Dynamics of sex chromosome evolution in a rapid radiation of cichlid fishes

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Abstract

Sex is a fundamental trait that is determined, depending on the species, by different environmental and/or genetic factors, including various types of sex chromosomes. However, while the functioning and evolution of sex chromosomes have been explored in species scattered across the eukaryotic tree of life, little is known about tempo and mode of sex chromosome evolution in closely related species. Here, we examined the dynamics of sex chromosome evolution in an archetypical example of adaptive radiation, the cichlid fishes of African Lake Tanganyika. Through inspection of male and female genomes of 244 cichlid taxa and the analysis of transcriptomes from 66 taxa, we identified the sex chromosomes in 79 taxa, involving 12 different linkage groups. We estimated that Tanganyikan cichlids have the highest rates of sex chromosome turnover and heterogamety transitions known to date. That the recruitments of chromosomes as sex chromosomes is not at random and that some chromosomes have convergently emerged as sex chromosomes in cichlids, provides empirical support for the limited options hypothesis of sex chromosome evolution.

Introduction

Sex chromosomes – referred to as Z and W in female and X and Y in male heterogametic sex determination (SD) systems – define, through their properties and combinations, the sex of an individual[1]. The evolutionary trajectories of sex chromosomes differ from those of autosomes: Due to the restriction of one of the two gametologs to one sex (W to females in ZW, Y to males in XY SD systems), their sex-specific inheritance (e.g., XY-fathers pass on their X exclusively to daughters and their Y to sons), and their reduced levels of recombination, sex chromosomes accumulate mutations more rapidly than autosomes, potentially leading to accelerated functional evolution[2,3].

The functioning of a chromosome as sex chromosome is often short-lived in evolutionary time scales. This relative instability of sex chromosomes is due to turnovers (i.e., changes of the actual chromosome pair in use as sex chromosome) caused by a new sex-determining mutation on a previously autosomal locus[4] or the translocation of the ancestral SD gene to another chromosome (e.g.,[5]). Sex chromosome turnovers may be accompanied by a transition in heterogamety[6]. Heterogamety can also change without transition to another chromosome, which in this case likely involves a turnover of, or a mutation within, the actual SD gene[7].

The assumed major driving forces underlying turnovers of sex chromosomes are deleterious mutational load[8 9], sexually antagonistic loci linked to a newly invading SD gene[10,11], selection on restoring sex-ratios[12] and genetic drift[6,13,14]. These drivers are predicted to differ in their respective outcome: turnovers induced by mutational load tend to preserve heterogamety[8,9], while sexually antagonistic selection driven turnovers more readily induce a change of heterogamety[10].

50 Finally, the gene repertoire on previously existing sex chromosomes can also be
51 extended by chromosomal fusion with an autosome, which then becomes sex-linked itself,
52 leading to the formation of a neo-sex chromosome[15].

53 The frequency of occurrence of these different paths of sex chromosome evolution
54 varies substantially across animal clades[16]. For example, in some vertebrates (mammals and
55 birds) the same sex chromosomes are shared across the entire class[1] (but see[12]). Models[13] as
56 well as empirical observations[15] suggest that sex chromosomes such as those of mammals
57 and (most) birds have differentiated to a degree that makes turnovers unlikely; these sex
58 chromosomes are in an "evolutionary trap"[17]. This is because a sex chromosome turnover
59 requires the fixation of one of the previous sex chromosomes as an autosome, which becomes
60 more deleterious and thus unlikely the more specialised and/or degenerated the sex
61 chromosomes are[18]. In other vertebrate lineages (amphibians, reptiles, and fish), frequent
62 turnover events and continued recombination led to many different and mostly non-
63 degenerated (homomorphic) sex chromosomes (e.g.[19,20]).

64 As of to date, empirical studies on the dynamics of sex chromosome evolution are
65 limited and scattered across different taxa. In an amphibian system with rapid rates of sex
66 chromosome turnover, the true frogs Ranidae, mutational load seems to be the major driving
67 force of sex chromosome turnover[20]. In geckos, a high rate of sex chromosome changes with
68 heterogametic transitions potentially supports sexual antagonism as a key mechanism of these
69 changes[10,11,21]. However, an in-depth analysis of sex chromosome turnovers over short
70 evolutionary timescales and with a broad taxon sampling is currently lacking (reviewed in[16]).

71 Here, we examined sex chromosome evolution in an archetypical example of rapid
72 organismal diversification, the adaptive radiation of cichlid fishes in African Lake
73 Tanganyika[22] (LT). Teleost fishes are generally known for their species richness[23], but
74 cichlids stand out in this clade on the basis of the "explosive" character of several of their

75 adaptive radiations, giving rise to a total estimated number of over 3000 species[23]. Rapid
76 speciation in adaptive radiations is usually attributed to ecological specialisation and thus
77 diversification in eco-morphological traits[24]. Here, we were interested if sex determination is
78 keeping pace with other traits in cichlids by determining the diversity of SD systems and by
79 investigating the dynamics of sex chromosome turnover across the entire LT cichlid
80 radiation. The available data from about 30 African cichlid species (reviewed in [25,26]) suggest
81 that sex chromosomes are not conserved in this group with both simple and polygenic sex
82 determination systems being known from the different species investigated. An emerging
83 picture is that certain chromosomes have recurrently been recruited as sex chromosomes in
84 cichlids. However, available studies supporting the convergent recruitment of sex
85 chromosomes have been based on cichlid species belonging to different lineages and the
86 observed patterns have rarely been assessed in a phylogenetic framework, which makes
87 inferences about rates of evolution as well as of convergence *versus* common ancestry
88 difficult (but see[25]). Importantly, as of yet, no inclusive analysis of sex chromosome
89 evolution exists for a cichlid adaptive radiation (nor for radiations in other fish families).

90 In this study, we inspected genomic and transcriptomic information from 229 Lake
91 Tanganyika cichlid taxa as well as 18 cichlid species belonging to the Haplochromini and
92 Lamprologini lineages, phylogenetically nested within the LT radiation[22,24,27] for signatures
93 of sex chromosomes. Based on this nearly complete taxon sampling of the LT radiation and
94 an available genome-wide phylogenetic hypothesis[24], we estimated the amount and direction
95 of sex chromosome turnovers in a young species flock. This allowed us to test for a possible
96 contribution of sexual antagonism in the evolution of sex chromosomes in LT cichlids.
97 Sexual antagonism has been suggested as a driving force of sex chromosome turnovers in
98 sexually dimorphic cichlids of the Lake Malawi radiation[28,29]. However, unlike the cichlid
99 adaptive radiation in Lake Malawi, which is composed solely of cichlids of the

100 Haplochromini lineage, the endemic LT cichlid assemblage consists of 16 cichlid lineages
101 (corresponding to the taxonomic assignment into tribes)[22], some of which are sexually
102 dimorphic while others are not.

103 To assess the dynamics of sex chromosome turnover in fish on a larger scale, we
104 expanded our comparative analyses to other fish systems as well. In particular, we
105 investigated sex chromosome turnovers in ricefishes, another model system for the evolution
106 of sex chromosomes[30].

107 Sex differences in the recombination rate could contribute to the differentiation of sex
108 chromosomes[31]. Unlike in the extremely heterochiasmic frogs of the family Ranidae[20] and
109 some fish model organisms[32,33], recombination rate along chromosomes do not systematically
110 nor drastically differ between the sexes in cichlids[33,34]. In ricefishes, reduced rates of
111 recombination have been linked to maleness in some species[35] but this does not seem to be a
112 general pattern in this group of fishes[36]. Ricefishes and cichlids may hence have differing,
113 probably lower, rates of sex chromosome degeneration than the heterochiasmic frogs, in
114 which mutational load resulting from sex chromosome degeneration caused by suppressed
115 recombination mainly drives turnover. In general, we expect fewer – if any – cichlid species
116 to be in the "evolutionary trap" of degenerated sex chromosomes and more sex chromosome
117 turnovers caused by sexual antagonism than mutational load[16]. Finally, with the identification
118 of sex chromosomes in genetically very closely related species we pave the way for the
119 subsequent identification of sex-determining genes and/or the causal mutations leading to sex
120 chromosome turnover.

121 # Results

122 **Sex chromosomes in LT cichlids**

123 To identify sex chromosomes in LT cichlids, we screened male and female genomes of 244
124 taxa[24] as well as six transcriptomes of 66 taxa[27] for sex-linked regions, applying three
125 complementary approaches: genome-wide association study (GWAS on the genomic data,
126 approach 1, see Methods), identification of sex-specific SNPs in the genomic data (approach
127 2) and allele frequency difference tests on the transcriptome data (approach 3). Genomic
128 locations of sex chromosomes refer to linkage groups (LGs) of the used reference genome of
129 a phylogenetically equidistant outgroup to the cichlid species of the LT radiation, the Nile
130 tilapia (*Oreochromis niloticus*). To estimate sex chromosome turnovers (see below), we used
131 two different datasets, a "permissive dataset" including all sex chromosomes identified with
132 approaches 1-3 and a "stringent dataset" excluding sex chromosomes that had support only in
133 approach 2, i.e. lacking transcriptome data or support for small sex-linked and potentially
134 non-expressed regions in the transcriptome data and occurring in tribes too small to be
135 investigated with approach 1.

136 By combing the result of approaches 1-3, we detected sex chromosomes in 78
137 endemic LT cichlid taxa as well as in the riverine Haplochromini *Orthochromis indermauri*
138 (Figs. 1 and 2; Supplementary Tables 1-4, Supplementary Figs. 1-4).

139 Approach 1 (GWAS), which was applied to the larger (that is, more species-rich)
140 cichlid tribes from LT only, revealed the presence of sex chromosomes shared among several
141 species of their respective tribes. We thus identified an XY SD system on LG19 in
142 Haplochromini/Tropheini, (thereby confirming an XY system previously known from one
143 species in this clade, *Tropheus* sp. "black"[37]), an XY and ZW system on LG05 in
144 Cyprichromini (confirming a ZW system previously described in *Cyprichromis leptosoma*[37]),
145 and an XY system on LG15 and LG20 in Lamprologini. Surprisingly, with this approach, we

146 did not detect a shared sex chromosome within the second-most species-rich cichlid tribe of
147 LT, Ectodini.

148 Approach 2, the inspection of the genomes within tribes for an accumulation of sex-
149 specific SNPs (i.e., XY or ZW SNPs) and outlier regions thereof revealed two XY systems
150 on LG19 within Haplochromini/Tropheini, one covering the first ~22 Mb of LG19 (in the
151 genus *Tropheus* and in *O. indermauri*) and a second one located at the end of LG19 co-
152 occurring with XY SNPs at the beginning of LG05 (in the second Tropheini clade grouping
153 all genera but *Tropheus*, that is, 15 species belonging to the genera *Pseudosimochromis*,
154 *Petrochromis,* and *Interochromis,* Fig. 1, Supplementary Fig. 4). We also recovered the
155 narrow sex-linked region on LG20 detected with GWAS in Lamprologini, corroborating the
156 effectiveness of this approach.

157 As in approach 1, we did not detect a sex-differentiated region shared across species
158 in Ectodini with approach 2.

159 When applied to the smaller tribes, approach 2 revealed rather narrow but clear outlier
160 regions that were shared between subsets of species in Benthochromini (XY LG10, two
161 species), Trematocarini (ZW LG04, two species) and Cyphotilapiini (XY LG16, two species)
162 as well as in all members of the Eretmodini (XY, LG07 and LG10). We also detected a less
163 pronounced and smaller ZW-outlier region on LG09 in the same two Cyphotilapiini species,
164 a pattern potentially explained by variation in X-linked markers across the different species
165 while simultaneously lacking sites on the Y (hemizygosity in males). Due to this uncertainty
166 and the stronger signal on LG16, we excluded the ZW LG09 system of Cyphotilapiini in the
167 subsequent analysis (in the permissive and stringent datasets). Within Bathybatini and
168 Perissodini, we identified a chromosome-wide increase of ZW SNPs on LG07 and of XY
169 SNPs on LG19, respectively, which however failed our thresholds for the permissive dataset
170 (see Methods). Upon inspection of XY-ZW differences per species within these tribes

171 (Supplementary Fig. 5), this pattern turned out to be caused by only one species in each tribe
172 (*Hemibates stenosoma* and *Plecodus paradoxus*), which both showed signs of a differentiated
173 sex chromosome across the entire length of the respective LG. Note that a ZW system on
174 LG07 has previously been described in *H. stenosoma*[37] and that we could also confirm the
175 XY system spanning the full length of LG19 with approach 3 (see below) in *P. paradoxus* as
176 well as in another Perissodini species, *Plecodus straeleni*, for which we did not have whole
177 genome data of both sexes. We hence included the sex chromosomes of these species in all
178 the down-stream analyses.

179 Approach 3, the species-specific investigations of sex-specific SNPs based on
180 replicate transcriptome data, confirmed all sex-differentiated regions that spanned larger
181 chromosomal regions (i.e., the XY systems on LG19 and LG05/LG19 in
182 Haplochromini/Tropheini, the XY and ZW systems on LG05 in Cyprichromini, the XY
183 system in Eretmodini). With this approach we also detected a ZW system on LG15 in two
184 Ectodini species (*Xenotilapia boulengeri* and *Enantiopus melanogenys*). Approach 3 further
185 permitted us to identify sex-linked LGs unique to eight additional species and not shared with
186 their respective sister species. For example, we detected an XY system on LG23 in the
187 Ectodini *Callochromis pleurospilus,* and a ZW system on LG20 in the Benthochromini
188 *Benthochromis horii* (Fig. 1, Supplementary Table 2). In another four species, the RNA data
189 showed a significant overrepresentation of either XY or ZW-SNP windows that could not be
190 placed unambiguously on reference LGs (Fig. 1, Supplementary Table 2).

191 Overall, in nine of the 13 investigated tribes of the cichlid radiation in LT, several
192 species shared the same SD system (chromosomal region and heterogametic type); however,
193 we did not find a shared sex chromosome across members of different tribes.

194 We detected sex linkage on 12 out of the 23 reference LGs (Fig. 2). Eight of these
195 reference LGs were sex-linked in species belonging to different tribes (Fig. 2a). Two

196 reference LGs (LG14 and LG18) that we did not identify as sex chromosomes within any of
197 the LT cichlid species, have respectively been identified as sex chromosomes in labstrains
198 and one natural population of the haplochromine cichlid *Astatotilapia burtoni* (occurring in
199 LT and affluent rivers)[38,39]. In addition to the published data for *A. burtoni*, we also included
200 the XY LG07 sex chromosome of *Pseudocrenilabrus philander* (Lake Chila)[25] in our
201 subsequent analyses, a haplochromine species integrated in the phylogenetic reconstruction
202 used here (Fig. 1) but represented with a single individual in the WGS dataset.

203 In 62 of the LT cichlids (79.5% of the LT species with a sex chromosomal signal), the
204 sex linkage was compatible with an XY system (Fig. 2b).

205

206 **Sex chromosome evolution in LT cichlids**

207 Next, to determine when particular sex chromosomes emerged and to trace heterogamety
208 transitions in the course of the cichlid adaptive radiation in LT, we performed ancestral state
209 reconstructions along a time-calibrated species tree[24]. We performed these analyses on the
210 permissive as well as on the stringent dataset.

211 We reconstructed 30 sex chromosome turnovers in the radiation and LG04 as the
212 likely sex chromosome at its root (permissive dataset; 27 turnover events with the stringent
213 dataset), translating into an estimated rate of 0.186 turnovers per Myr (Fig. 1, Supplementary
214 Fig. 7, permissive dataset; turnover rate stringent dataset 0.187). On average, we therefore
215 expect one sex chromosome turnover event between two species that diverged ~2.7 Ma. This
216 rate-estimate was ten times higher than the one that we calculated for ricefishes
217 (Adrianichthyidae; 0.02 transitions per Myr; Supplementary Fig. 9; 19 species investigated,
218 see Methods).

219 The distribution of sex chromosomes in LT cichlids differed from random
220 expectations (Fig. 2d). There was no correlation between the size of a reference LG, the

221 number of genes on a reference LG, or the number of known sex-candidate genes on a
222 reference LG and the frequency at which these LGs appeared as sex chromosome in LT
223 cichlids (Fig. 2d). Our findings thus corroborate that SD is a rapidly and non-randomly
224 evolving trait in cichlids. We further found that the number of turnovers in a tribe is
225 correlated with its species richness (Fig. 2c, pGLS: P=0.0043, coeff=0.039), suggesting that
226 the turnover rate has been relatively constant throughout the radiation.

227 Our heterogamety reconstructions further suggested that XY is the most likely
228 ancestral state in the cichlid adaptive radiation in LT (Supplementary Fig. 8). Subsequently,
229 11 transitions occurred from XY to ZW (permissive dataset; 11 towards ZW and one towards
230 XY in the stringent dataset). Heterogamety transitions are predicted to have a directional bias
231 towards new dominant sex chromosomes[13], suggesting that in cichlids from LT – just like in
232 cichlids from Lake Malawi[28,29] – the new W chromosomes are dominant over ancestral Ys.

233 When integrating over the reconstructed transitions in heterogamety and sex
234 chromosomes, we found heterogamety changes that were uncoupled from turnovers in LGs
235 and that were hence not captured in our rate estimate of sex chromosome turnover: A
236 transitions from XY to ZW was detected on LG05 in Cyprichromini and on LG04 in
237 Trematocarini and in Bathybatini (*H. stenosoma*) (Fig. 1; Supplementary Figs. 7-8).

238 The overlap of heterogametic and sex chromosome turnovers also showed that the
239 majority (23 *versus* seven) of the observed sex chromosome turnovers in LT cichlids
240 preserved the heterogametic state, suggesting that mutational load, predicted to preserve
241 heterogametic state[20], might be a major driver of sex chromosome turnover in cichlids as
242 well. The transitions with a change in heterogamety offer the possibility to investigate the
243 actual potential of sexual antagonistic selection between very young species (the divergence
244 time between e.g. *Paracyprichromis* and *Cyprichromis* between which a turnover has
245 occurred is ~3.8 Ma). The heterogametic status of the four species for which we could not

246 identify the sex-linked LG (see above) lead to additional heterogamety transitions not
247 captured in the sex chromosome turnover rate.

248 Overall, the heterogamety transition rate in LT cichlids (0.028 transitions per Myr
249 permissive dataset; 0.031 per Myr stringent dataset) was about four times higher than in
250 ricefishes (0.007 transitions per Myr; ancestral state: ZW). To explore heterogamety changes
251 on a greater taxonomic scale, we also calculated heterogamety transition rates for all ray-
252 finned fishes available in the Tree of Sex database (http://www.treeofsex.org/) that were
253 included in a recent comprehensive phylogeny[40] (543 species analysed in total). Our analysis
254 revealed a rate of 0.009 transitions per Myr for ray-finned fishes as a whole and identified
255 XY as the ancestral state (Supplementary Table 6; Supplementary Fig. 9). Across the ray-
256 finned fish phylogeny, transitions from XY to ZW were significantly younger than those
257 from ZW to XY (Supplementary Fig. 9b, P=0.01428).

258

259 **Chromosome fusions and novel sex chromosomes**

260 Novel sex chromosomes can be created by chromosome fusions[41], which can contribute to
261 reproductive isolation and eventually drive speciation over miss-segregation at meiosis,
262 changes in recombination rates, novel physical combinations of loci, and changes in gene
263 expression[42,43,44]. The here identified signatures of sex linkage suggest that several sex-
264 chromosome/autosome fusions have occurred in the course of the cichlid radiation in LT or
265 that autosome/autosome fusions occurred prior to the recruitment of these fused autosomes as
266 sex chromosome (Fig. 1). The distribution of sex-differentiated genomic regions indicated a
267 fusion (or large chromosomal translocations) between LG05 and LG19 in
268 Haplochromini/Tropheini and between LG15 and LG20 in Lamprologini (Fig. 1,
269 Supplementary Fig. 1). There was also some support for the previously described genome
270 rearrangements in the tribe Eretmodini[45], which showed an increase of XY SNPs on several

271 LGs (Fig. 1, Supplementary Fig. 3). Additional sex-differentiated regions point to species-
272 specific fusion events (e.g. LG11 and LG15 in *Gnathochromis pfefferi,* LG05 and LG13 in *C.*
273 *leptosoma*). Our analyses also confirmed the reported sex linkage of LG04 as well as of
274 LG07 in *H. stenosoma*[37] (Supplementary Fig. 5). Chromosome fusions have previously been
275 implicated with the evolution of novel sex chromosomes in other taxa, as well as in the
276 riverine haplochromine cichlid *A. burtoni*[38,39].

277 Interestingly, the so far only karyotypically investigated member of the tribe
278 Tropheini, *Ctenochromis horei*, has a reduced number of chromosomes in a male and an
279 unsexed individual (2n=40) compared to other Haplochromini, which usually feature
280 2n=42[45]. We did not detect the LG05/LG19 XY system found in many other Tropheini in *C.*
281 *horei*. Hence, while the karyotype of this species indeed supports chromosomal fusions in the
282 Haplochromini/Tropheini, this data cannot help to resolve when and how these events
283 occurred. The data at hand are sparse but it might be that several large chromosomal
284 rearrangements occurred before the novel chromosomes were recruited as sex chromosomes,
285 making inferences of the driving forces of these fusions difficult.

286

287 **Convergent evolution of sex chromosomes**

288 On some LGs, the regions that showed sex linkage largely overlapped between members of
289 different tribes (Supplementary Fig. 10), which can either be explained by common ancestry
290 or by the independent (convergent) recruitment of those LGs as sex chromosome. In
291 particular on LG19, several closely related species including six *Tropheus* species
292 (Haplochromini/Tropheini), the riverine haplochromine *Orthochromis indermauri*, and *P.*
293 *paradoxus,* and *P. straeleni* (Perissodini) feature an XY system in the same chromosomal
294 region (Supplementary Fig. 10). Our ancestral state reconstruction suggested an independent
295 origin of the LG19 SD system in Perissodini and *Tropheus*, in each case early in their tribe's

296 evolutionary history, and another independent origin in the terminal branch leading to *O.*
297 *indermauri* (Supplementary Fig. 7). Phylogenetic inference from Y- and X-haplotypes indeed
298 supported the independent evolution of LG19 as XY sex chromosome in Perissodini (Fig. 3),
299 while grouping together the Y-haplotypes of the *Tropheus* species and *O. indermauri*. This
300 suggests common ancestry of the XY system in the two haplochromine clades with an origin
301 either early on in haplochromines (implying several losses later in the evolution of this tribe;
302 likely because of this, such a scenario was not supported by ancestral state reconstruction) or
303 a later origin and inheritance of the sex chromosomal system in *Tropheus* and *O. indermauri*
304 from an extinct or unsampled taxon.

305 The XY sex chromosome system on LG05/LG19 found in the second clade of
306 Haplochromini/Tropheini (grouping all genera except *Tropheus*) must be derived from
307 another independent evolutionary event, since the regions on LG19 that show XY alleles in
308 the two Haplochromini/Tropheini clades are not overlapping (Supplementary Fig. 10) and
309 also do not group together in the phylogenetic tree of LG19 (Fig. 3). Other convergent cases
310 of sex chromosome recruitment supported by our ancestral state reconstruction involved
311 LG05 (in Cyprichromini and the haplochromine *A. burtoni*[38,39]) and LG07. LG07 has
312 independently been recruited as a sex chromosome in *H. stenosoma* (Bathybatini)[37], in
313 Eretmodini, in the lamprologine *Neolamprologus cylindricus* (Fig. 1 , Supplementary Fig. 7),
314 in several Lake Malawi cichlids[28,29] (Haplochromini), as well as in *P. philander*[25]
315 (Haplochromini), making it the most widespread sex-linked LG known in cichlids to date.

316

317 **Sex chromosome differentiation**

318 A comparison of the proportion of sex-specific sites on the different sex-linked LGs revealed
319 a continuum of sex chromosome differentiation in the cichlid adaptive radiation in LT (Fig.
320 4, Supplementary Fig. 10), ranging from a few kb (LG20 in Lamprologini) to almost full

321 chromosomal length (LG05 in Cyprichromini, LG19 in *Tropheus* and Perissodini). Varying
322 lengths of sex-differentiated regions were even detected within the same LG when being used
323 as sex chromosome by different lineages (e.g., the sex-differentiated region on LG05 spans
324 only 8 Mb in Tropheini versus the entire LG in Cyprichromini).

325 The canonical model of sex chromosome evolution predicts progressing
326 differentiation of sex chromosomes with time[2]. Contrastingly, we found no correlation
327 between the estimated age of origin of a sex chromosome and its degree of differentiation
328 (Fig. 4, pGLS: P=0.9049, coeff=0.0011). Some very young sex chromosomes showed signs
329 of differentiation, i.e., sex-specific sites, along almost the full length of an LG, suggesting
330 wide-spread suppression of recombination along their sex chromosomes.

331 The amount of sex-specific sequences in cichlids of the LT radiation inferred from a
332 "subtraction pipeline" (approach 4) of expressed male and female sequences[46] was somewhat
333 higher in XY than in ZW systems (P=0.058), but not when accounting for phylogenetic
334 signal (phylogenetic ANOVA: P=0.2, Supplementary Fig. 11a). The observed pattern could
335 suggest that Y-chromosomal genes are more highly expressed in adult (gonadal) tissues of
336 closely related species than W-chromosomal genes. The difference in the amount of sex-
337 specific contigs between XY and ZW systems does not reflect a difference in the degree of
338 differentiation between the two heterogamety types (Supplementary Fig. 11b, phylogenetic
339 ANOVA: P=1).

340

341 **Candidate genes of sex determination in LT cichlids**

342 Our inspection of known genes implicated in SD revealed that such genes were located on all
343 LGs, including those, for which no sex linkage was detected, with no particular
344 overrepresentation on certain LGs (Supplementary Fig. 12). The regions with the strongest
345 signal for being sex-differentiated did not contain any of these genes (Supplementary Table

346 2). However, through the inspections of the regions with the strongest signs of sex linkage we
347 identified promising new candidate genes for SD in these regions, such as *tox2* in
348 Lamprologini, a HMG-box transcription factor involved in the hypothalamo-pituitary-
349 gonadal system. *Tox2* resembles the mammalian master SD gene *Sry*[47], which also codes for
350 an HMG-box protein.

351 In cichlids from lakes Malawi[28] and Victoria[48], sexually antagonistic colour genes
352 underlying a characteristic orange-blotched colour pattern are linked to SD genes, creating
353 the potential for speciation by sexual selection. In LT cichlids, which in general do not
354 feature the orange-blotched phenotypes, we did not find any obvious pattern in the
355 localisation of colour genes on sex-linked LGs (Supplementary Fig. 12).

356 **Discussion**

357 Here we report the identification of sex chromosomes for 79 taxa of cichlid fishes, most of
358 which belonging to the cichlid adaptive radiation of LT, based on the analysis of whole-
359 genome data from virtually all cichlid species of the radiation[22,24] and transcriptome data
360 from a representative set of 66 taxa[27].

361 Models[13] and empirical observations[17] suggest that, beyond a certain degree of
362 differentiation, sex chromosome turnover becomes unlikely. On the other hand, frequent
363 turnovers, sex reversal, and continued recombination can contribute to counteract sex
364 chromosome differentiation[9,49]. Our analyses revealed that, in the cichlid adaptive radiation
365 of LT, sex chromosome turnovers seem to have occurred very frequently (Fig. 1), indicating
366 that the cichlids' sex chromosomes have not (yet) reached a threshold preventing turnover,
367 but that their sex chromosomes remain dynamic instead.

368 Sex chromosome recruitment in LT cichlids is non-random with respect to the
369 recruited chromosome (Fig. 2), which is compatible with the hypothesis that certain
370 chromosomes are more likely to become sex chromosomes than others[20,50]. This pattern
371 becomes even more apparent when the LT cichlids are compared to other African cichlid
372 species (Fig. 5), revealing that some LGs (in particular LG05, LG07, and LG19) emerged
373 multiple times as sex chromosomes whereas others never appeared as such. This corroborates
374 the hypothesis that particular chromosomes are preferentially[50] or even cyclically[9,49] recruited
375 as sex chromosomes. Within LT cichlids, sex chromosome turnovers have likely been driven
376 by a combination of mutational load and sexual antagonism. However, we detected a
377 prevailing persistence of male heterogamety in LT cichlids, which is a common pattern in
378 fish[51], suggesting a smaller role for sexual antagonism than previously postulated. The
379 observed prevalence of XY systems is compatible with models of speciation driven by sexual

380 selection and sex-ratio distortion in cichlids that predict higher probabilities for the
381 maintenance of male heterogamety[52].

382 The evolution of a novel sex determiner driven by linkage to a sexually antagonistic
383 colour locus has previously been documented in Haplochromini from Lake Malawi[28], which
384 are characterised by pronounced levels of sexual dimorphism. In our set of mostly riverine
385 Haplochromini and Tropheini (the LT representatives of this clade) species, we found that a
386 single sex chromosome system prevails, XY on LG05/LG19, which was probably established
387 after a turnover from the rather strongly differentiated XY LG19 system present in the genus
388 *Tropheus*. It thus appears that in the Tropheini, in which sexual dimorphism is much less
389 pronounced (and even absent in some species) compared to the radiations of Haplochromini
390 in lakes Malawi and Victoria, sexual antagonism does not play a prominent role as a driving
391 force for sex chromosome turnover. Still, several Tropheini species seem to have lost the XY
392 LG05/LG19 SD system and we were unable to detect most of the a new system that replaced
393 it based on the available data, probably because the sex-linked chromosomal regions are
394 rather small. These species will be particularly interesting to investigate further for the
395 presence and the drivers of very young, novel sex chromosomes with a potential role for
396 sexual antagonism impacting sex chromosome turnover. In addition, the observed cases of
397 young homologous sex chromosome turnovers between closely related species (e.g. in the
398 genus *Cyprichromis* on LG05 or in Trematocarini on LG04), which are indeed compatible
399 with a role for sexual antagonism as driving force in cichlid sex chromosome evolution[10],
400 open the route for further analysis of the causal mutations driving sex chromosome turnovers.
401 Especially the presence of several ZW as well as XY species in Cyprichromini, potentially
402 caused by a single transition event on the same chromosome, will allow in the future to trace
403 which alleles have been affected by a heterogamety change. Such analyses may eventually

404 reveal the causal mutation(s) (supposedly within the SD gene) of the heterogamety turnover
405 and the dominance relationships between XY and ZW systems.

406 We failed to detect sex-linked LGs in several of the LT cichlids, which certainly can,
407 to some extent, be explained by our limited sample size per species and the limited power to
408 detect small sex-specific regions, especially when using transcriptome data, as well as
409 complex polygenic SD systems. While the limited per species sample size in the currently
410 available data certainly leaves some SD regions undetected, we found it particularly
411 intriguing that we could identify sex-linked regions only in three species across the second-
412 most species-rich tribe, Ectodini. Some species of this tribe display an impressive level of
413 sexual dimorphism, suggesting similar or even more pronounced sexual antagonistic
414 selection compared to tribes such as the Haplochromini/Tropheini, which show relatively
415 strongly differentiated sex chromosomes. It will thus be interesting to examine if Ectodini
416 (and also members of other LT cichlid tribes) have very small if any sex-specific genome
417 regions that our approaches failed to detect. This would further reveal if selective forces on
418 SD differ within the radiation and if our sex chromosome turnover rate is indeed
419 underestimating the true dynamics of sex chromosome change in LT cichlids.

420 Although the sex chromosomal status of many species remains to be identified in LT
421 cichlids, our ancestral state reconstructions estimated a sex chromosome turnover rate ten
422 times higher than the one in ricefishes, another group of fishes with an astonishing diversity
423 of sex chromosomes, as well as the one published for true frogs, which was previously
424 considered the fastest sex chromosome turnover rate known in vertebrates[20]. Note that
425 extremely high numbers of SD system turnovers have also been described in geckos[21], but
426 these have so far not been quantified using a comparable rate estimate.

427 Chromosome fusions could drive speciation through incompatibilities in genome
428 structure[42,43,44] and cytogenetic analyses have indeed provided evidence for chromosome

429 fusion and fissions in some cichlid species[45]; however, their impact on cichlid diversification
430 has not yet been assessed. Sex-chromosome/autosome fusions generating an odd number of
431 chromosomes in one sex and leading to the formation of neo-sex chromosomes can be driven
432 by altering expression of genes on the translocated chromosome[53,54], sexually antagonistic
433 selection resolving conflict by restricting an antagonistic allele to a sex chromosome[55], or
434 meiotic drive[56]. Until now, differences in chromosome number between male and female
435 cichlids have not been reported, with the notable exception of copy number variations in
436 female-determining B chromosomes in Lake Victoria and Lake Malawi cichlids[57,58]. For the
437 limited number of cytogenetically investigated cichlid species, males and females have the
438 same number of regular chromosomes and across cichlids in general, chromosome numbers
439 differ little[45]. Overall, our analyses provide support for several large chromosomal
440 rearrangements between the identified sex-linked LGs, suggesting that structural changes in
441 the genome and the emergence of sex chromosomes are coupled in cichlids. The causality of
442 this relationship remains to be investigated, just as the impact of genome rearrangements on
443 reproductive isolation and eventually diversification. The available data on rearrangements
444 are sparse, but it might be that several large chromosomal rearrangements occurred before the
445 novel chromosomes were recruited as sex chromosomes, making inferences of the driving
446 forces of these fusions difficult.

447 A next necessary step will be the identification of sex-determining genes and
448 mutations causing sex chromosome turnover, facilitated by the close relatedness of the LT
449 cichlids allowing the generation of interspecies hybrids and the opportunity to study multiple
450 sex chromosome turnover events and directions, including the repetitive occurrence of
451 heterogamety transitions without sex chromosome change. While the repeated recruitment of
452 the same LG as sex chromosome indicates a particularly powerful core set of SD genes on
453 the one hand, several transitions to novel LGs on the other hand question their dominance.

454 Although this could represent recycling of sex chromosomes to some extend, we lack the
455 molecular and most importantly functional evidence for any master SD gene in cichlids of the
456 Great Lakes radiations.

457 In conclusion, the estimated rapidity of sex chromosome turnover within (LT)
458 cichlids supports the hypothesis that SD mechanisms, albeit serving the unifying function of
459 sex determination, can be extremely labile. It remains to be tested if sex chromosome
460 turnovers simply keep pace with other fast evolving traits in radiating cichlid fishes or if they
461 are particularly frequent, potentially contributing to speciation.

462 # Methods

463 **Sequencing data**

464 We used whole genome sequencing (WGS) data in the form of mapped reads in BAM files as
465 well as variant call format from Ronco *et al.*[24] and raw transcriptome data from El Taher *et*
466 *al.*[27] (see Supplementary Table 1 for details on species included and per species sample
467 sizes). Based on a recent compilation of LT cichlid species[22], the WGS data included 225
468 taxa (174 described species with 4 of those represented with two local variants/populations
469 each, and 47 undescribed species). The data further included 16 non-LT radiation
470 haplochromine cichlid taxa (13 described species one of which represented with two local
471 variants, and two undescribed species) and three riverine non-LT Lamprologini taxa (two
472 described and one undescribed species) summing to a total of 244 taxa and 469 individual
473 genomes (Supplementary Table 1). The transcriptome data that we used, were comprised of
474 66 taxa of LT cichlids (4 undescribed species, 61 described species one of which represented
475 with two regional variants), with typically three males and three females per species (details
476 are provided in Supplementary Table 1; 7 out of the 66 species had differing replicate
477 numbers) and three tissues per individual (brain, gonad, gills; details on read numbers
478 provided in Supplementary Table 3). In total, the dataset comprised 248 cichlid taxa.

479

480 **Variant calling for WGS data**

481 Mapped reads in coordinate-sorted BAM format were derived from Ronco *et al.*[24] (for
482 mapping coverage statistics see Extended Data Table 1 in Ronco *et al.*[24]), which are based on
483 mapping against the Nile tilapia (*Oreochromis niloticus*) genome (NBI RefSeq
484 GCF_001858045.1_ASM185804v2). Unplaced scaffolds of the reference genome were
485 concatenated lexicographically into an "UNPLACED" super chromosome.

486 In addition to the variant file containing all species derived from Ronco *et al.*[24],
487 variants were called for each tribe separately with GATK's[59] (v.3.7) HaplotypeCaller (per
488 individual and per chromosome) and GenotypeGVCFs (per 1 Mb window), and merged with
489 GATK's CatVariants. Variants were further filtered with BCFtools
490 (https://github.com/samtools/bcftools, v.1.6), applying the settings ReadPosRankSum<-0.5,
491 MQRankSum<-0.5, FS<20.0, QD>2.0, MQ>20.0 and placing tribe-specific thresholds on
492 minimum and maximum read depths to account for varying sample sizes (Bathybatini: 50-
493 300; Benthochromini: 25-100; Cyphotilapiini: 50-200, Cyprichromini: 100-400; Ectodini:
494 250-1500; Eretmodini: 50-200; Tropheini/Haplochromini: 375-1375; Lamprologini: 700-
495 3000; Limnochromini: 50-300; Trematocarini: 50-300). For the tribes Lamprologini,
496 Tropheini/Haplochromini, Ectodini, and Limnochromini we further applied
497 InbreedingCoeff>-0.8.
498 Indels were normalised with BCFtools's norm function, monomorphic sites were
499 excluded, and SNPs around indels were masked depending on the size of the indel: for indels
500 with a size of 1 bp, 2 bp were masked on both sides, and 3, 5, and 10 bp were masked for
501 indels with sizes of 3 bp, 4-5 bp, and >5 bp, respectively. Individual genotypes were then
502 masked with VCFtools[60] (v.0.1.14) if they had low quality (--minGQ 20) or depth (--minDP
503 4). Filtered variants were phased and missing genotypes were imputed with Beagle[61] (v.4.1).
504 We then retained only biallelic sites that had no more than 50% missing data prior to phasing.
505 For sites that were polymorphic but no individual had the reference genome allele, we set the
506 first alternative allele as reference allele.
507
508 **Approach 1 Tribe-wise association tests for sex on WGS data using GWAS**
509 In total, we applied four approaches to identify sex-linked genomic regions (approach 1-4).
510 Approaches 1 and 2 were applied on the tribe-level, the taxonomic rank above genus, but

511 below family and which, in the case of the LT radiation, groups species with divergence
512 times of 9.7-6.2My.

513 For approach 1 (Supplementary Figs. 1 and 2), the phased sets of variants for tribes
514 with at least 10 species (Lamprologini: sample size of 196 individuals representing 100
515 species; Ectodini: sample size of 81 individuals representing 40 species; Haplochromini
516 including the LT-endemic Tropheini: sample size of 99 individuals of 55 species; and
517 Cyprichromini: sample size of 21 individuals of 11 species) were each transformed into bim
518 and bed format with PLINK[62] (v.1.90b). Next, we ran association tests (GWAS) for sex on
519 these tribe-specific variant files using the univariate linear mixed model integrated in
520 GEMMA[63] (v.0.97) accounting for population stratification. After visual inspection of
521 GWAS results for potentially sex-associated regions (i.e., peaks or shifts of increased
522 significance), genotypes of the 100 most significantly with sex associated sites for
523 Haplochromini and Cyprichromini (broad signal for sex-association along the entire length of
524 LG19 and LG05, respectively) and of outlier SNPs (narrow peak regions on LG15, LG20 and
525 unplaced contigs comprising 51 SNPs with a -log10(*P*-value) > 3 ; extraction of the top 100
526 most significantly sex-associated SNPs revealed same clustering and no further sex-
527 associated region since those SNPs were scattered across the genome) were clustered and
528 visualised with the R package Pheatmap (v.1.0.12, https://cran.r-
529 project.org/web/packages/pheatmap/index.html) in R (v.3.5.2) and inspected for grouping by
530 sex.

531

532 **Approach 2 Tribe-wise tests for an accumulation of sex-specific SNPs**

533 Approach 2 was applied to tribes that contain more than a single species (Supplementary
534 Table 1 for all sample sizes): We here tested for an accumulation of sites with sex-specific
535 alleles, referred to as XY and ZW sites depending on the heterogametic sex (Supplementary

536 Figs. 3 and 4), under the assumption that a sex chromosomal region will show an
537 accumulation of sex-specific alleles due to linkage caused by suppressed recombination. To
538 this end, we subsetted the unphased, filtered sets of variants per tribe and included only
539 species for which we had individuals of both sexes (Supplementary Table 1). We then
540 removed indels and sites with more than 20% missing data and more than two alleles with
541 VCFtools (v.0.1.14). The resulting files were loaded into R (v.3.5.0) with VCFR[64] (v.1.8.0.9).
542 Sex-specific sites were classified as follows: Each variant site was recoded per species as a
543 "nosex" site if the male and the female individual had the same genotype; as "noinfo" if one
544 or both individuals had no genotype call; as "XY" if the male was heterozygous and the
545 female homozygous; and as "ZW" if the female was heterozygous and the male homozygous.
546 Next, we calculated within each tribe the sum of nosex, ZW, and XY sites in windows of 10
547 kb with a slide of 2 kb as well as the difference between XY and ZW sites per window. Next,
548 we calculated the mean genomewide percentage of nosex, ZW, and XY sites over all
549 windows and multiplied these values with the number of called sites per window to obtain
550 expected values for XY, ZW, and nosex under the assumption that most variant sites across
551 the genome show no particular sex difference. The expected values per window were
552 compared to the observed values using a Fisher's Exact test with the exception of the tribe
553 Lamprologini in which the large counts of sites per window rendered exact calculations with
554 a Fisher's Exact test impossible so that we applied a Pearson's Chi-squared test. These tests
555 will indicate windows that significantly differ from the genome-wide mean. We next
556 inspected how windows differed from the genome-wide mean and designated and plotted a
557 window with its corresponding *P*-value as (i) XY if the observed XY value was greater than
558 the expected one, and the observed ZW value smaller than the expected one and, as (ii) ZW if
559 the observed ZW value was greater than the expected and the observed XY value smaller
560 than the expected one. If both, the observed XY and observed ZW values were larger than the

561 expected value, a window was declared ambiguous and not further considered. If both,
562 observed XY and observed ZW values were equal or smaller than the expected values, a
563 window was declared nosex and not considered further. Fisher's Exact test and Pearson's Chi-
564 squared *P*-values of XY and ZW windows were plotted together (on -log10 and log10 scale
565 respectively) and with an overlay of the calculated XY-ZW difference for each window
566 normalised by dividing the obtained value through the number of species analysed. The
567 obtained plots were inspected for the presence of LG-wide or regional shifts in XY-ZW
568 difference and outliers from the expected XY or ZW sites. We also calculated and visualised
569 the XY-ZW difference in each window at the species level. In order to assess a false-
570 discovery threshold, we permutated the observed data within each tribe 100 times by
571 randomly assigning the SNPs to the observed genomic positions. We recalculated the XY-
572 ZW difference per window as well as the expected values. We assessed from each
573 permutation the highest absolute XY-ZW difference of a window and the smallest *P*-value
574 for XY/ZW sites. The largest absolute XY-ZW difference normalised by species number
575 across all permutations within each tribe was then used as minimal threshold to define the
576 sex-linked regions in the observed data. The lowest *P*-value of all XY/ZW windows across
577 all permutations was -log10(*P*-value)=5.04 in Haplochromini/Tropheini, which corresponds
578 to a FDR ~4 after Bonferroni correction. To minimise the possibility of false positives after a
579 comparison of all observed data across all tribes, we finally retained only drastic XY/ZW-
580 outlier regions that in addition of exceeding the tribe-wise threshold of XY-ZW difference
581 derived from each tribes permutation, also had a -log10(*P*-value)>20 (corresponding to a
582 FDR of 2.30×10^{-26} after Bonferroni correction).

583 Upon a first inspection of sequence content of sex-linked regions, we noticed in the
584 empirical RNA and DNA data XY and ZW peaks in different tribes/species within the same
585 region on LG02 of the reference genome. This region (21.36 Mb - 21.93 Mb) is annotated

586 with 26 protocadherin tandem gene copies. We suspect that this array of similar genes
587 impacts mapping and hence masked this region from our sex chromosomal call. Furthermore,
588 it has previously been shown that LG03 is a sex chromosome in *Oreochromini* and that the
589 assembly quality of this region is poor due to presence of repetitive elements leading to
590 difficulties in the identification of sex-linked regions on this LG[65]. This is also reflected in
591 our data by an increase of missing data on this LG and hence less reliable SNP data. We
592 therefore also excluded outlier regions on LG03 as potential sex chromosome.

593 Since approach 2 was run on the tribal level we next needed to identify how many and
594 which species are responsible for the sex chromosome signals detected within a tribe, i.e.
595 identify the sex chromosomes on the species level from this approach. To this aim, we
596 visualised, per window, species level XY-ZW differences in the outlier regions and clustered
597 individual genotypes (with the possible values "homozygous reference", "homozygous
598 alternative", "heterozygous") with divisive hierarchical clustering based on a pairwise
599 dissimilarities matrix of Gower's distances calculated with the R package FSA (v.0.8.30)
600 (https://github.com/droglenc/FSA). Resulting dendrograms were inspected for grouping by
601 sex rather than species and boxplots of species-specific XY-ZW difference for support by
602 increased absolute XY-ZW difference. Due to the reduced sample size, final calls based on
603 the outlier regions were only made if at least two species within a tribe shared the same sex
604 linkage (Supplementary Table 2).

605

606 **Approach 3 Species-specific association tests for sex on transcriptome data**

607 For approach 3, species-specific association tests (Supplementary Fig. 6), we pooled tissue-
608 specific transcriptomes of brain, gonad and gills into one transcriptome per individual and
609 quality filtered and trimmed these with Trimmomatic[66] (v.0.33) with a 4 bp window size, a
610 required window quality of 15 and a minimum read length of 30 bp resulting in typically six

611 multi-tissue transcriptomes per species (Supplementary Table 3 for read numbers). The
612 following analysis was then run per species: We performed reference-free *de novo* variant
613 calling with KisSplice[67] (v.2.4.0) with settings "-s 1 -t 4 -u" and "--experimental". The
614 identified SNPs were placed on the Nile tilapia genome assembly with STAR[68] (v.2.5.2a)
615 (settings "--outFilterMultimapxNmax 1 --outFilterMatchNminOverLread 0.4 --
616 outFilterScoreMinOverLread 0.4"). The genome index used for this mapping was generated
617 with the corresponding STAR parameters: --runMode genomeGenerate, --sjdbOverhang 124,
618 --sjdbGTFfeatureExon exon and the genome annotation file (RefSeq
619 GCF_001858045.1_ASM185804v2). Kiss2Reference[67] was used to classify KisSplice
620 variants aligned to the Nile tilapia reference genome, and kissDE[67] (v.1.4.0) was applied to
621 determine variants that differed between the two sexes. The resulting files were loaded into
622 R. The KisSplice events were filtered with the following attributes: Only SNPs were kept;
623 SNPs placed on mitochondrial DNA or on unplaced scaffolds of the reference genome were
624 removed; only SNPs with significant *P*-values for an allele difference between the sexes
625 ($P \leq 0.05$ after adjustment for multiple testing following the Benjamini and Hochberg
626 method[69]) were retained. Significant SNPs were classified as (i) "XY" if they had zero read
627 counts in all females and a minimum of one count in at least two males and as (ii) "ZW" if
628 they had zero counts in all males and a minimum of one count in at least two females. Next,
629 the density of these XY and ZW SNPs was assessed in 10 kb non-overlapping windows
630 (Supplementary Fig. 6 first plot).

631 The difference between XY and ZW SNPs per 10 kb window was then calculated and
632 only outlier windows were kept (Supplementary Fig. 6 second and third plot). These outlier
633 windows were defined as windows with a difference of XY-ZW SNPs greater than the 75th
634 percentile value + 1.5 times the inter quartile range. We then compared the distribution of XY
635 and ZW SNPs in all outlier windows with a paired two-sided Mann-Whitney test

636 (Supplementary Fig. 6 fourth plot). If the two distributions were significantly different from
637 each other (*P*-value<0.05), the heterogametic system was defined as the distribution (XY or
638 ZW) with the higher total amount of SNPs. As a last step, we quantified XY or ZW SNPs of
639 outlier windows (depending on the previously defined heterogametic system) per reference
640 LG (corrected by chromosome size) and defined as potential sex chromosome the LG(s) with
641 an amount of SNPs higher than the 75th percentile value + 3 times the inter-quartile range. In
642 order to keep only the most extreme outliers and to avoid false positives, only the LGs with a
643 number of SNPs higher than the standard deviation were kept for this final call. In species
644 for which a heterogametic system was identified, we further visualized all SNPs of the outlier
645 windows of that system along the genome (Supplementary Fig. 6 fifth plot).
646
647 **Approach 4 Inference of heterogamety from sex-specific "sequence subtraction"**
648 We applied approach 4 to all taxa of the LT radiation, which had both transcriptome and
649 assembled WGS data of a female and a male available. For this, we adapted a "subtraction
650 pipeline" from[46] to infer (expressed) sex-specific contigs. *De novo* assembled draft genomes
651 from[24] were used as species-specific references. For each species, we pooled all male and
652 female transcriptome data of all three tissues and quality filtered them with Trimmomatic[66]
653 (v.0.33) with a 4 bp window size, a required minimum window Phred score quality of 15, and
654 a minimum read length of 80 bp. Next, we modified the pipeline as follows[46]: In step 1, we
655 used STAR (v.2.5.2a) to map RNA reads of one sex to the DNA *de novo* assembly of the
656 opposite sex[24] (--outFilterMultimapNmax 10 --outFilterMatchNminOverLread 0.4 --
657 outFilterScoreMinOverLread 0.4 --outFilterMismatchNmax 100 --seedSearchStartLmax 20 --
658 seedPerReadNmax 100000 --seedPerWindowNmax 1000 --alignTranscriptsPerReadNmax
659 100000 --alignTranscriptsPerWindowNmax 10000). In step 2, we assembled all
660 transcriptome reads that did not map to the genome of the opposite sex as previously

661 described[46]. In step 3, we used GMAP-GSNAP[70] (v.2017-08-15) with a minimum trimmed
662 coverage of 0.9 and a minimum identity of 0.98, to map the sex-specific *de novo* assembled
663 transcripts (generated in step 2) to the genome of the opposite sex. In step 4, we used STAR
664 and BEDTools[71] (v.2.26.0) to remove presumed sex-specific contigs that had more than 50%
665 of their length covered with RNA-reads from the opposite sex. In step 5, we used the CD-
666 HIT-EST function of Cd-hit[72] (v.4.6.4) to merge and extend sex-specific contigs. In step 6,
667 we mapped male and female genomic reads to sex-specific contigs as described in the
668 original pipeline[46]. In step 7, we used RSEM[73] (v.1.2.31) to calculate RPKM (reads per kb of
669 transcript per million mapped reads) values. We did not apply steps 8 (a repeat filter) and 9 (a
670 transcript length filter). We tested for a correlation between the type of heterogametic system
671 and the difference in the number of sex-specific contigs with a phylogenetic ANOVA using
672 phytools[74] (v.0.6-67).
673
674 **Final sex chromosome systems definition**
675 Sex-linked chromosomes, sex-differentiated regions and heterogametic state (XY/ZW) per
676 species were inferred from sex-association in GWAS (approach 1), the sex-specific allele test
677 (approach 2) and species-specific sex-differentiated site accumulations identified by allele
678 differences test based on transcriptomes (approach 3). For approach 1 and 2, in which we first
679 report the result per tribe, we defined sex chromosomes on the species level as follows: We
680 required the same sex-linked region to be present in at least two species to base a sex
681 chromosomal call on WGS data only. This might underestimate the presence of sex
682 chromosomes in our dataset but further reduces the number of false positives. Based on
683 approach 3, which includes more individuals per species and was run on the species-level, we
684 could confirm the sex chromosomes with larger sex-differentiated regions identified by
685 approach 1 and 2. We failed to detect some of the rather small regions with approach 3, such

686 as the narrow ~5 kb region in Lamprologini, which we think is due to a combination of the
687 low number of genes present in these regions and probably low levels of expression of these
688 genes in adults. However, approach 3 allowed us to identify and confirm species-specific sex
689 chromosomes that we would not call/identify otherwise (note that similar sample sizes and
690 transcriptome approaches have previously been used to identify sex chromosomes e.g.[46,75]).
691 The effectiveness of our method is evidenced by our ability to recall all three previously
692 identified sex chromosomes of LT cichlids[37].

693 Still, given the particularly reduced sample sizes for the small tribes in approach 2, we
694 further decided to generate two sex chromosome call sets, a permissive dataset retaining all
695 sex chromosomes identified by either approaches 1, 2 and 3 or combinations thereof and a
696 stringent dataset, excluding all sex chromosomes that were exclusively identified in approach
697 2. We performed all subsequent analyses with both sets and report the results.

698 Approach 4 had previously been established for flies using a comparable sequencing
699 set-up[46]. Within LT cichlids, we noted that this method seemed to have different levels of
700 sensitivity depending on the type of heterogamety. We thus cautiously decided to not base
701 our final sex chromosome call on this approach, but still report the outcome.

702

703 **Reconstruction of sex chromosome turnovers in cichlids**

704 In order to reconstruct sex chromosome evolution across the LT radiation, we coded the final
705 sex chromosome set as a probability matrix which included 14 different LGs identified in at
706 least one species as sex-linked, incorporating the published data for two labstrains and a cross
707 derived from a natural population of *A. burtoni*[38,39] and a population of *P. philander*[25]
708 (permissive dataset; stringent dataset 13 LGs). Note that *P. philander* was present in the
709 current dataset with a single individual only and the *A. burtoni* samples included here derive
710 from two different populations not allowing the confirmation of previously published data.

711 Species, for which we could not identify a sex-linked LG and none was published to the best
712 of our knowledge, were included in the analysis and attributed equal probability for all 14
713 LGs (permissive dataset; 13 LGs in the stringent dataset). We placed these sex chromosome
714 identities onto a time-calibrated phylogeny of LT cichlids[24], which we pruned to include only
715 the species studied here, using phytools. We followed the approach described in Jeffries *et*
716 *al.*[20] and inferred ancestral sex chromosome states using a stochastic mapping approach
717 implemented in phytools. We compared the likelihood scores (based on the Akaike
718 Information Criterion (AIC)) for three different transition rate models, equal rates (ER),
719 symmetrical (SYM), and all rates different (ARD), which identified ARD as the best model
720 for transition rates between states. We simulated 1000 stochastic character maps along the
721 phylogeny. In addition, we ran stochastic mapping for each chromosome separately, coding
722 the use of the chromosome as a sex chromosome in a given species as a binary (yes/no) trait
723 to account for the fact that some tips of the phylogeny are in two or more states (i.e., two or
724 more reference LGs showed sex-linkage likely due to chromosomal rearrangements/fusions)
725 rather than having the equal probability of being in one out of two states. Note, that for *A.*
726 *burtoni,* even four different LGs have been reported as sex chromosome[38,39]. We then
727 combined the 14 separate reconstructions (permissive dataset; 13 in the stringent dataset) into
728 one phylogenetic representation. The results obtained with the two approaches were very
729 similar and we hence continued calculations with the binary reconstructions.

730 We determined the timepoints of sex chromosome turnover events as points on
731 branches where the inferred probability of using a given chromosome as a sex chromosome
732 dropped below 0.5 for the first time starting from the tips of the phylogeny with the function
733 densityMap of phytools. Based on Jeffries *et al.*[20] we did not consider species that had no
734 detectable sex chromosome as having losses but only considered transition events that led to
735 the emergence of a new sex chromosome, i.e., we only considered gains.

736 Likewise, we ran a second independent analysis with 1000 stochastic mappings to
737 reconstruct ancestral states for the type of heterogamety (XY/ZW). In addition to the
738 reconstructed turnover points, we here added a turnover on the terminal branch leading to *A.*
739 *burtoni*, since for this species, both XY and ZW sex chromosomes have been described[39].
740 To test if gene content or chromosome size drives the observed pattern of sex
741 chromosome recruitment in LT cichlids, we randomly picked 30 times (the number of sex
742 chromosome recruitments derived from ancestral state reconstruction) a window of 10 kb of
743 the reference genome and attributed the LG containing this window as sex chromosome to a
744 species. We simulated this operation 10000 times and counted how many times each LG was
745 recruited in each simulation. We than counted in how many simulations nine or more LGs
746 were not recruited, as this was the observed pattern.
747 We then tested for a correlation of the number of sex chromosome turnovers leading
748 to the tips of each tribe with the number of species investigated in each tribe with a
749 phylogenetic generalized linear model (pGLS) using the R package ape[76] (v.5.2).
750
751 **Reconstruction of sex chromosome turnovers in other teleosts**
752 We then ran the same two analyses for ricefishes (Adrianichthyidae), which, to the best of
753 our knowledge, are the only fish family with detailed data on sex chromosomes with synteny
754 inference based on a comparison to a common reference genome (*Oryzias latipes*).
755 Information on sex chromosomes was taken from Hilgers and Schwarzer[30] and placed on a
756 time-calibrated phylogeny of the family Adrianichthyidae (19 species, Supplementary Table
757 5), extracted from a recent comprehensive ray-finned fish phylogeny[40]. We could not include
758 sex chromosome data of three species (*Oryzias sakaizumii*, *Oryzias wolasi*, and *Oryzias*
759 *woworae*), as these were not included in the phylogeny and no other comprehensive time-
760 calibrated tree comprising these fishes was available to us.

761 To compare our data on a larger scale, we calculated transition rates for ray-finned
762 fishes of the Tree of Sex database (http://www.treeofsex.org/). We used all Tree of Sex
763 species that were also included in the recent comprehensive ray-finned fish phylogeny[40]
764 (Supplementary Table 6). As several species names were not initially included in the
765 phylogeny[40], we inspected species names of Tree of Sex for typos, older versions of species
766 names and synonyms in FishBase (www.fishbase.org) and Eschmeyer's Catalog of Fishes
767 Online Database (https://www.calacademy.org/scientists/projects/eschmeyers-catalog-of-
768 fishes), and corrected the names accordingly. This allowed us to map SD data for 472 species
769 from the Tree of Sex database onto the phylogeny. We further added published data for
770 cichlids[25,29,37,38,39] and this study, resulting in an additional 72 species. Sex determination data
771 from the Tree of Sex database were simplified and coded as a probability matrix with three
772 states, namely "XY" (including species classified by Tree of Sex as "XY heteromorphic",
773 "XY homomorphic", "XO", "XY polygenic"), "ZW" (including species classified by Tree of
774 Sex as "ZW heteromorphic" and "ZW homomorphic", "ZO", "ZW polygenic") and
775 "NonGSD" (including species classified by Tree of Sex as "apomictic", "hermaphrodite",
776 "ESD_other", "pH", "size", "density", "TSD", "other"). The final matrix is provided in
777 Supplementary Table 6. Similar to our approach described above, all other species with no
778 information on sex determination were included with an equal probability for all three states.
779

780 **Convergent evolution of sex chromosomes**

781 We detected the same region on LG19 as sex-linked in species belonging to the tribes
782 Haplochromini and Perissodini. Within Haplochromini, this sex-linked region was present in
783 six species of the genus *Tropheus* (tribe Tropheini, the endemic LT Haplochromini) as well
784 as in *O. indermauri* (a distantly related riverine haplochromine from the Lufubu river, which
785 drains into LT). Our ancestral state reconstruction suggested an independent origin of the

786 LG19 SD system in Perissodini, *Tropheus* and *O. indermauri.* To further investigate the
787 hypothesis of convergence, we extracted all SNPs from LG19 which were XY in at least one
788 of the species with a positionally overlapping XY-system from a variant call file containing
789 all species investigated in the present study[24] with VCFtools. In addition to a male and a
790 female of these eight species, we included representatives without this XY-LG19 system
791 from the other tribes (a male and a female of each of *Xenotilapia flavipinnis*, *Plecodus*
792 *elaviae*, *Petrochromis trewavasae*, *Eretmodus cyanostictus*, *Benthochromis tricoti*, *C.*
793 *leptosoma*, *Limnochromis auritus*, and *Cyphotilapia frontosa*) as well as other haplochromine
794 species (a male *P. philander*, a male and a female *A. burtoni*, a male *Ctenochromis polli*). We
795 only kept variants with less than 10% missing data. We next extracted the two haplotype
796 sequences of each individual for all variants in FASTA format. Assuming that the variant
797 phasing with beagle was not error-free across whole chromosomes, we inspected the
798 haplotypes and corrected the phasing for the eight LG19-XY species. This was done such that
799 for sites where an XY male was heterozygous while the corresponding XX female was
800 homozygous, the allele of the male shared with the female was designated as haplotype 1 (the
801 presumed X-allele) and the other allele as haplotype 2 (the presumed Y-allele). We then
802 inferred a phylogenetic tree by maximum likelihood with IQ-TREE[77] (v.1.7-beta12) under
803 the GTR+F+ASC substitution model to account for ascertainment bias and assessing branch
804 support with 1000 ultrafast bootstrap approximations[78]. We rooted the obtained phylogenetic
805 tree in accordance with the species tree (Fig. 1).
806
807 **Defining the degree of sex chromosome differentiation, potential sex-determining**
808 **regions, and candidate genes**
809 On the above-defined sex chromosomes, we characterised species-specific sex-differentiated
810 regions by counting the numbers of XY- and ZW-SNPs in windows of 10 kb. The density of

811 XY- or ZW-windows is shown in Supplementary Fig. 10. We defined the size of the sex-
812 differentiated region as the proportion of the LG covered by windows that have a density of
813 sex-specific SNPs that is more than twice as high as the genome-wide mean over all windows
814 such that the sum of all sex-differentiated windows defines the cumulative length of the sex-
815 differentiated regions and the minimum and maximum window coordinates define the range
816 of the sex-differentiated region on the LG. We tested for a correlation between sex
817 chromosome differentiation and the estimated age of origin of the sex chromosome derived
818 from the turnover point with a phylogenetic generalized linear model (pGLS) using the R
819 package ape. From the results of approaches 1-3, we identified sex-differentiated regions
820 shared between several species and overlaid these with candidate genes involved in sex
821 determination and pigmentation. Pigmentation genes in the reference genome were defined
822 over gene ontology annotations including the term "pigmentation" and its child terms. We
823 also retrieved orthologous sequences of the Nile tilapia to the medaka pigmentation genes
824 defined by Braasch *et al.*[79] over Biomart, Ensembl release 96 (www.ensembl.org). Since this
825 Nile tilapia genome is a different genome release than the reference genome used by us, we
826 searched the NCBI database for the obtained Ensembl gene IDs and translated them to the
827 assembly version that we used with the NCBI Genome Remapping Service. Candidate genes
828 for SD included genes previously identified through a literature search[80,81] and a gene
829 ontology analysis based on a GO annotation matching the word "sex" (list of gene IDs of
830 candidate genes for SD and pigmentation in Supplementary Table 7). We further investigated
831 all annotated genes that were partially or fully included in the window(s) with the maximum
832 number of sex-specific SNPs on the sex chromosome (Supplementary Table 2).

833 ## Data availability

834 Sequencing data used in this study were published elsewhere[24,27]. All other data are provided
835 in this paper and its Supplementary Information. Data were analysed with publically
836 available software and their included functions as detailed in the methods section.

837

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1022

1023 ## Acknowledgements

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Author Contributions

A.B. designed the study with input from A.E., F.R., and W.S.; A.E. and A.B. analysed all data, M.M. performed variant calling and phylogenetic reconstructions for convergent sex chromosome evolution, and helped with ancestral state reconstructions as well as with statistics, F.R. helped in analysing data for ancestral state reconstructions and sex-specific site identifications, A.B. and A.E. wrote the manuscript with final contributions from all authors. All authors read and approved the final manuscript.

Competing interest declaration

The authors declare no competing interests.

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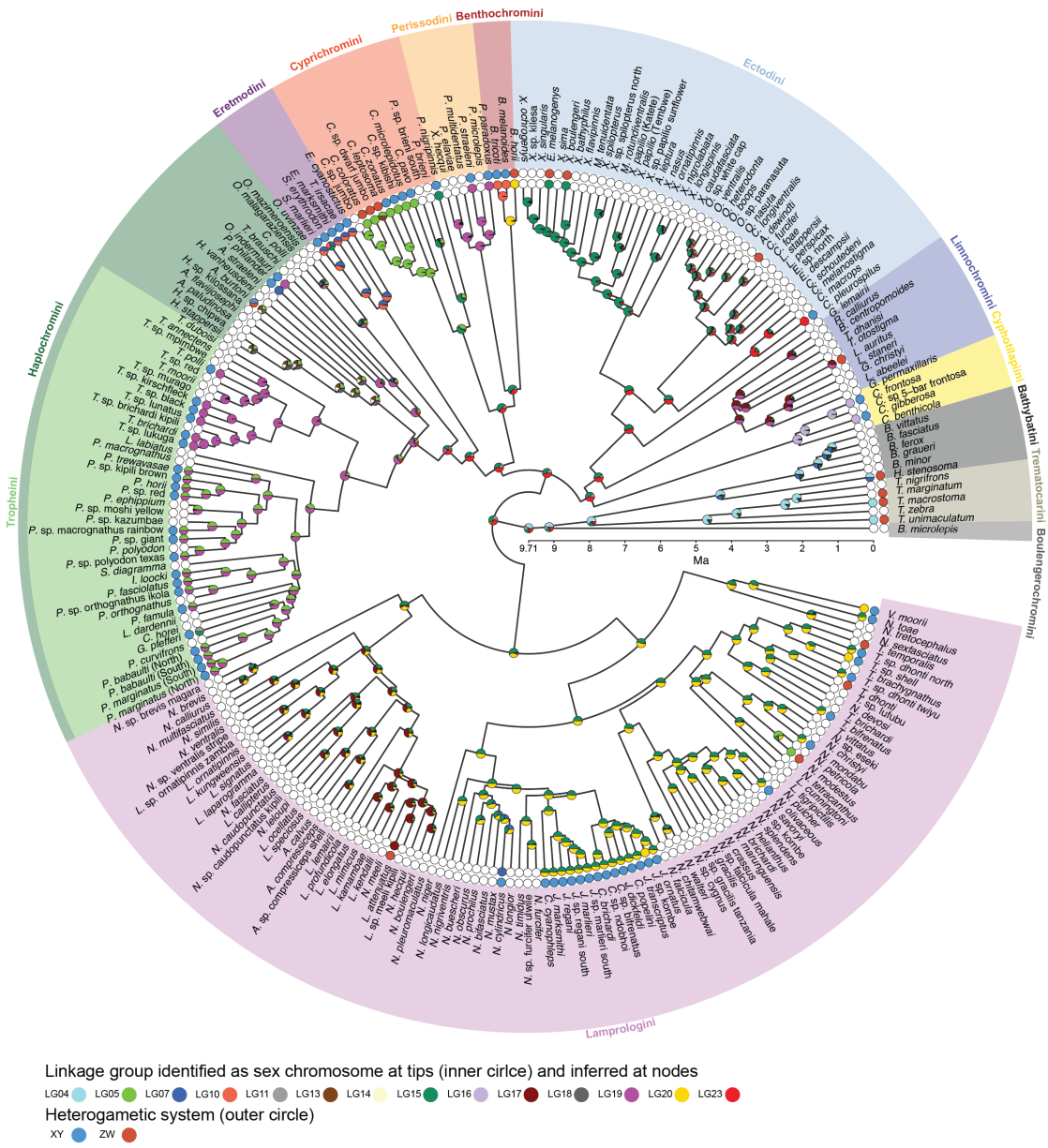
1045 **Figures**



1046 **Fig. 1: Sex chromosome evolution in the cichlid adaptive radiation in Lake Tanganyika.**

1047 Sex chromosome state and ancestral state reconstruction in LT cichlids are placed on a time-

1048 calibrated species tree[24] with tribe-grouping indicated by colour shading. The inner circle at -

1049 tips shows identified sex-linked LGs. Colours refer to LGs of the reference genome; two- or

1050 more coloured symbols at tips indicate sex chromosomal signals that were detected on two or

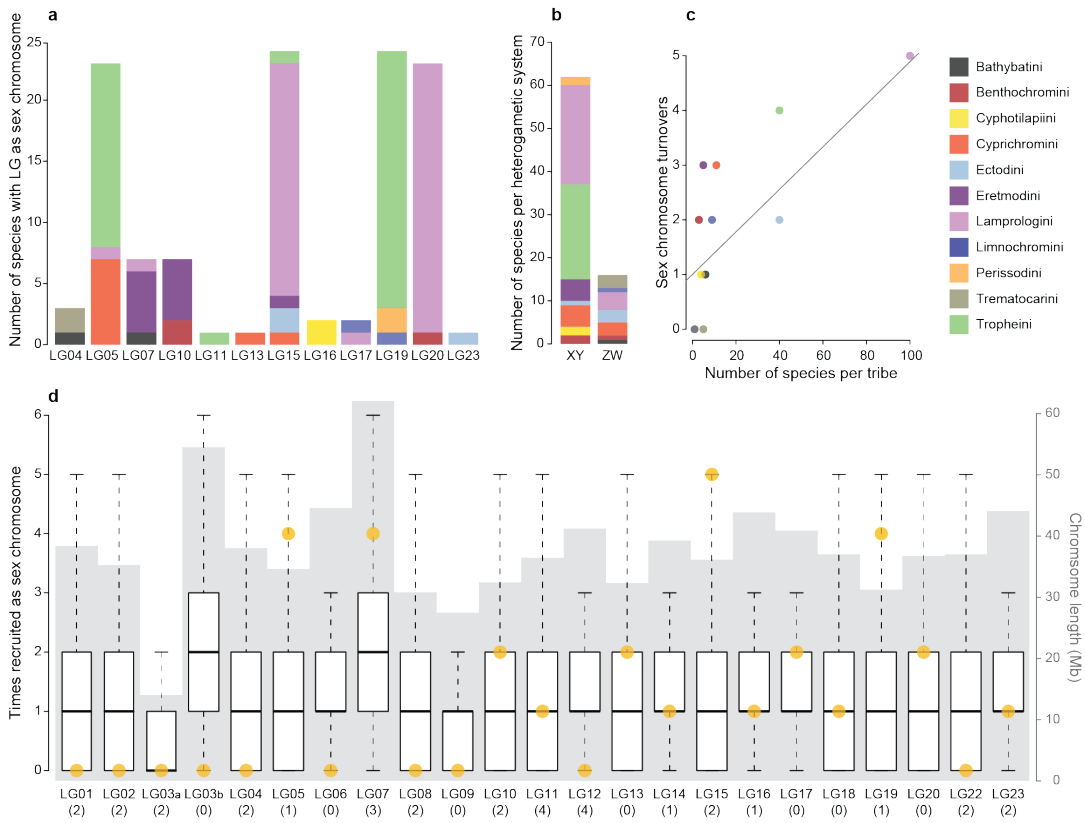
1051 more reference LGs suggesting chromosomal rearrangements. The outer circle indicates the

1052 heterogametic status of each species (blue: XY; red: ZW). White circles at tips indicated that

1053 no sex chromosome could be identified. Pie charts at nodes represent the probability for an

1054 LG being a sex chromosome at this time derived from ancestral state reconstructions.

1055



1056

1057 **Fig. 2: Non-random sex chromosome distribution in Lake Tanganyika cichlids.**

1058 **a** Use of different LGs as sex chromosomes. Bars represent the number of times an LG has

1059 been detected as sex-linked at the species level and are coloured according to tribe. **b** The

1060 occurrence of sex determination (SD) systems. Bars represent how often an XY or ZW SD

1061 system was identified at the species level and are coloured according to tribe. **c** Correlation

1062 between species richness and sex chromosome turnover. The number of sex chromosome

1063 turnovers leading to the tips of each tribe is correlated with the number of species

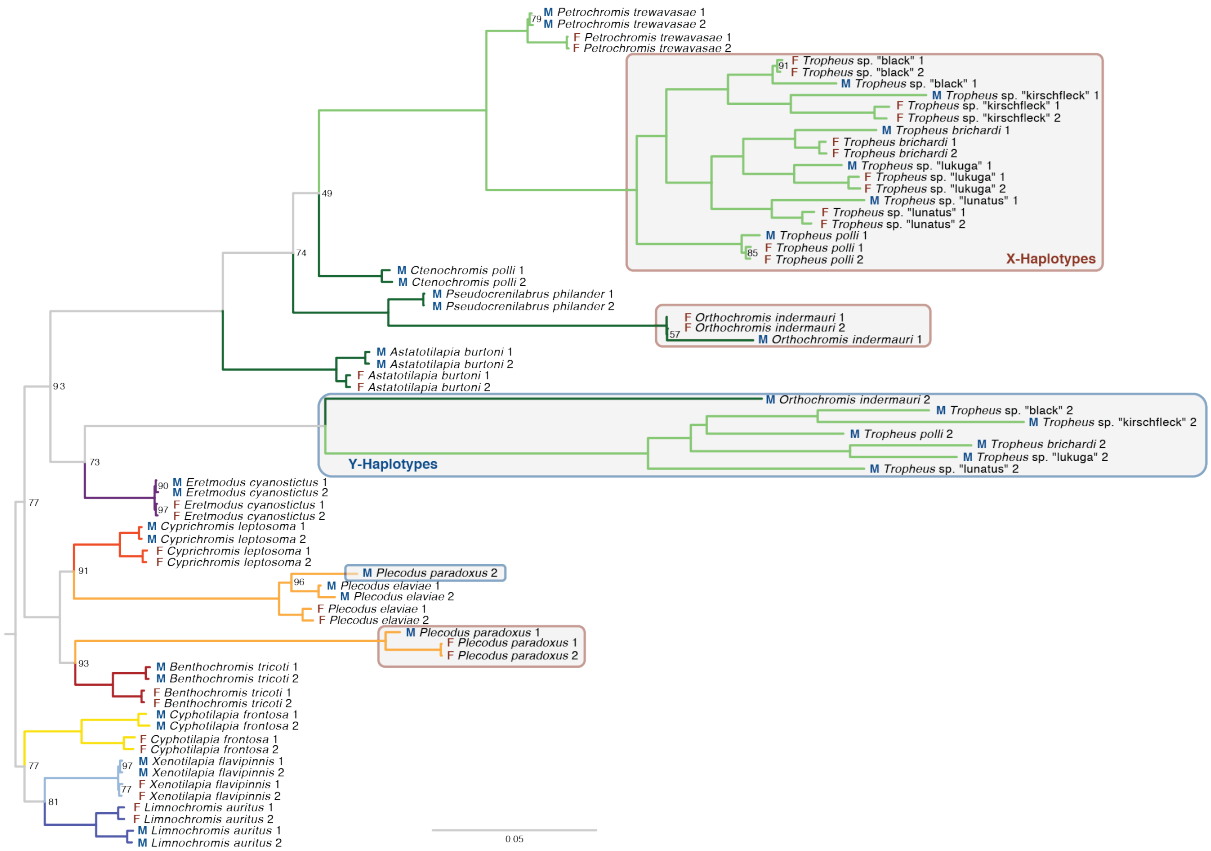
1064 investigated in each tribe (pGLS: P=0.0043, coeff=0.039). Dots are coloured according to

1065 tribes; the line represents the linear model fitted to the data. **d** Boxplots showing the expected

1066 number of sex chromosome recruitments if recruitment was at random (10000 permutations).

1067 Boxplot centre lines represent the median, box limits the upper and lower quartiles, and

1068 whiskers the 1.5x interquartile range. Outliers are not shown. Ten reference LGs were never
1069 implicated in a turnover event in LT cichlids. Under random recruitment in the simulations
1070 this pattern occurred only in 2.01 % of all simulations. Yellow dots indicate the number of
1071 observed sex chromosome recruitments per LG derived from ancestral state reconstructions,
1072 grey background shading represents chromosome length in Mb and numbers below each
1073 boxplot indicate the number of previously described sex-determining genes on these LGs.
1074

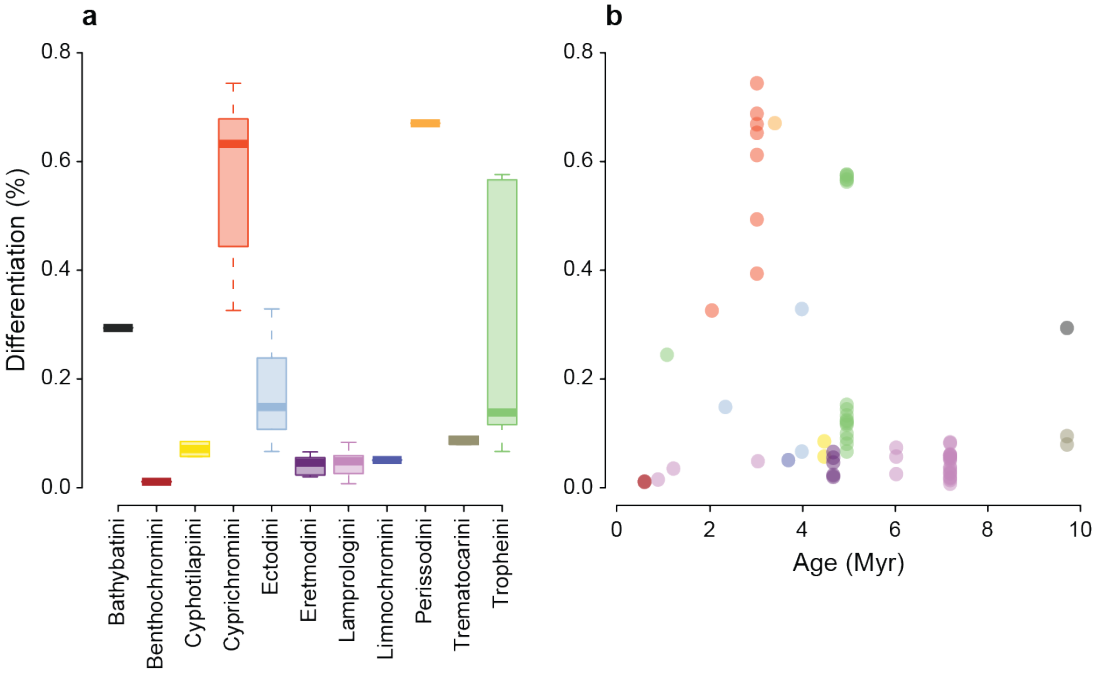


1075

1076 **Fig. 3: Convergent evolution of LG19 as XY sex chromosome in two Tanganyikan**
1077 **cichlid tribes**

1078 Phylogenetic tree of X- and Y-haplotype sequences does not group the *P. paradoxus* Y-
1079 haplotype with the *Tropheus* Y-haplotypes but supports the species tree, suggesting
1080 convergent evolution. The Y-haplotype of the non-LT riverine haplochromine *O. indermauri*
1081 groups with the Y-haplotypes of the *Tropheus* species, supporting monophyly of this sex

1082 chromosomal system. The scale bar indicates the number of substitutions per site; values at
1083 nodes represent bootstrap support (% of 1000 bootstraps, if no value is shown the node
1084 support was 100%).
1085



1086

1087 **Fig. 4: Sex chromosome differentiation in Lake Tanganyika cichlids**.
1088 **a** Size distribution of sex-differentiated regions. The size of these regions corresponds to the
1089 proportion of the LG with windows that have more sex-specific SNPs than two times the
1090 mean across all windows. **b** Per-species proportion of the chromosome(s) showing sex
1091 differentiation and corresponding estimated ages of the sex chromosomal system based on
1092 ancestral state reconstructions on a time-calibrated species tree. The degree of differentiation
1093 is not correlated with the estimated age of origin (pGLS: P=0.9049, coeff=0.0011).

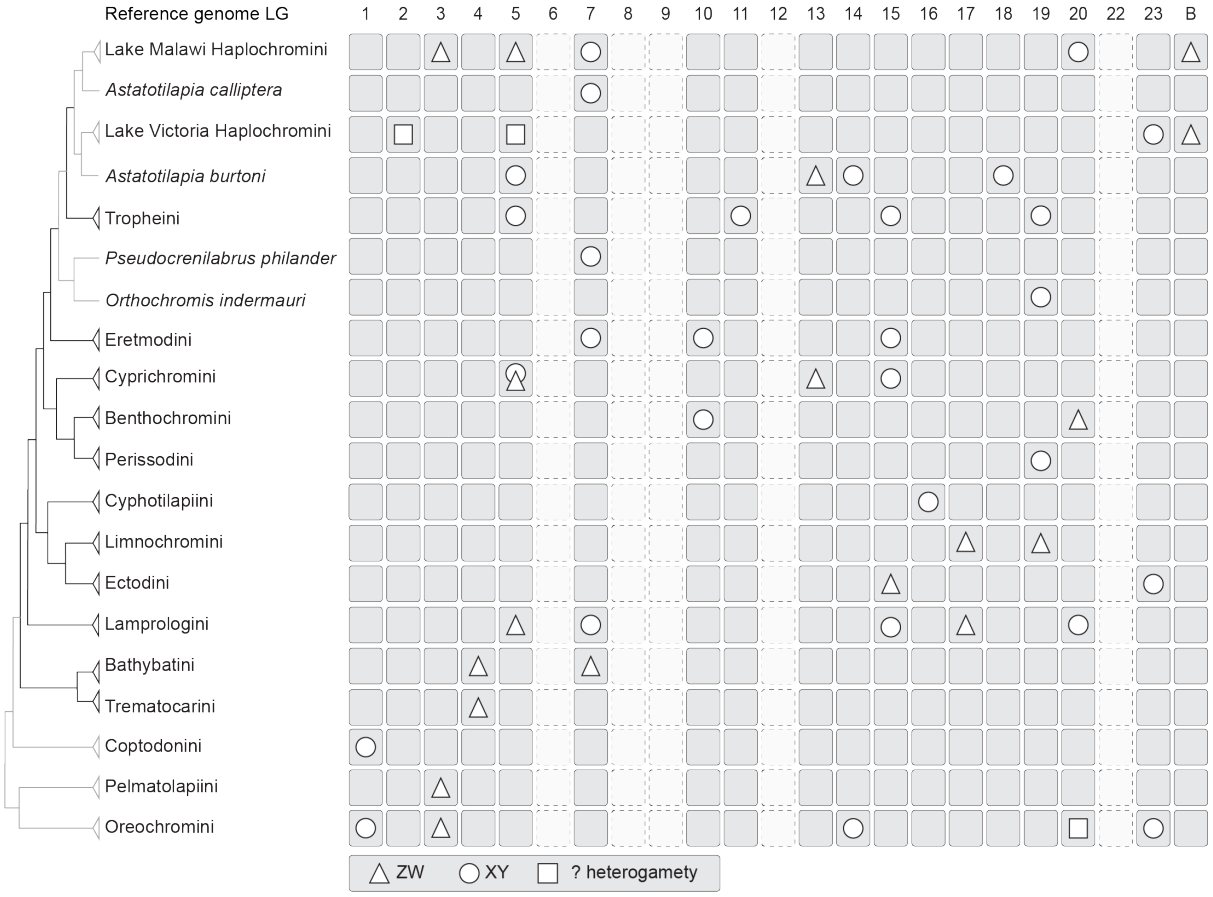


Fig. 5: Sex chromosome evolution in African cichlids. Phylogenetic relationships[24,25] and sex chromosome occurrence with reference to the genome of the Nile tilapia (*O. niloticus*) in African cichlids. Cichlid lineages found in Lake Tanganyika are indicated in black, cichlids from other lakes or rivers in grey. Sex chromosome information is derived from this study, and previously published summaries[25,26].