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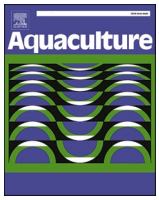

Screening and characterization of sex-linked DNA markers in Mozambique tilapia (*Oreochromis mossambicus*)

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ABSTRACT

The Mozambique tilapia (*Oreochromis mossambicus*) is an important aquaculture fish. It shows sexual dimorphism in growth, with males growing much faster than females. Thus, production of all-male fingerlings of Mozambique tilapia would improve aquaculture efficiency. However, the lack of accurate sex-specific markers has hampered the mono-sex breeding and the study of the molecular mechanisms of sex determination in this species. Recently, we performed genome sequencing and re-sequencing of female and male Mozambique tilapia and identified the sex chromosome and sex determining region. In the present study, two sex-linked SNP markers were identified and validated. Male specific contigs were assembled from the unmapped reads after aligning male re-sequencing data against the published XX genome. Based on these male-specific contigs, three sex-linked DNA markers, Marker-1, Marker-2 and Marker-3, were developed and correctly identified the genetic sex of 16 fish. The universality of these three male-specific markers was then tested in a panel of 144 individuals, which showed a discrepancy of 5.5% (8/144) between genotypic sex and phenotypic sex. In conclusion, we developed three sex-linked markers to identify different sex genotypes in Mozambique tilapia. This study lays a foundation for the establishment of MAS-GMT (marker-assisted selection of genetically male tilapias) technique and sex-controlled breeding of Mozambique tilapia, as well as for the identification of the sex determining gene.

1. Introduction

The study of fish sex determination has direct commercial applications because of the sexual dimorphism for many economic traits, including the rate of growth and body color (Mei and Gui, 2015). Significant size dimorphism between sexes has been observed in dozens of species. For example, the female grows faster and reaches a larger size than males in some species, including half-smooth tongue sole (*Cynoglossus semilaevis*) (Chen et al., 2007) and common carp (*Cyprinus carpio*) (Wu and Gui, 1999). In contrast, males grow faster and to a larger body size in Nile tilapia (*Oreochromis niloticus*) (Mair et al., 1995) and yellow catfish (*Pelteobagrus fulvidraco*) (Wang et al., 2009). Therefore, production of mono-sex populations is favored in aquaculture, and the identification of sex-specific markers in these species is a priority for aquacultural research. Despite of the commonly existence of sexual dimorphism in phenotypic traits, sex chromosomes in most fishes are homomorphic and indistinguishable by cytological analyses. These homomorphic sex chromosomes still recombine, so only closely linked genetic markers will remain associated with the sex locus (Charlesworth et al., 2005).

Decades ago, different techniques were used to identify sex-specific markers, such as restriction fragment length polymorphism (RFLP), amplified polymorphic DNA markers (RAPD), and Bulked segregant analysis (BSA). For example, the first sex-linked DNA marker was isolated in salmon (*Oncorhynchus tshawytscha*) by RFLP (Devlin et al., 1991). Two sex-linked markers were identified by RAPD in African catfish (*Clarias gariepinus*) (Kovacs et al., 2000). Ten simple sequence repeats (SSRs) markers were identified by BSA in Nile tilapia (Lee et al.,

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2003). With the development of the sex-linked markers in commercial fishes, marker-assisted selection (MAS) has been widely used in aquaculture (Mylonas et al., 2010). However, these techniques are time-consuming, labor-intensive and costly, which has hampered the identification of sex-linked markers in more species. In the past 10 years, the rapid application of high-throughput sequencing techniques, such as *de novo* genome sequencing, RAD-Seq (restriction site associated DNA sequencing) and whole-genome re-sequencing, has greatly promoted the identification of sex-linked markers and the establishment of MAS in fishes (Gui et al., 2021; Ramos and Antunes, 2022; Wong et al., 2022; You et al., 2020). Based on the comparison of re-sequencing data between females and males, W chromosome-specific fragments and ZW sex determining system were identified in Chinese tongue soles (*C. semilaevis*) (Zhang et al., 2019) and blue tilapia (*O. aureus*) (Wu et al., 2021). With similar sequencing strategy and bioinformatic pipelines, Y chromosome-specific fragments and XY sex determining system were identified in grass carp (*Ctenopharyngodon idellus*) (Zhang et al., 2017), snakehead (*Channa argus*) (Yang et al., 2020), Southern catfish (*Silurus meridionalis*) (Zheng et al., 2020), mandarin fish (*Siniperca chuatsi*) (Han et al., 2020), ayu (*Plecoglossus altivelis*) (Li et al., 2021a), Pacific abalone (*Haliotis discus hannai* Ino) (Luo et al., 2021), golden pompano (*Trachinotus ovatus*) (Guo et al., 2021), jade perch (*Scortum barcoo*) (Suntronpong et al., 2022), fugu (*Takifugu bimaculatus*) (Wang et al., 2022b), spotbanded scat (*Selenotoca multifasciata*) (Jiang et al., 2022) and large yellow croaker (*Larimichthys crocea*) (Yu et al., 2022).

Tilapia is one of the most important groups of fish in world aquaculture. The global production of tilapia in 2020 was estimated at 6 million tons, making it the second most farmed fish species. China is the largest producer for tilapia to meet both domestic and international markets (FAO, 2020). Tilapia display an extraordinary diversity of sex determining systems (Gammerdinger and Kocher, 2018). Sex-linked markers and sex determining genes on different sex chromosomes have been successfully identified in different species and even different strains of the same species. AFLP markers on LG1 were developed using BSA of the mapping families in Nile tilapia from stocks of Stirling origin (Lee et al., 2011). Five sex-linked markers were identified on LG23 (Sun et al., 2014) and *amhy* was identified to be the male sex determining gene in Nile tilapia from stocks of Japanese origin (Li et al., 2015) and later validated in two wild populations (Triay et al., 2020). W-linked markers associated with sex determination have also been successfully detected on LG3 (Wu et al., 2021) in blue tilapia and Wami tilapia (Zhu et al., 2016). Recently, a sex-linked marker has been successfully isolated on LG1, which laid a foundation to produce all male amelanotic red tilapia (Lu et al., 2022). Genome-wide significant QTL intervals associated with sex were later identified on LG1 and LG23, and four microsatellite markers located within the QTL were successfully developed for MAS in red tilapia (Zhu et al., 2022).

Mozambique tilapia, which is endemic to southeastern Africa, is an important fish species for aquaculture. Moreover, compared with other tilapia, Mozambique tilapia more easily adapts to salt water (Nitzan et al., 2017; Yan et al., 2013) and lower temperatures (Sardella et al., 2004). Thus, rearing Mozambique tilapia and its hybrids is helpful for selective breeding programs. Like other tilapia, Mozambique tilapia shows obvious differences of growth rate and mature size between males and females, with males growing faster than females. Thus, all-male breeding is of high commercial value in aquaculture. Previously, a major XY sex-determining locus has been identified on LG1 using a hybrid population of Mozambique and Red tilapia (Liu et al., 2013). Later, an XX/XY sex determining system and the sex determining region (10 Mb) were identified on LG14 in Mozambique tilapia based on the whole genome re-sequencing of pools of males and females (Gammerdinger et al., 2018). Recently, we performed genome sequencing of a female Mozambique tilapia. Genome re-sequencing of 5 females and 5 males individually, identified sex-linked SNPs and narrowed the sex determining region to 3 Mb on LG14 (Tao et al., 2021b). These results provide valuable resources for future studies in Mozambique tilapia. In the present study, we developed sex-linked SNP markers and PCR-based markers for diagnosis of genetic sex in Mozambique tilapia.

2. Materials and methods

2.1. Ethics statement

Animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

2.2. Experimental fish

The Mozambique tilapia, introduced from the United States in 2000, were cultured in the farm of the Pearl River Fisheries Research Institute of the Chinese Academy of Fisheries Science. The experimental fish were maintained in large aerated aquarium within a re-circulating freshwater system, with a density of 50 fish per cubic meter of water, at a temperature of 27 °C, under a natural photoperiod (14 L, 10D). pH was at 7.1 ± 0.1. Concentration of ammonia nitrogen was less than 0.1 mg/L, and dissolved oxygen was over 5.8 mg/L. Pieces of residual bait at the bottom of the tank were sucked out, and the lost water was replenished in a timely manner. Fish were fed twice per day using the tilapia commercial diet (Guangdong HAID Group Co., Ltd). In total, 160 fish were sampled at 180 dah (days after hatching).

2.3. Phenotypic sex identification

The phenotypic sex of Mozambique tilapia at 180 dah was individually determined by stripping of gametes. If there were no gametes, histological examination of gonad tissue was performed as described (Tao et al., 2018). Briefly, the gonads were collected from fish at 180 dah, fixed in 4% paraformaldehyde (PFA) solution and embedded in paraffin. Later, the paraffin embedded tissues were cut into sections on a microtome (Leica, RM2235, Germany), and subsequently stained with hematoxylin and eosin. The phenotypic sex was determined on Olympus BX51 light microscope (Olympus, Tokyo, Japan).

2.4. DNA extraction

The caudal fins of 160 sampled fish were put into 100% ethanol and stored in −30 °C until use Genomic DNA was extracted from caudal fin clips by proteinase K digestion and subsequent phenol/chloroform extraction (Sambrook et al., 1989). The quality and yield of DNA were assessed by 1% agarose gel electrophoresis and NanoDrop 2000 spectrophotometry (Thermo Scientific, Waltham, MA, USA). The extracted genomic DNA was used for subsequent isolation and validation of sex-linked makers.

2.5. Isolation of sex-linked SNPs and identification of male-specific sequences

Genomic DNA from 5 male and 5 female fish from the Pearl River Fishery Research Institute were individually re-sequenced previously on an Illumina platform (Bioproject PRJCA004934). Pooled resequencing of 8 males and 12 females from a previous study was performed on an Illumina platform (Bioproject PRJNA432420). Sex-linked SNPs within the located region were called as described (Tao et al., 2021b), and the 700 bp flanking sequences of 4 male heterogametic SNPs on LG14 were extracted to design primers using Primer Premier5.0 (Table S1). PCR products were checked by agarose gel electrophoresis on a 1% agarose gel, and then Sanger sequencing was performed with the forward primer to evaluate the accuracy of these sex-linked SNPs. Sex-specific fragments were identified following the pipeline described previously (Ou et al., 2017; Zheng et al., 2020). Briefly, Y chromosome-specific sequences

were obtained by the following procedures (Fig. 1). The male reads were concatenated from re-sequencing data of 5 male individuals (Tao et al., 2021b) using seqtk (v1.2) (https://github.com/lh3/seqtk). The XX Mozambique tilapia genome assembled to the chromosome level (Tao et al., 2021b) was used as the reference. BWA (Li, 2013) with the default parameters was used to align the male reads against the female reference genome. The male reads which did not align to the female reference genome were extracted using SAMtools (Li et al., 2009). IDBA (Peng et al., 2011) was used to assemble the unmapped male reads into contigs by connecting reads with overlapping nucleotides. Subsequently, the concatenated clean reads of 5 female individuals (Tao et al., 2021b) were mapped to the assembled male contigs using BWA. Finally, the assembled male-specific (Y-specific) contigs were verified in female and male pooled libraries from previously reported Family 1 of Mozambique tilapia (Gammerdinger et al., 2018).

2.6. Confirmation of male-specific fragments with PCR amplification

To obtain the sequences flanking the male-specific region, develop co-dominant genetic markers and identify the candidate sex-determining gene, the genome of an XY Mozambique tilapia was sequenced by PacBio high-fidelity (HiFi) sequencing (unpublished data). Based on the Y-specific sequences and sequences flanking the male-specific region, primers were designed by Primer Premier 6.0 (premierbiosoft.com/primerdesign/index.html) and synthesized by a commercial company (BGI, Beijing, China). The feasibility and universality of the male-specific markers were first tested by PCR of 16 individuals, and later validated in an additional 144 individuals. Each PCR mixture (20 μL) contained 10 μL 2 × Taq MasterMix (Vazyme Biotech, Nanjing, China), 0.4 μL forward primer and 0.4 μL reverse primer (10 μM), 1 μL DNA (100 ng) and 8.2 μL ddH_2O. The PCR cycling conditions were as follows: one cycle at 95 °C for 3 min; 30 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for the time according to the length of the amplified sequences; one cycle of 72 °C for 10 min. The amplified PCR products were visualized by gel electrophoresis on 1.5% agarose gels stained with Nucleic Acid Stain (Dingguo, Beijing, China).

3. Results

3.1. Phenotypic sex of Mozambique tilapia

Sexual dimorphism in body size is apparent in Mozambique tilapia (Fig. 2 A-B). To determine the phenotypic sex of Mozambique tilapia, histological analyses were performed with gonads from fish at 180 dah. Oocytes of different phases were observed in the gonad of female fish, while spermatogonia, spermatocytes, spermatids and sperms were identified in the gonad of male fish. The morphology, histology of gonads from one female and one male fish were taken as examples (Fig. 2 C-F).

3.2. Identification of sex-linked SNPs

Two out of 4 sex-specific SNP markers, named SNP_36563933 and SNP_38350519, were correlated with phenotypic sex by Sanger sequencing using the forward primers. These SNP markers were homozygous (T/T) in 100% of females (79/79) and heterozygous (T/C) in 90.1% of males (73/81) (Fig. 3). These two sex-linked SNPs were located in intergenic regions by BLAST search in the Mozambique tilapia reference genome, with an E value of $1e^{-5}$.

3.3. Identification of sex-specific sequences

In total, the clean data from 5 male individuals was 209 Gb, including 1393 million reads (150 bp). These clean reads were aligned to the female reference genome, with 1384 million reads matched and discarded. The remaining unmapped 9 million reads should be male-specific. They were used to assemble male-specific contigs using IDBA, and 6827 male-specific contigs were obtained. The length of the assembled contigs ranged from 200 to 9793 bp. Most of them were 200–1000 bp in length, accounting for 86.13% of the total. Only 935 contigs had a length greater than 1000 bp. The concatenated reads from 5 female individuals were aligned to the assembled 6827 male-specific contigs using BWA. SAMtools was used to calculate the coverage of each male contig, and the sequences that could not be covered by any female reads were identified as candidate male-specific sequences. The assembled contigs were mostly mapped by the female reads, with only 3 contigs not covered by the female reads (Supplementary file 1). Finally, 2 of 3 contigs displayed a male-specific pattern with respect to the SNPs in the previously reported Family 1 of Mozambique tilapia (Gammerdinger et al., 2018).

3.4. Development and validation of male-specific markers

Two pairs of primers were designed on the two male-specific contigs, respectively. PCR results showed that these markers displayed different but stable patterns between sexes, and the representative profiles of electrophoresis of 8 males and 8 females is shown in Fig. 4. As expected, Marker-1 and Marker-2 produced Y-specific bands of 515 bp and 351 bp, respectively, with no bands observed in females. Sanger sequencing of PCR products gave the same sequences as the assembled male-specific contigs. Marker-3 was developed by aligning these male-specific sequences to the haplotype-resolved PacBio HiFi assembly of an XY fish, which spanned the interval between Marker-1 and Marker-2 and produced a 2870 bp Y-specific band and 1840 bp X-specific band in males. These markers were used to amplify the genomic DNA from another 144 individuals with known phenotypic sex to test their universality. Discrepancy between genotypic sex and phenotypic sex was observed in 8 phenotypic males (Fig. 5, Fig.S1 and Fig.S2). Compared to male-specific markers, the developed co-dominant marker improved the efficiency, which laid a foundation for production of all-male population and for isolation of sex-determining gene.

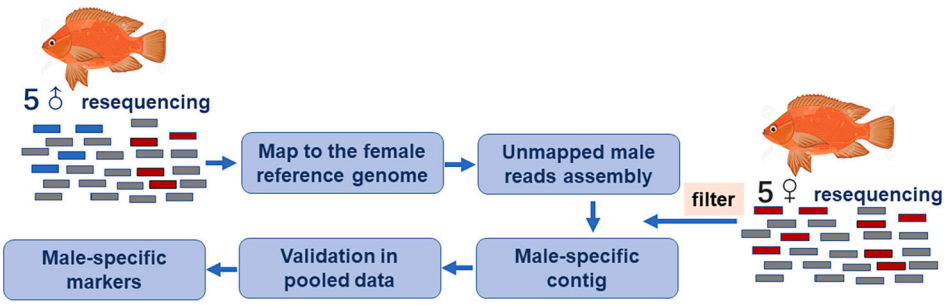

Fig. 1. The workflow for screening the Y-specific markers in Mozambique tilapia.

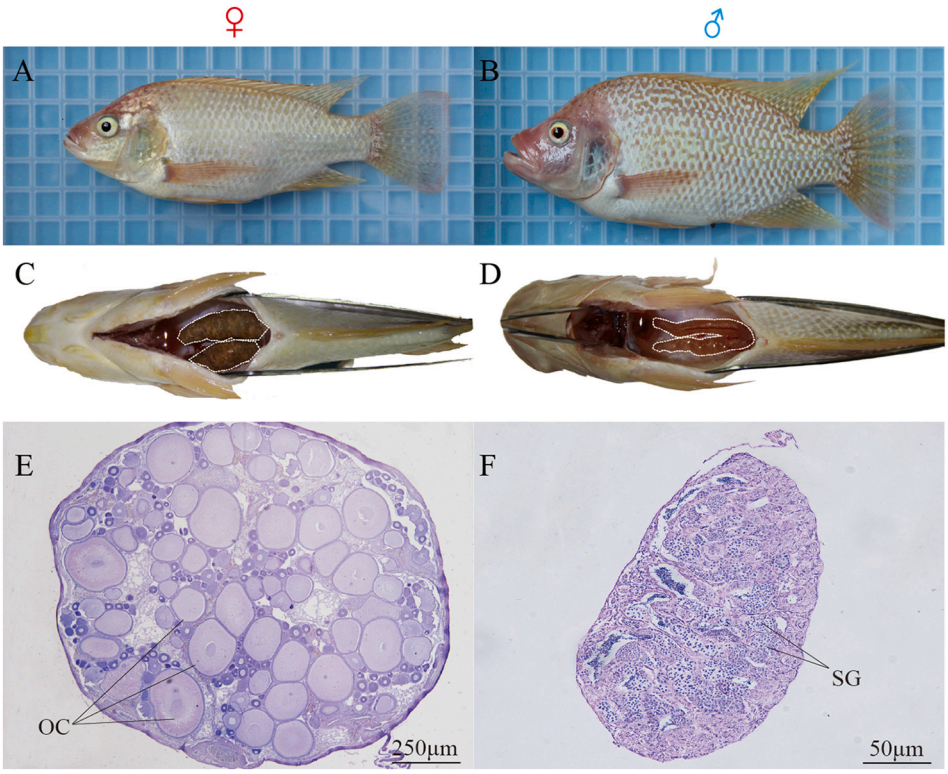

Fig. 2. Identification of phenotypic sex in Mozambique tilapia at 180 dah. A-B, sexual dimorphism in body size of Mozambique tilapia. C-D, morphology of gonads in female and male fish. *E*-F, histological sections of Mozambique tilapia gonads stained with hematoxylin and eosin. SG, spermatogonia; OC, oocyte. dah, days after hatching.

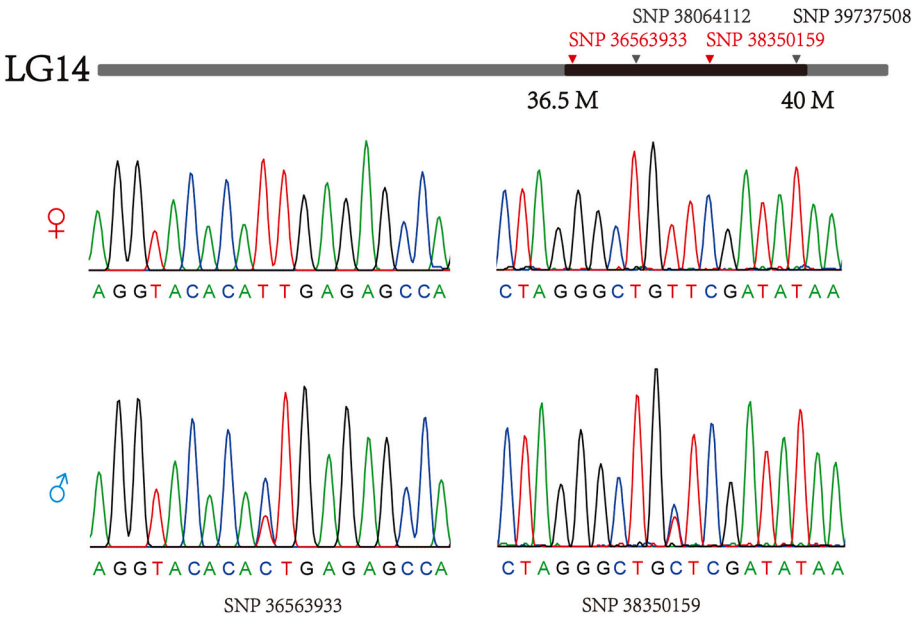

Fig. 3. Location and sequencing chromatograms of 2 sex-linked SNP markers in Mozambique tilapia.

4. Discussion

Aquaculture has played a vital role in increasing global food production, and fish compose one of the most important animal protein sources (Samples, 2014). Tilapia is the second most farmed fish in world aquaculture. It possesses substantial sexual dimorphism in growth rate and body size. Thus, males are preferred in aquaculture, and a global consensus on tilapia industry is mass production of GMT. Previously, exogenous hormone treatment and thermal treatment during key period of sex differentiation, and hybridization between different tilapia species were used to increase the proportion of males in Nile tilapia (Baroiller and D'Cotta, 2001; Baroiller et al., 2009; Beardmore et al., 2001). However, the use of these approaches was not fully acceptable because of potential environmental pollution and public health issue or variable success in generating all-male progenies. Thus, identification of molecular markers and generation of supermales is an ideal way to produce

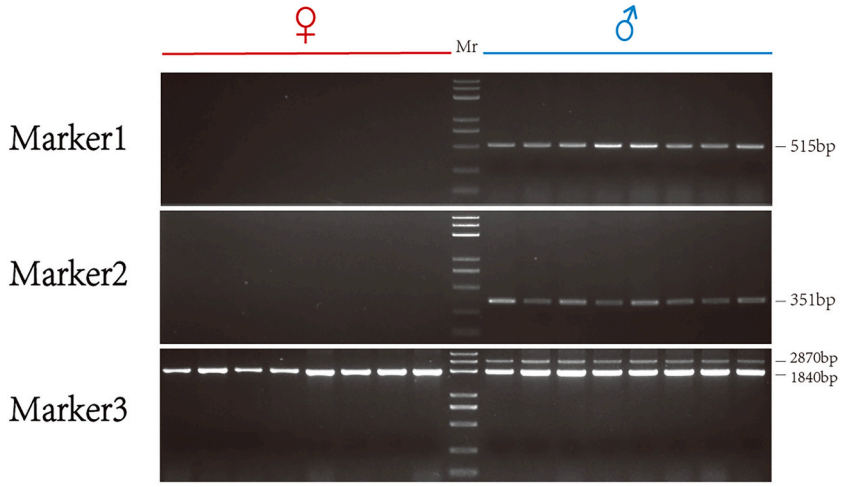

Fig. 4. PCR amplification results in Mozambique tilapia with Marker 1–3. Mr., DNA Marker DL2000.

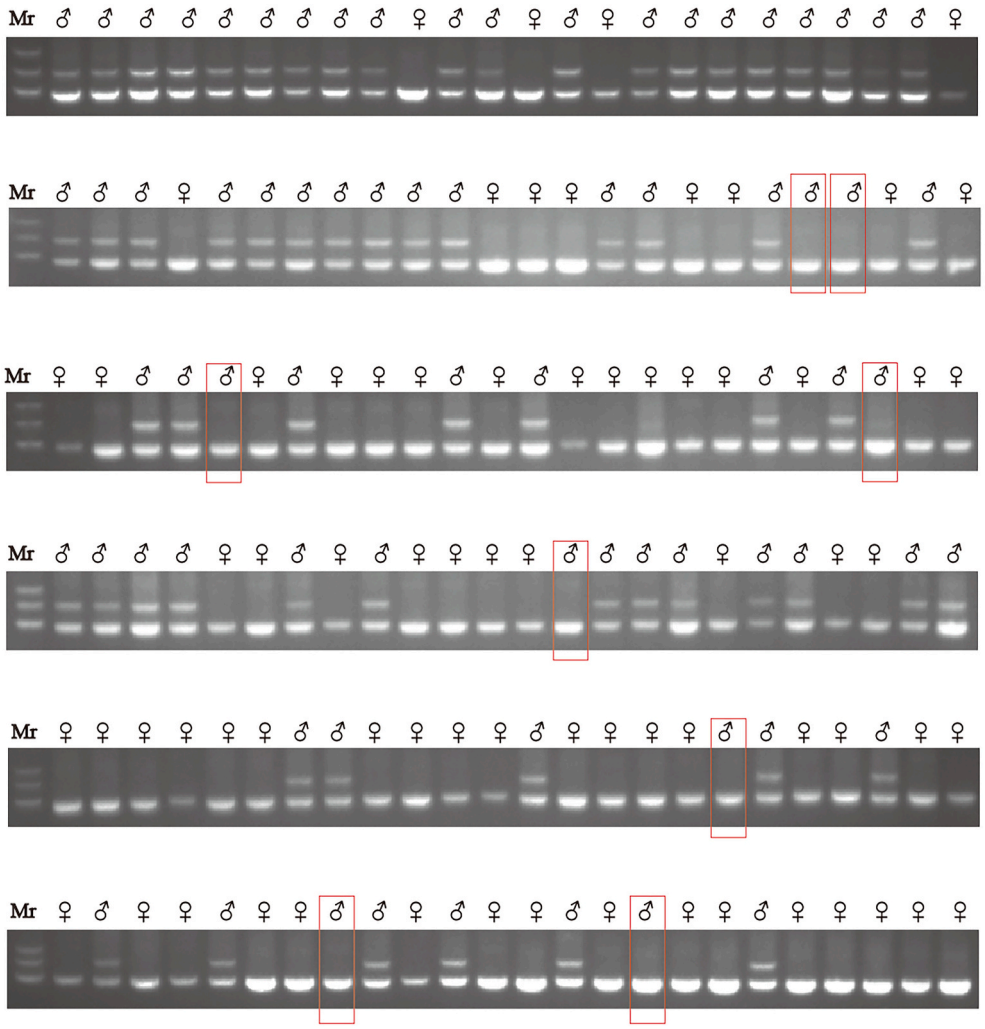

Fig. 5. PCR validation of Marker 3 in another 144 individuals of Mozambique tilapia. Individuals in red rectangle displayed discrepancy between phenotypic and genetic sex. Mr., DNA Marker DL2000. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

all-male population. Both XX/XY and ZZ/ZW sex determination systems have been identified in closely related tilapias (Campos-Ramos et al., 2001; Cnaani et al., 2008; Tao et al., 2021a; Yanez et al., 2020). Sex-linked markers have been identified in different tilapias on LG1 (Lee et al., 2003; Liu et al., 2013; Palaiokostas et al., 2013), LG3 (Wu et al., 2021), LG20 (Palaiokostas et al., 2015) and LG23 (Sun et al., 2014), indicating the complexity and diversity of the sex determination in tilapia. However, existing studies were mainly focused on the widely

farmed Nile tilapia and blue tilapia, few studies were conducted to identify sex-linked markers in Mozambique tilapia. In the present study, sex-linked markers on LG14 were isolated and verified in Mozambique tilapia. Previously, sex determining loci have been identified on LG1 in salt tolerant tilapia (*O. mossambicus* x *Oreochromis spp.*) (Liu et al., 2013). The discrepancy might be attributed to the rapid transition of sex chromosome during hybridization, as reported in swordfish (Franchini et al., 2018) and tree frogs (Jeffries et al., 2018).

Sex-linked markers are essential tools for marker-assisted breeding and elucidating the genetic basis of sex determination and differentiation (Liu and Cordes, 2004). Accumulated sequence divergence within the sex-determining region could be developed as molecular markers for discriminating genetic sex. Identification of sex chromosome and sex-linked markers has been difficult, due to the limited sequence divergence on homomorphic sex chromosomes. Previously, reduced representation sequencing (RAD-Seq, dd-RAD and SLAF-Seq), with DNA digested by restriction enzymes, has been extensively used to identify sex-linked markers in teleosts (Drinan et al., 2018; Xue et al., 2020). However, these techniques only produced limited sequence data of the digested fragments. Therefore, many sex-specific regions were inevitably omitted, which seriously hampered the identification of sex-linked markers. With the development and decreased cost of whole genome re-sequencing technology, sex-linked markers have been developed in more and more fishes. Similar workflows have been developed to align genome sequences or compare re-sequencing depth between sexes, in order to screening for sex-specific molecular markers (Han et al., 2020; Yang et al., 2020; Zheng et al., 2020). In the present study, reciprocal comparisons of re-sequencing data of the males and females were adopted to screen Y-specific sequences in Mozambique tilapia. The mapping rate and the sequencing depth of re-sequencing reads to the reference genome were high enough to identify Y-specific sequences, which enabled us to develop sex-linked markers in Mozambique tilapia.

Our examination of 160 fish with the three sex-linked markers in Mozambique tilapia, revealed a concordance rate of 95% between genotypic and phenotypic sex. Similar results have also been observed in other species. For example, 13 out of 800 tested large yellow croakers (Lin et al., 2021), 11 out of 94 tested fighting fish (Wang et al., 2022a) and 3 out of 39 tested fugu (Wang et al., 2022b) displayed inconsistent results between phenotypic sex and genetic sex. The discrepancy may be explained by three alternative hypotheses. First, sex reversal may have occurred due to external factors (hormones, temperature, and social factors). Notably, all the 8 mismatched fish in the present study were phenotypic males. Increased male proportion was observed in Mozambique tilapia at 32 °C (Wang and Tsai, 2000), and genotypic female-to-phenotypic male sex reversal was also observed in different populations of Nile tilapia due to elevated egg incubation temperature (Baroiller et al., 2009; Sissao et al., 2019; Zhao et al., 2020). Recently, a comprehensive database named ASER (Animal Sex Reversal) including the sex reversal-associated genes and regulatory network was constructed, which is helpful to explore the molecular mechanism of sex reversal in tilapia (Li et al., 2021b). Second, the discrepancy might be due to recombination between sex chromosomes, translocation of sex-specific sequences to autosomes or mutation of the sex-determining gene, as reported in other species (Nemeshazi et al., 2020). If the discrepancy was caused by recombination, the distance between these identified markers and sex determining locus should be relatively far from each other. If so, it is still hard to explain why the SNP markers and the co-dominant marker displayed the same concordance rate. Third, additional sex determiners on LG1, LG3 and LG23 were possible because tilapia MAS programs starting from hybrid populations. We have tested the unmatched individuals with makers developed on LG1 (Lu et al., 2022), LG3 (Wu et al., 2021) and LG23 (Sun et al., 2014). However, none of these markers worked to identify the genotypic sex of these unmatched individuals. Therefore, our results favored the first hypothesis.

5. Conclusion

In conclusion, we identified two sex-linked SNP markers and three sex-specific markers in sex determining region on LG14 of Mozambique tilapia based on the assembled genome and re-sequencing data from females and males. A total of 160 individuals were used to test these markers, which gave a concordance rate of 95% between phenotypic and genotypic sex. The identified sex-specific markers are helpful for sex control breeding and isolation of the sex determining gene in Mozambique tilapia.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738331.

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