Diversity of sex chromosomes in Sulawesian medaka fishes

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AUTHOR CONTRIBUTIONS

37 S.A., K.Y., and J.K. conceived and designed the research. S.A., J.M., A.J.N., K.Y., and J.K.
38 performed the experiments. S.A. analyzed the data. K.W.A.M. contributed materials. S.A.
39 and J.K. wrote the manuscript. All authors have read and approved the final manuscript.

40

41 CONFLICT OF INTEREST

42 The authors have no interests or relationships, financial or otherwise, that influence our
43 objectivity.

44

45 DATA AVAILABILITY STATEMENT

46 All sequence reads will be available from the DDBJ Sequence Read Archive (SRA): short
47 sequence reads for pool sequencing (DRAXXXXXX) and ddRAD-seq reads for a family of
48 *Oryzias eversi* (DRAXXXXXX).

49

50 **Abstract**

51 Recent genetic and genomic studies have revealed tremendous diversity in sex
 52 chromosomes across diverse taxa. Although theoretical studies predict that sex
 53 chromosome evolution can drive the evolution of sexual dimorphism, empirical studies are
 54 still limited. A taxonomic group that shows diversity in both sex chromosomes and sexual
 55 dimorphism provides excellent opportunity to investigate the link between the evolution of
 56 sex chromosomes and sexual dimorphism. The medaka fishes (family Adrianichthyidae)
 57 exhibit both rapid sex chromosome turnovers and diversification of sexually dimorphic traits.
 58 In the present study, we investigated the sex chromosomes of 13 *Oryzias* species from
 59 Sulawesi, Indonesia, that have diversified in sexual dichromatism. Using pooled sequencing
 60 we found sex chromosomes in 9 species that all had XY systems, with a few species being
 61 possibly modified by multiple loci. Seven species (*O. woworae*, *O. asinua*, *O. wolasi*, *O.*
 62 *matanensis*, *O. celebensis*, *O. hadiatyae*, and *O. dopingdopingensis*) share linkage group
 63 (LG) 24 as sex chromosomes; however, they differed in the length and magnitude of
 64 sequence divergence between the X and Y chromosomes. The sex chromosome of *O.*
 65 *eversi* was LG4, which has not been reported as a sex chromosome in any other medaka
 66 species. In *O. sarasinorum*, LG16 and LG22 are associated with sex. Although LG16 was
 67 found to be sex-linked in another medaka species previously examined, the sex-determining
 68 regions did not overlap. Frequent turnovers and the great diversity of the sex chromosomes
 69 make Sulawesi medaka species a model system for investigating the roles of sex
 70 chromosome evolution in the diversification of sexual dimorphism.

71

72 **KEYWORDS**

73 Adrianichthyidae, *Oryzias*, pool-seq, sexual dimorphism, Sulawesi

74

75 1 INTRODUCTION

76

77 Recent genetic and genomic studies have revealed tremendous diversity in sex
 78 chromosomes across diverse taxa (Bachtrog et al., 2014). In several taxa, closely related
 79 species or even populations use different chromosomes for sex determination
 80 (Gammerdinger and Kocher, 2018; Miura, 2017; Myosho et al., 2015; Ross et al., 2009).
 81 Such sex chromosome turnover may be associated with the evolution of sexual dimorphism
 82 (Kitano and Peichel, 2012; Mank, 2009; Rice, 1984). A sexually antagonistic allele at an
 83 autosomal locus can drive the transposition of a pre-existing sex determining gene or the
 84 evolution of a new sex determining gene near that locus because it can resolve the
 85 intra-locus sexual conflict by enabling males and females to have different genotypes
 86 (Charlesworth and Charlesworth, 1980; van Doorn and Kirkpatrick, 2007). Although a few
 87 examples demonstrate that genes responsible for sexual dimorphism are located on newly
 88 evolved sex chromosomes (Kitano et al., 2009; Roberts et al., 2009), this hypothesis
 89 remains to be rigorously tested.

90 Alternatively, genes responsible for sexual dimorphism may evolve after the
 91 evolution of sex chromosomes. Recombination suppression is a universal feature of sex
 92 chromosomes, although the drivers of recombination suppression remain controversial
 93 (Beukeboom and Perrin, 2014; Jeffries et al., 2021; Lenormand and Roze, 2022; Ponnikas
 94 et al., 2018). Once sex chromosomes stop recombination, X and Y (or Z and W)
 95 chromosomes start to diverge in regulatory sequences, amino acid sequences, and/or the
 96 composition of genes (Lenormand and Roze, 2022; Yoshida et al., 2014; Zhou and
 97 Bachtrog, 2012). These differences may lead to the evolution of sexual dimorphism.
 98 Particularly, mutations that can resolve intra-locus sexual conflict may tend to accumulate in
 99 such non-recombining regions (Rice, 1987). Therefore, there may be a link between the
 100 evolution of non-recombining regions and sexual dimorphism.

101 Even when different taxa share sex chromosomes, they often differ in the size of
 102 non-recombining regions and sequence divergence between X and Y (or Z and W)
 103 chromosomes. For example, although sex chromosomes are relatively conserved in
 104 mammals, different species have different gene contents (Cortez et al., 2014). These
 105 differences may arise through differences in the timing of recombination suppression
 106 (Bergero and Charlesworth, 2009), the strength of sexually antagonistic selection (Bergero
 107 and Charlesworth, 2009; Wright et al., 2016), or population sizes (Engelstädter, 2008).
 108 However, few studies have examined whether variation in the non-recombining regions is
 109 linked to variation in sexual dimorphism. For example, previous studies on freshwater fishes
 110 belonging to the genus *Poecilia* indicate a possible association between male phenotypes

111 and the magnitude of divergence between X and Y chromosomes (Sandkam et al., 2021;
112 Wright et al., 2017).

113 A taxonomic group containing many closely related species that show diversity in
114 both sex chromosomes and sexual dimorphism provides an excellent opportunity to
115 investigate the link between the evolution of sex chromosomes and sexual dimorphism. The
116 family Adrianichthyidae, commonly referred to as medaka fishes, shows frequent turnovers
117 of sex chromosomes (Myosho et al., 2015). Since *DMY* gene was discovered as a
118 sex-determination gene (Matsuda et al., 2002), a total of 3 sex-determining genes have
119 been identified and 8 chromosomes have been reported to be sex-linked thus far (Myosho
120 et al., 2015, 2012; Nagai et al., 2008; Takehana et al., 2014, 2008, 2007). Furthermore,
121 medaka fishes, particularly in Sulawesi Island, Indonesia, show great diversity in sexual
122 dimorphism in body coloration and fin shapes (Mokodongan and Yamahira, 2015; Sumarto
123 et al., 2020). These fishes enable us to test whether the sex chromosome evolution is
124 associated with the evolution of sexually dimorphic traits. Previous studies have shown that
125 red and white pigmentation in the Japanese medaka fish *Oryzias latipes* (Aida, 1921; Wada
126 et al., 1998) and blue coloration in the Sulawesi medaka *Oryzias woworae* (Ansai et al.,
127 2021) are linked to their sex chromosomes. However, other sexual dimorphisms are
128 autosomal (Ansai et al., 2021; Kawajiri et al., 2015, 2014). Therefore, it remains elusive
129 whether sex chromosome evolution plays an important role in the evolution of sexual
130 dimorphism in this family.

131 As the first step toward making the medaka fishes a model for research on the link
132 between the evolution of sex chromosomes and sexual dimorphism, we investigated the sex
133 chromosomes of 13 species of *Oryzias* from Sulawesi. We have three specific aims. First,
134 we analyzed the sex chromosomes of species that either had not yet been investigated (*O.*
135 *sarasinorum*, *O. nigrimas*, *O. nebulosus*, *O. orthognathus*, and two genetically distinct
136 populations of *O. marmoratus*) or were recently described as a new species (*O. hadiatyae*,
137 *O. eversi*, and *O. dopingdopingensis*). Second, because previous studies on the sex
138 chromosomes of Sulawesi medaka fishes used strains that have been maintained in the
139 laboratory for multiple generations, we analyzed sex chromosomes of wild-derived fish,
140 except for two species. Domesticated stocks can change sex chromosomes. For example,
141 the zebrafish has a ZW sex-determination system in the wild but lost the master
142 sex-determining locus during domestication (Wilson et al., 2014), making the sex
143 determination of laboratory strains polygenic (Liew et al., 2012). Finally, we investigated
144 variation in the magnitude of divergence between the X and Y chromosomes among species
145 that share the same sex chromosome.

146

147 2 MATERIAL AND METHODS

148

149 2.1 Fish

150 Thirteen species of *Oryzias* endemic to Sulawesi, Indonesia, were analyzed for their sex
151 chromosomes (Figure 1; Table S1). For *O. marmoratus*, we used two populations: Lake
152 Lantoa and Lake Mahalona. Specimens of two of the species, *O. everisi* and *O. sarasinorum*,
153 were derived from a full-sib family for each species, whose parents were taken from a
154 closed colony maintained in the lab for about 6 and 8 years, respectively. For the other
155 species, specimens were caught from the wild. All fish were euthanized with ethyl
156 3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich), and then fixed in ethanol or
157 RNAlater solution (Thermo Fisher). Genomic DNA was isolated from muscle tissue of the
158 fixed specimens using the DNeasy Blood & Tissue Kit (Qiagen), and the DNA concentration
159 was determined with a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher).

160

161 2.2 Pooled sequencing

162 We used a pooled sequencing approach for finding the sex chromosomes (Palmer et al.,
163 2019). For each population or species, equal molars of DNA (9–10 individuals per sex) were
164 mixed to make a pooled DNA sample of 500 ng. Libraries were constructed using NEBNext
165 Ultra II DNA Library Prep Kit for Illumina (NEB) with each pool indexed with a unique dual
166 barcode (NEBNext Multiplex Oligos for Illumina, NEB). The libraries were sequenced with
167 150-bp paired-end reads on the Illumina HiSeq X instrument at Macrogen Japan, Tokyo,
168 Japan. Average sequencing coverage ($\pm$ SD) per pool was 34.4X ($\pm$ 7.8) (Table S1). The
169 coverages of all pools exceeded a recommended sequencing depth for a
170 heterozygosity-based approach to sex chromosome identification (Palmer et al., 2019).

171 The sequenced reads were trimmed using Trim Galore 0.6.4_dev with Cutadapt
172 2.6 and then mapped to a reference assembly of a previously constructed *Oryzias*
173 *celebensis* female (OryCel_1.0) (Ansai et al., 2021) by BWA-MEM v0.7.17 with default
174 parameters. The alignment files were sorted by genomic coordinates using *sort* in samtools
175 v1.9, and the PCR duplicates were removed using *MarkDuplicates* in Picard Tools v2.26.9
176 (<https://broadinstitute.github.io/picard>).

177 Genomic differentiation between the female pool and the male pool was analyzed
178 using PSASS v3.1.0 (<https://github.com/SexGenomicsToolkit/PSASS>). Briefly, a pileup file
179 made for each sex of each population was generated using uniquely mapped reads (MAPQ
180 ≥ 10). Next, the values of F_{ST} between two sexes and the number of sex-specific
181 heterozygous SNPs (i.e., heterozygote in one sex while homozygous in the other sex) were
182 calculated for each 100-kb non-overlapped window with default parameters and the group

snps (--group-snps) option: in this option, consecutive SNPs are counted as one. For the subsequent analysis, windows that were not anchored to any chromosomes or have low depth of mapped reads (absolute depth < 10) were removed.

2.3 Identification of sex chromosomes

We first searched for windows that showed both high intersexual F_{ST} and high density of sex-specific heterozygous SNPs, either male-specific or female-specific. These values were judged as statistically significantly high when they exceeded the 99.9th percentile of genome-wide distributions. Therefore, chromosomes with two or more candidate windows were defined as sex chromosome(s). When no sex chromosomes were identified with this criterion, we reduced the genome-wide significant threshold to the 99.5th percentile (see Table S3 for the criteria used for each sex chromosome). The linkage group (LG) number of each region followed that of *O. latipes*; that is, the correspondence between the LG of *O. latipes* and the chromosome number of the reference *O. celebensis* assembly was previously determined by synteny analysis using 1-to-1 orthologous genes (Ansai et al., 2021).

To identify the sex chromosome of *O. eversi*, we additionally conducted double-digest restriction site-associated DNA sequencing (ddRAD-seq) of a lab-raised family. The ddRAD data were obtained from parents and their F₁ offspring (25 females and 25 males), and ddRAD-seq was conducted using EcoRI and BglII, as described previously (Sutra et al., 2019). Briefly, the library was sequenced with 150-bp paired-end reads on Illumina HiSeq X at Macrogen Japan (Table S2). In total, 16,641,036 and 25,212,292 reads (2,512,796,436 and 3,807,056,092 bp) were sequenced from the G₀ female and the G₀ male, respectively. For the F₁ progeny, 5,236,056 ± 1,296,817.6 reads (790,644,456 ± 195,819,458 bp) were sequenced on average (± standard deviation). The reads were trimmed and then mapped to the *Oryzias celebensis* reference assembly (OryCel_1.0) as described in Sutra et al. 2019. Variant sites were called from uniquely mapped reads (MAPQ ≥ 10) using *mpileup* and *call* in bcftools v1.5 (Li, 2011). Using VCFtools v0.1.13 (Danecek et al., 2011), we selected only SNPs with high genotyping quality (GQ ≥ 30), a high read depth (DP ≥ 10), and genotyping in ≥90% of individuals in the family. We removed non-informative sites that were missing in either parent or were homozygous in both parents or showed significant segregation distortion (χ^2 test, $P < 0.05$ with Bonferroni correction), resulting in 222 SNPs. Finally, association between sex and genotypes were tested using Fisher's exact test with Bonferroni correction in R v4.1.0.

2.4 Gene ontology analysis

To investigate what kinds of genes are enriched around the sex-determining locus on LG24 in *O. hadiatyae*, we collected protein-coding genes within the region from a gene annotation dataset of the *O. celebensis* reference assembly (Ansai et al., 2021). Using BLASTN, we identified orthologs of *O. latipes* (Hd-rR strain). Using the Ensembl gene ID of *O. latipes* (Hd-rR strain), gene ontology (GO) enrichment analysis was conducted using ShinyGO v0.75 (Ge et al., 2020).

3 RESULTS

We first examined whether sex chromosomes previously identified in laboratory stocks were the same as those detected in our analysis using wild-caught fish. Four species (*O. celebensis*, *O. matanensis*, *O. woworae*, and *O. wolasi*) were previously reported to have XY sex-chromosome systems with LG24 being sex-linked (Myosho et al., 2015). In all of these species, LG24 showed signatures of sex chromosomes in our analysis too (Figure 2; Table S3). For example, in *O. celebensis*, three windows on LG24 showed high levels of intersexual F_{ST} and accumulation of male-specific heterozygous SNPs (Figure 2a; Table S3). These windows were close to marker genes that were previously reported to be sex-linked (*sox7*, chr24:14,112,606–14,115,342; *pgm3/PGM*, chr24:15,397,090–15,404,121; *hsd1l*, chr24:17,575,446–17,579,140). On LG24 of all these four species, the numbers of male-specific SNPs were elevated, indicating that these have XY systems. Interestingly, *O. wolasi* exhibited another peak on LG10 (Figure 2c; Table S3), suggesting the possible presence of a minor-effect locus in this species in the wild.

Although a previous study showed that *O. marmoratus* in the laboratory stock derived from Lake Touwti has a XY system with LG10 sex-linked (Myosho et al., 2015), we found different results. Fish collected from Lake Lantao showed signatures of sex chromosome on three chromosomes (LG10, LG12, and LG16). However, we did not find any signatures of sex chromosomes on any chromosomes in the *O. marmoratus* Lake Mahalona population (Figure 2g,h; Table S3). Currently, we do not know whether these differences reflect a difference between laboratory stocks and wild fish or among different geographical populations.

In addition to the previously reported four species (see above), three species newly analyzed in this study (*O. asinua*, *O. hadiatyae*, and *O. dopingdopingensis*) showed signatures of the XY sex chromosome on LG24 (Figure 2b-e; Table S3). The regions with signatures of sex chromosomes overlapped among these seven species, suggesting the possibility that the master sex-determining gene may be shared. These seven species with sex-linkage on LG24 enable us to investigate variation in the extent of X–Y divergence

around the sex-determining loci among species. We found that they differed in the length of genomic regions with signatures of sex chromosomes. Three closely related species (*O. woworae*, *O. wolasi*, and *O. asinua*) had much broader regions of X–Y divergence than the other four species. Even these three species varied: *O. asinua* and *O. woworae* had more windows (27 windows in the 12.3–16.9 Mb region and the 12.4–16.9 Mb region, respectively), with excess of male-specific heterozygous SNPs than in *O. wolasi* (19 windows in the 13.2–16.7 Mb region). The other four species, *O. matanensis*, *O. celebensis*, *O. dopingdopingensis*, and *O. hadiatyae*, exhibited intersexual divergence in much more limited windows (12, 11, 10, and 6 windows, respectively). We did not find significant differences in mapped read coverage between two sexes for any of these seven species (Figure S1), suggesting that degeneration of the Y chromosome is small. *Oryzias hadiatyae* had the narrowest region with signatures of sex chromosomes within a 800-kb region (14.1–14.9 Mb) on LG 24. This region includes 31 genes according to the annotation of *O. latipes* Ensembl. No significant enrichment for any GO terms was found.

New sex chromosomes were identified in two species. *Oryzias sarasinorum* showed sex chromosome signatures on LG16 and LG22 (Figure 2j). Whereas 4 outlier windows at 25.9–26.4 Mb on LG16 were enriched for male-specific heterozygous SNPs, 3 windows at 34.6–35.4 Mb of LG22 were enriched for female-specific heterozygous SNPs (Figure 4a, b; Table S3). These results suggest the possibility that this species may have a mixture of XY and ZW systems, as observed in cichlids (Roberts et al., 2016). *Oryzias eversi*, which diverged from *O. sarasinorum* at approximately 170,000 years ago (Horiwa et al., 2021), did not show any significant signatures of sex chromosomes based on our criteria (Figure 2k); however, male-specific heterozygous SNPs tended to accumulate in 5 windows located at the 5'-end of LG4 (i.e., 0.3, 2.5, 2.7, 3.1, and 7.4 Mb; >99.9th percentile) (Figure 4c). To test the sex-linkage of this locus, we genotyped a lab-raised family with ddRAD-seq. We found three SNPs significantly associated with sex (chr4:141,042, chr4:8,924,730, and chr9:530,418; $P < 0.05$, Fisher's exact test with Bonferroni correction). One of them on LG4 (chr4:141,042) was perfectly associated with sex, with all 25 males being heterozygote and all 25 females being homozygote (Table S4). These results suggest that LG4 may be XY chromosomes in *O. eversi*.

The three species endemic to Lake Poso (*O. nigrimas*, *O. nebulosus*, and *O. orthognathus*) did not exhibit any significant signatures from sex chromosomes on any chromosome (Figure 2l–n).

289 4 DISCUSSION

290

291 We found great diversity in sex chromosomes among Sulawesi medaka species, both in the
 292 linkage groups associated with sex and the magnitude of differentiation between X and Y
 293 sex chromosomes. LG24 is the most frequently observed linkage group associated with sex,
 294 with seven of the medaka species using LG24 as XY sex chromosomes. These seven
 295 species belong to 4 of 6 different major clades of Sulawesi medaka fishes, and their
 296 geographic ranges on the island are quite distant (apart) from each other (Figure 1). There
 297 are two possibilities for the origin of LG24 sex chromosomes: these sex chromosome
 298 evolved once at an early stage of Sulawesi medaka diversification or they evolved multiple
 299 times. Although we cannot exclude either possibility, the patterns of divergence between X
 300 and Y seem to be similar among the monophyletic group *O. woworae*, *O. wolasi*, and *O.*
 301 *asinua* (Figure 3), suggesting that the sex chromosomes of this clade may have the same
 302 origin. Identification of sex-determining mutations is a possibility in other medaka species
 303 (Matsuda et al., 2002; Myosho et al., 2015, 2012; Takehana et al., 2014), and verification
 304 would help to answer which scenario, the single or multiple origins of LG24 sex
 305 chromosomes, is more likely.

306 The seven species with sex-linked LG24 differed in the length and magnitude of
 307 X–Y divergence. One possible mechanism for different divergence levels is the different
 308 timing of the evolution of recombination suppression. In several species, such as among
 309 mammals and sticklebacks, there are strata on sex chromosomes, which is thought to have
 310 arisen through multiple events of chromosomal rearrangements on the sex chromosomes.
 311 Further comparative analysis of chromosomal structures and recombination rates is
 312 necessary to test this possibility. Sexually antagonistic selection can promote the evolution
 313 of recombination suppression and X–Y divergence (Bergero and Charlesworth, 2009; Rice,
 314 1987; Wright et al., 2016). Our previous analysis revealed that a quantitative trait locus for
 315 blue coloration on the lateral body surface, which is a sexually dimorphic trait found in *O.*
 316 *woworae* males, is located on LG24 (Ansai et al., 2021). Given that the blue body coloration
 317 is shared by *O. wolasi* and *O. asinua* and that these three species have larger regions of
 318 X–Y divergence than the other four species known to possess the sex-linked LG24, it is
 319 possible that sexually antagonistic selection could play a role in X–Y divergence. However, it
 320 should be noted that red coloration, another sexually dimorphic trait, was mapped on
 321 autosome (Ansai et al., 2021). Further identification of loci responsible for potential sexually
 322 antagonistic traits in Sulawesi medaka species would help us test the link between the
 323 evolution of sexual dimorphism and sex chromosomes.

324 This study identified new sex chromosomes in two closely related species: *O.*

325 *sarasinorum* and *O. eversi*. In *O. sarasinorum*, LG16 and LG22 are associated with sex.
 326 Although a previous study showed that LG16 is sex-linked in *O. javanicus* (Takehana et al.,
 327 2008), the sex-determining regions did not overlap. Interestingly, the divergent window
 328 (25.9–26.4 Mb) on LG16 contains genes located on the SD locus of Atlantic cod *Gadus*
 329 *morhua* (*cep76*, chr16:25,931,989–25,950,574; *ptpn2a*, chr16:25,953,059–25,965,311)
 330 (Star et al., 2016). A recent study demonstrated that the Y-specific sequence of Atlantic cod
 331 contains a single gene named *zkY* (Kirubakaran et al., 2019), suggesting that investigating
 332 whether a similar gene is located on the Y sequence of *O. sarasinorum* is promising. The
 333 sex chromosome of *O. eversi* was found to be LG4, which has not been reported as a sex
 334 chromosome in any other medaka species. In *O. eversi*, a SNP at the 5' end of LG4
 335 (chr4:141,042) was perfectly associated with sex. Near this locus (chr4:219,486–226,125),
 336 there exists the *amh* gene, which encodes anti-Müllerian hormone that regulates gonad
 337 development and germ-cell proliferation in medaka fishes (Klüver et al., 2007; Morinaga et
 338 al., 2007; Nakamura et al., 2012). Recent studies have demonstrated that *amh* has
 339 repeatedly evolved as an SD gene in different teleost fishes, including the Patagonian
 340 pejerrey *Odontesthes hatcheri* (Hattori et al., 2012), Northern pike *Esox lucius* (Pan et al.,
 341 2019), threespine stickleback *Gasterosteus aculeatus* (Peichel et al., 2020), and *Sebastes*
 342 rockfish (Song et al., 2021). Further studies are needed to identify causal mutations on the Y
 343 allele and to analyze genetic diversity of the sex chromosomes in wild populations.

344 We found that sex chromosome systems in two wild populations of *O. marmoratus*,
 345 collected from Lake Mahalona and Lake Lantao, differ from that reported for a laboratory
 346 stock originally derived from Lake Towuti (Myosho et al., 2015). The first possible
 347 explanation for the discrepancy is geographical variation. Our recent study using
 348 genome-wide SNPs demonstrated that wild populations of *O. marmoratus* are not
 349 monophyletic (Mandagi et al., 2021). Therefore, a taxonomic species classified as *O.*
 350 *marmoratus* may be composed of several genetically distinct species. Another possibility is
 351 that domestication changed the sex chromosomes, as observed in the zebrafish (Liew et al.,
 352 2012; Wilson et al., 2014). Analysis of sex chromosomes in natural populations of Lake
 353 Towuti *O. marmoratus* is necessary to tell which theory is correct.

354 No apparent signatures of sex chromosomes were found in *Oryzias* species
 355 endemic to Lake Poso (*O. nigrimas*, *O. nebulosus*, and *O. orthognathus*). Currently, we are
 356 unsure whether these species lack sex chromosomes. Alternatively, it is possible that they
 357 have multiple sex chromosomes, but with each one having a small effect, and the small
 358 sample sizes in this study precluded the identification of such sex chromosomes.
 359 Genome-wide association studies using whole-genome sequencing datasets of more
 360 individuals may enable the identification of weak and/or polygenic signatures of the sex

361 chromosomes.

362 Although theoretical studies predict that sex chromosome evolution can drive the
363 evolution of sexual dimorphism, empirical studies are still limited (Dean and Mank, 2014;
364 Kitano and Peichel, 2012; Rice, 1984). A taxonomic group that shows diversity in both sex
365 chromosomes and sexual dimorphism can provide us with excellent opportunities to
366 investigate the link between the evolution of sex chromosomes and sexual dimorphism. Our
367 study demonstrates that frequent turnovers and the great diversity of the sex chromosomes
368 make the Sulawesi medaka a model system for investigating the roles of sex
369 chromosome evolution in the diversification of sexual dimorphism.

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574 **FIGURE 1** Sex-chromosome system in species of Adrianichthyidae endemic to Sulawesi,
575 Indonesia. A map of Sulawesi showing locations of wild habitats of the 13 species analyzed
576 in this study (map provided by Thomas von Rintelen). A chronogram shows divergence
577 times for Sulawesi Adrianichthyidae species, which was estimated in Ansai et al. (2021).
578 The x-axis indicates the time-scale in millions of years ago (Mya). The major lineages of
579 Sulawesi medaka species are shown as different colors. The names of species analyzed in
580 this study are shown in black font. Linkage groups (LG) are defined according to numbering
581 of the chromosomes in the Japanese rice fish *Oryzias latipes*, based on genomic synteny
582 between *O. latipes* and *O. celebensis* reference assemblies. A representative image of an
583 adult male of each species is shown on the right side of the panel
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589 FIGURE 2 Genomic differentiation between the two sexes estimated by pooled sequencing
590 in *Oryzias celebensis* (a), *O. woworae* (b), *O. wolasi* (c), *O. asinua* (d), *O. matanensis* (e), *O.*
591 *hadiatyae* (f), *O. marmoratus* (Lake Mahalona) (g), *O. marmoratus* (Lake Lantao) (h), *O.*
592 *dopingdopingensis* (i), *O. sarasinorum* (j), *O. eversi* (k), *O. nigrimas* (l), *O. nebulosus* (m),
593 and *O. orthognathus* (n). Intersexual F_{ST} and numbers of heterozygous single nucleotide
594 polymorphisms (SNPs) in either of the sexes are calculated. Closed arrowheads indicate
595 candidates for sex-determining loci identified in this study. Each dot shows a 100-kb
596 non-overlapped window. Continuous and dashed lines show the 99.9th percentile and 99.5th
597 percentile values, respectively. Brown and blue colors indicate numbers of heterozygous
598 SNPs in females and males, respectively
599

FIGURE 3 Intersexual differentiation on chromosome 24, which is a sex chromosome in 7 *Oryzias* species. Intersexual F_{ST} (a), and numbers of heterozygous single nucleotide polymorphisms (SNPs) in either of two sexes (b), are shown in each 100 kb of non-overlapped window. Continuous and dashed lines denote the 99.9th percentile and 99.5th percentiles, respectively. Brown and blue colors indicate numbers of heterozygous SNPs in females and males, respectively

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610 FIGURE 4 Intersexual differentiation on a sex chromosome of *Oryzias eversi* (a) and *O.*
611 *sarasinorum* (b, c). Intersexual F_{ST} , numbers of heterozygous single nucleotide
612 polymorphisms (SNPs) in either of the sexes (b), and read coverage $\log_2$ (female
613 reads/male reads) are shown in each 100 kb of non-overlapped window. Continuous and
614 dashed lines show the 99.9th percentile and 99.5th percentile values, respectively. Brown
615 and blue colors indicate numbers of heterozygous SNPs in females and males, respectively
616