific markers**

560 Using the identified 10.81 Mb Z-borne scaffolds and 7.10 Mb W-borne scaffolds of monitor
561 lizard (*V. acanthurus*), we first identified indels based on their alignment using LASTZ(v1.02)⁸¹
562 with default parameters. On two homologous scaffolds (ChrZ_scaf_189 and Chr_W_scaf_176),
563 we found three W-specific insertions with the lengths as 206 bp, 331 bp and 209 bp
564 (**Supplementary Table S8**). At the flanking regions of these insertions, we designed two PCR
565 primers spanning a predicted length of 1312 bp (assay 1) and 2479 bp (assay 2) sequence
566 fragments for validating the sex chromosome specificity in monitor lizard (*V. acanthurus*). Both
567 primer sets were amplified under the following conditions: initial denaturing at 95°C for 5
568 minutes followed by 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds and an extension
569 step of 72°C for 1 minute, with final extension of 72°C for 10 minutes. These two markers were
570 validated on 60 individuals; 22 females and 38 males from 5 different localities distributed
571 across the species distribution.

572

573 **Data Availability**

574 The genomic and transcriptomic data of worm lizard (*A. parapulchella*), river turtle (*E.*
575 *macquarii*), marbled gecko (*C. marmoratus*) and monitor lizard (*V. acanthurus*) have been
576 deposited in GenBank under the BioProject accession code PRJNA737594. The draft genome
577 and annotation of monitor lizard (*V. acanthurus*) have been deposited in Genome Warehouse
578 (GWH) under the BioProject accession code PRJCA005583.

579

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591

592 **Author contributions**

593 TE, KM, JG conceived the idea. KM, FS, JD conducted lab works. ZZ and QZ conducted
594 bioinformatic analyses. QZ, ZZ and TE wrote the first draft. All co-authors contributed
595 intellectually to writing and editing the draft multiple times.

596

597 **Ethics Statement**

598 Animal care and experimental procedures were performed following the guidelines of the
599 Australian Capital Territory Animal Welfare Act 1992 (Section 40) and conducted under
600 approval of the Committee for Ethics in Animal Experimentation at the University of Canberra
601 (Permit Number: CEAE 11/07 and CEAE 11/12).

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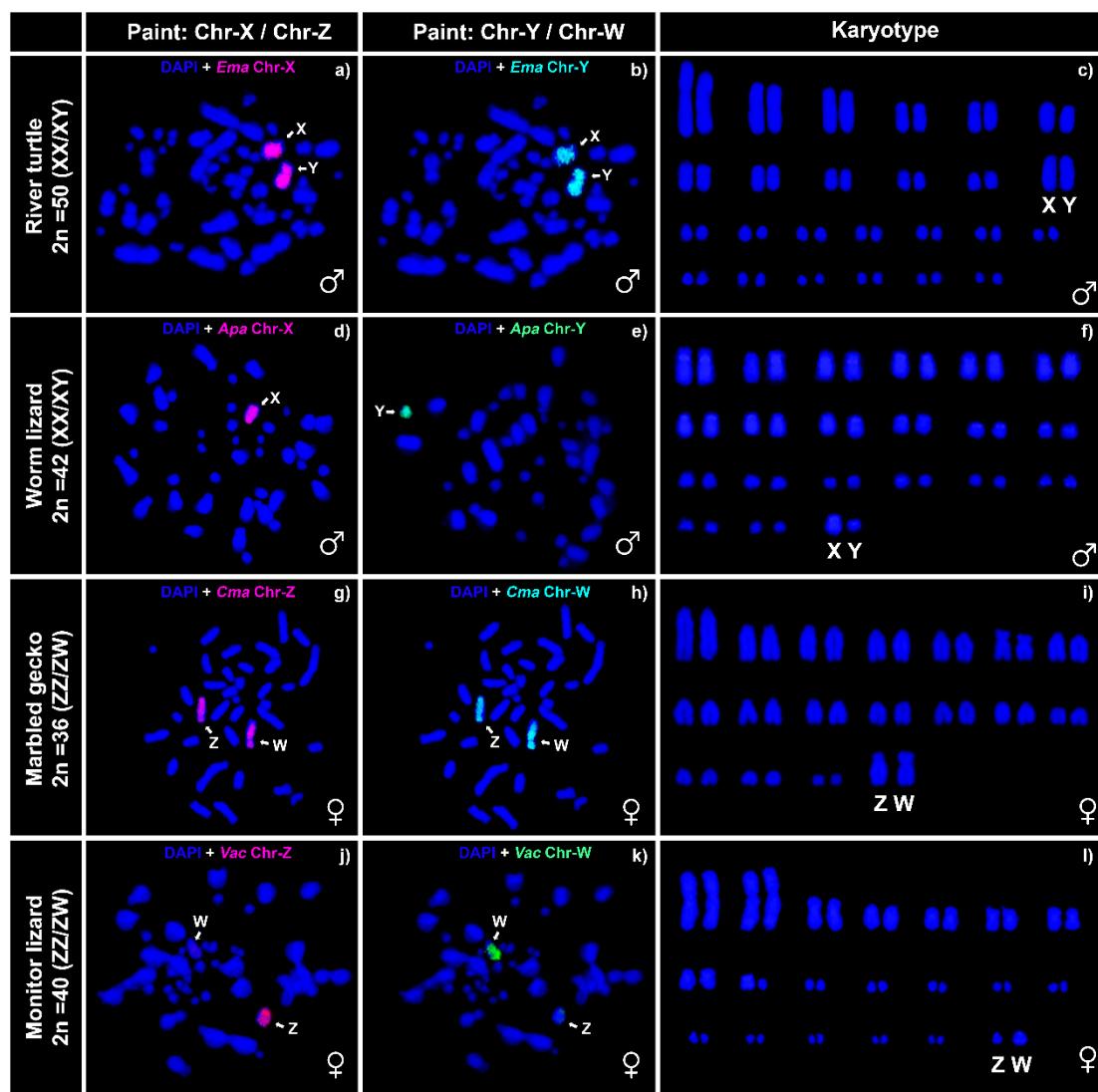
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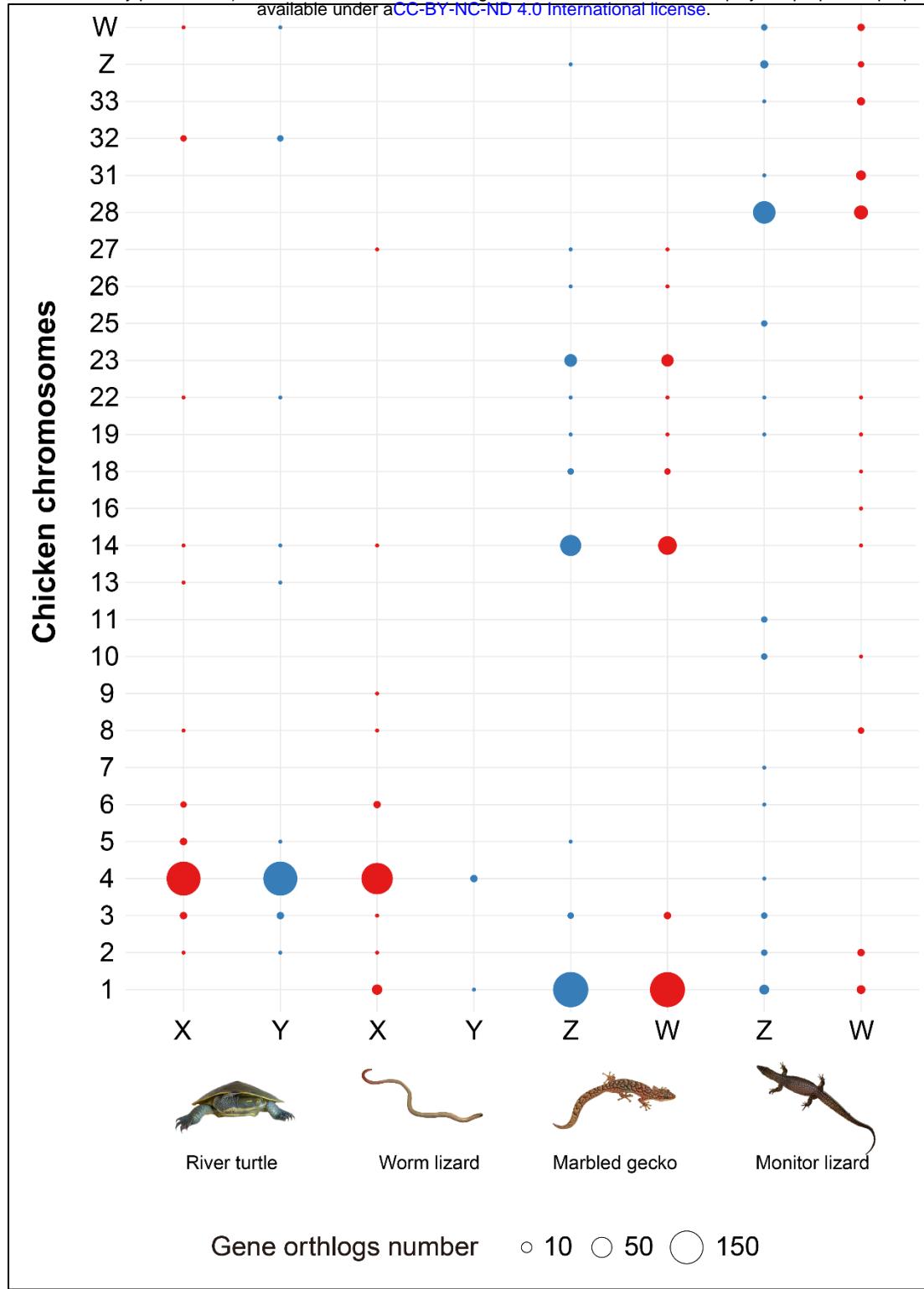
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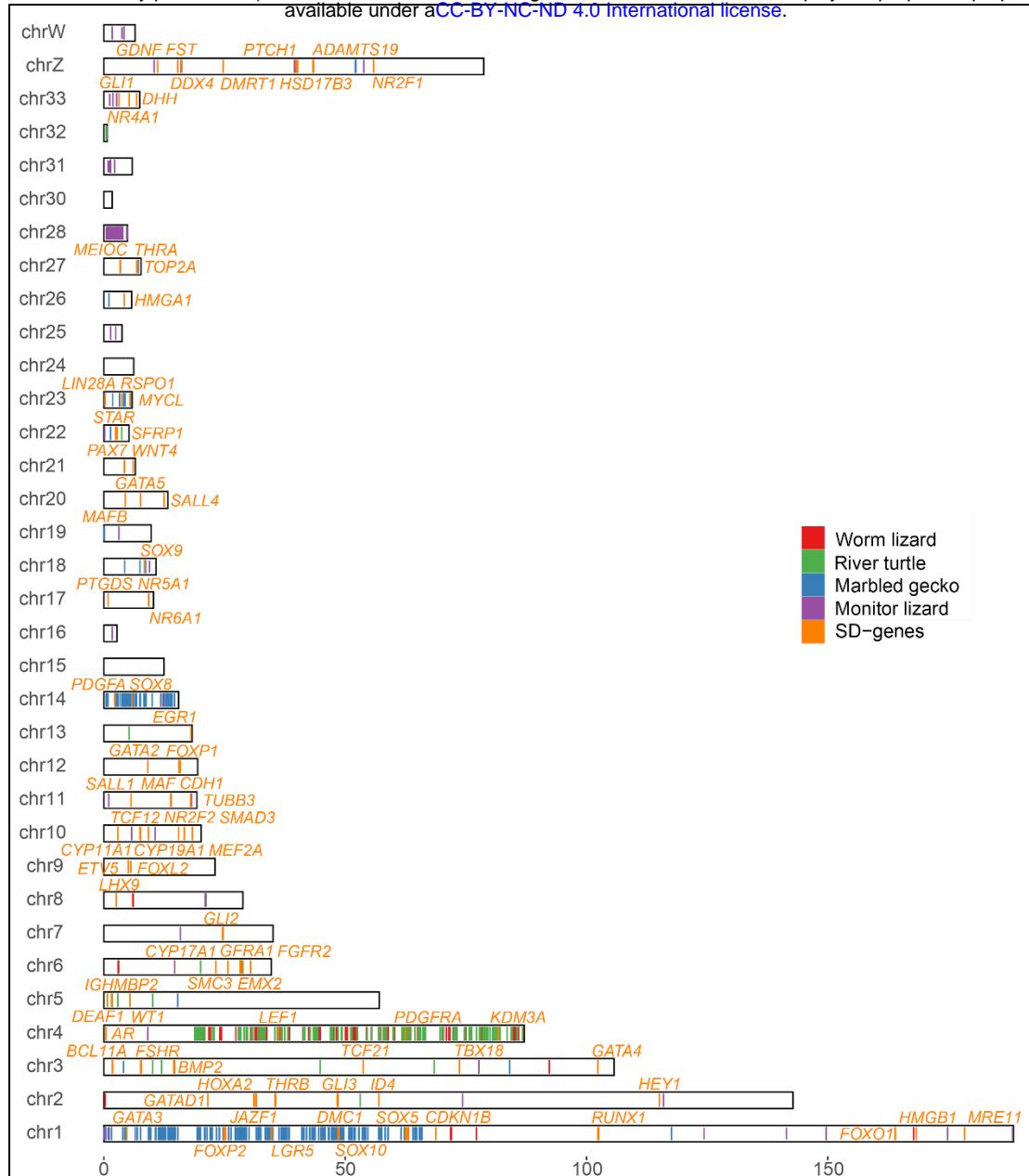
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Supplementary Figures Zhu et al.



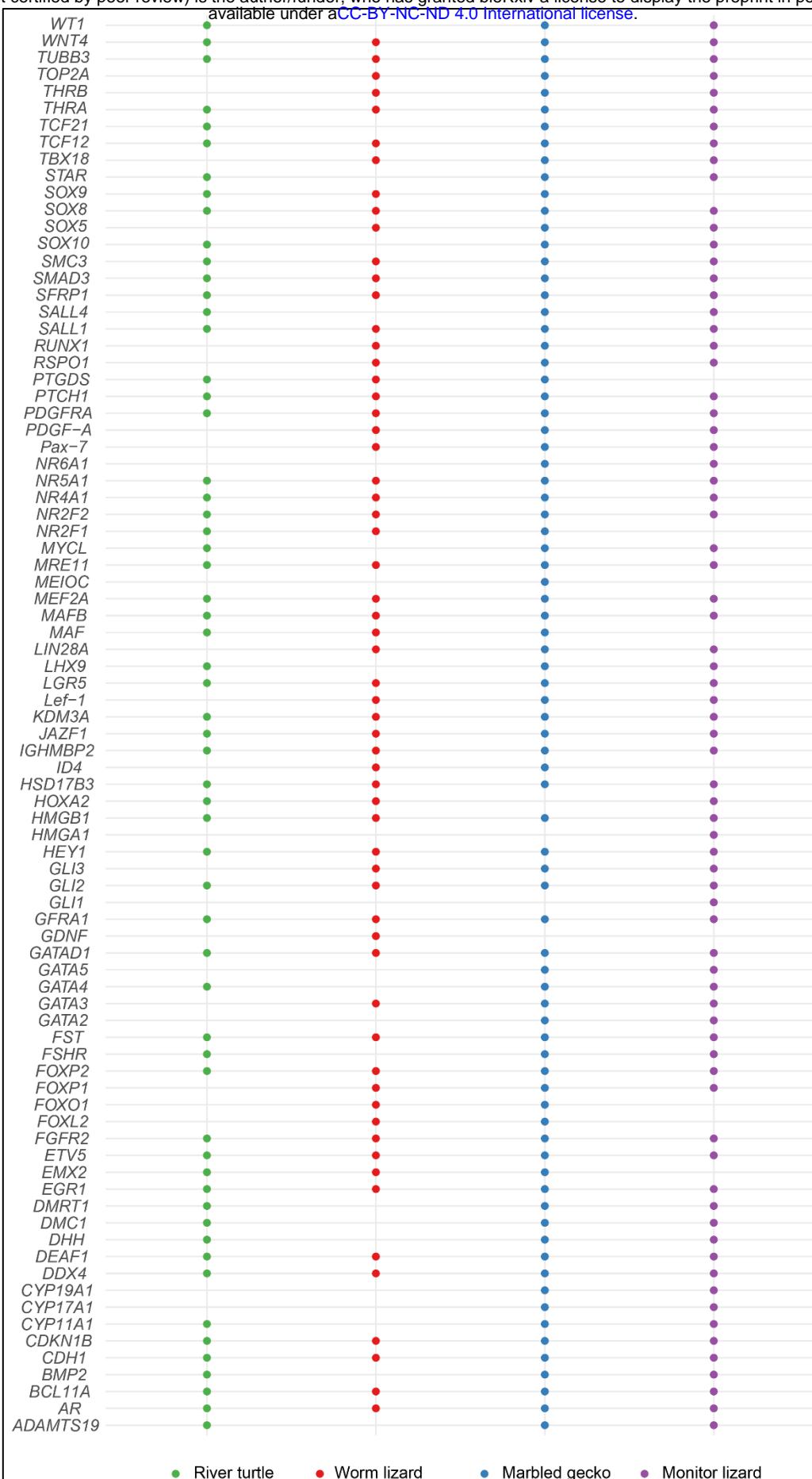
Supplementary Figure 1 | Florescence in situ hybridization (FISH) images of the four reptile species showing validation of microdissected chromosome probes.





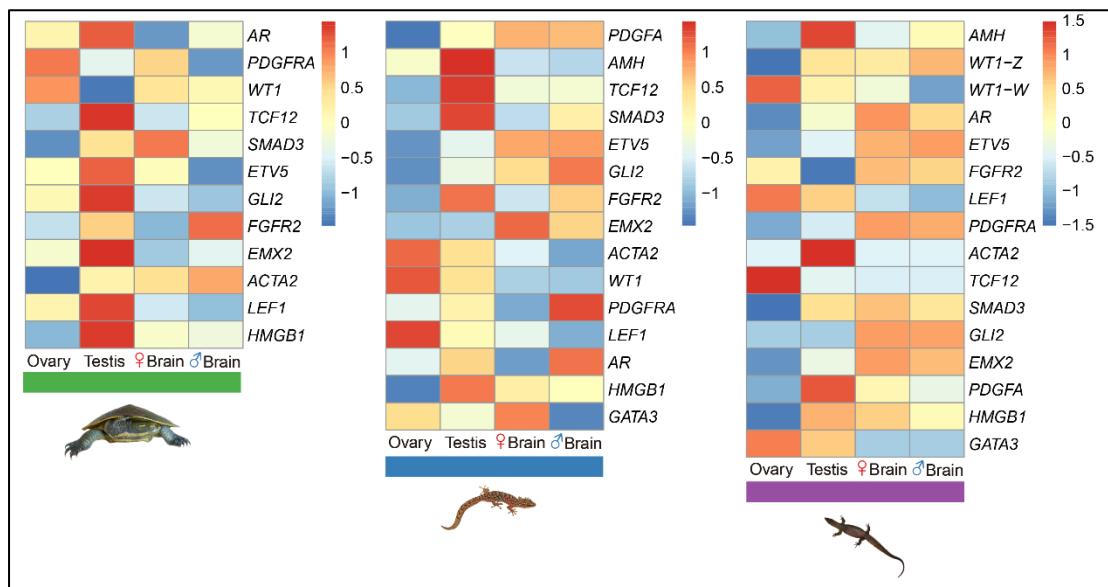
Supplementary Figure 3 | Location of orthologs of sex-determining genes on the homologous chicken chromosomes

We labelled the ortholog of known sex-determining genes in the chicken (Gga6a) genome. And different colors refer to genes of different species, with red to Worm lizard, green to River turtle, blue to Marbled gecko and purple to Monitor lizard. Lines in orange refers to the orthologs of sex-determining genes that have been studied. SD-genes: Candidate Sex determining genes



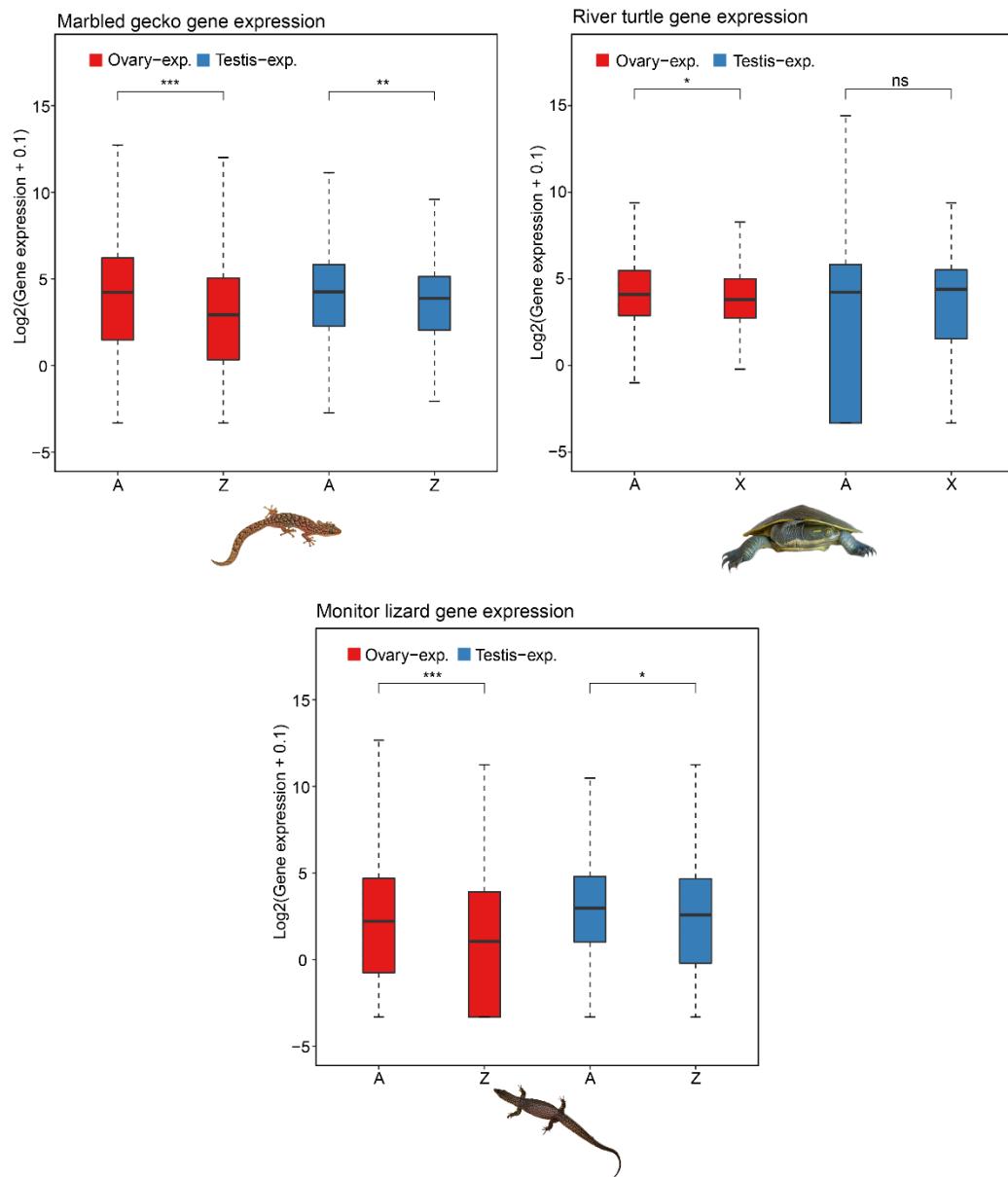
Supplementary Figure 4 | Orthologs of sex-determining genes ag, Ggaba in 4 species.

The figure shows the presence (shown as a dot in the figure, otherwise no dot) of assembled orthologs of known sex-determining genes in the four species. And different colors refer to different species, which are red to Worm lizard, green to River turtle, blue to Marbled gecko and purple to Monitor lizard.



Supplementary Figure 5 | Comparisons of sex-determining genes' expression patterns in different tissues. (Except for APA with only sexed somatic tissue transcriptomes.).

The figure shows the log2 values of expressions (TPM) of assembled orthologs of known sex-determining genes in the three species. And different colors refer to different species, which are green to River turtle, blue to Marbled gecko and purple to Monitor lizard.



Supplementary Figure 6 | Gene expression of River turtle, Marbled gecko and Monitor lizard.
 Each box shows the log₂ values of absolute gene expressions (TPM). A: autosomal genes; Z: Z-linked genes; X: X-linked genes. For XY species, to see masculinization, autosomal genes will generally have a higher testis expression. For ZW species, it is the opposite, and for ovary, it is all opposite.