Polymorphism in a sex-linked DNA marker located on LG23 in Hainan strain of Nile tilapia (*Oreochromis niloticus*)

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Abstract
Marker-5, which is linked to the major sex-determination gene *amhy* on LG23, has been used for sex genotyping of various strains of Nile tilapia. Marker-5 specifically amplifies fragments of approximately 1,422 and 982 bp of the X and Y chromosomes, respectively. Herein, we evaluated Marker-5 polymorphism in Nile tilapia of Hainan strain, China. Agarose gel electrophoresis showed that in 13 out of 85 female fish, Marker-5 amplified a larger band, distinct from the standard X-specific band. Sanger sequencing showed that this larger band was 1,688 bp in length and showed high similarity with both 1,422-bp X-specific and 982-bp Y-specific fragments. The larger band was proved to be a female sex-linked allele of Marker-5 and was labeled as X₉L. When the polymerase chain reaction (PCR) products of X₉LY fish, amplified by Marker-5, were tested by agarose gel electrophoresis, an unusual band, smaller than X₉ but larger than that of Y fragment, was observed. The unusual band could be a heteroduplex of the X₉ and Y fragments. In very few XXL male, *amhy* was found to be linked with 1,422-bp fragment amplified by Marker-5. Understanding the
Marker-5 is useful for its utilization in sex-controlled breeding in farmed Nile tilapia.

**KEYWORDS**
genetically male tilapia, heteroduplex, hybridization, sex control, sex-linked marker

1 | INTRODUCTION

Tilapia is the second most common farmed fish, with global production exceeding 5.6 million m.t. in 2018 (FAO, 2020). Nile tilapia (*Oreochromis niloticus*) is the most widely farmed species of tilapia. The two main factors that have contributed to the development of the all-male tilapia farming as the industry standard in all varieties of tilapia include sex dimorphism favoring males and the practice of mixed-sex farming, resulting in unwanted reproduction before harvest (Cnaani et al., 2008; Lozano, Gjerde, Ødegård, Rye, & Luan, 2014; Mair, Abucay, Beardmore, & Skibinskia, 1995). The all-male farming controls unwanted reproduction, results in an elevated growth rate, and guarantees the production of marketable-sized fish. The most common method currently used to obtain all-male fry is the oral administration of androgen hormones; however, this method is not environmentally friendly (Megbowon & Mojekwu, 2014). Some countries employ interspecific mating, an environmentally friendly method, to produce all-male tilapia fry, which yields a hybrid with a higher male proportion (MP). However, the presence of significant variation in MP among crosses of the same inter-specific hybrid combination is the main constraint against the use of hybrids (Lozano et al., 2014).

Previous studies have shown that Nile tilapia has an XX/XY male heterogametic system for sex determination, which is controlled by a major gene (Mair, Scott, Penman, Beardmore, & Skibinski, 1991). Based on crosses between XX females and YY males, researchers have developed genetically male tilapia (GMT) technology, an effective method for the large-scale production of all-male tilapia (Mair et al., 1995; Novelo, Gomelsky, Coyle, & Kramer, 2020). The fast and accurate identification of the sex genotype is critical for this method. Thus, sex-specific or sex-linked markers have been developed for several fish species, including Nile tilapia (Mei & Gui, 2015). There exist at least two major sex-determination genes on different sex chromosomes in Nile tilapia. A linkage analysis has mapped the sex chromosomes to LG1 in the Stirling strain of Nile tilapia (Ezaz et al., 2004; Lee et al., 2011; Palaiokostas et al., 2013). Sex-linked markers on LG23 are strongly associated with the sex-type in a number of unrelated populations of Nile tilapia (Caceres et al., 2019; Eshel et al., 2014; Sissao, D’Cotta, Baroiller, & Toguyeni, 2019; Sun et al., 2014). A major sex-determination gene, *amhy*, was cloned and identified on LG23 (Li et al., 2015). Thus, the LG23 sex-linked marker appears to be vital for controlling phenotypic sex in Nile tilapia.

Marker-5, one of the sex-linked markers on LG23 in Nile tilapia, is on the Fosmid clone, enabling the successful positional cloning of *amhy* (Li et al., 2015; Sun et al., 2014). Because Marker-5 is located on the same Fosmid clone as *amhy*, separated by a small physical distance, it is considered as a sex-specific marker in Nile tilapia. Marker-5 and derived or similar markers have been used to test the sex genotype of laboratory strains, cultured strains, as well as wild populations (Salirrosas et al., 2017; Sissao et al., 2019; Sultana, Khan, Hossain, & Alam, 2020; Zhao et al., 2020). Marker-5 could amplify large X-specific and small Y-specific bands, which could be distinguished by agarose gel electrophoresis. Also, Marker-5 could efficiently screen XX, XY, and YY fish. Thus, Marker-5 is an ideal marker for controlling phenotypic sex and GMT production of Nile tilapia. However, substantial variation in amplified X and Y fragments’ length would make sex genotyping difficult by Marker-5. The application of markers to new populations is expected to reveal new polymorphisms. Understanding polymorphisms in the sex-linked marker will provide a basis for the development of GMT in more strains via marker-assisted selection.
As mentioned above, a number of sex-linked markers have been developed for Nile tilapia. In this study, we used three markers (including Marker-5) to (a) examine marker polymorphism in the Hainan strain of Nile tilapia, (b) determine chromosomal locations of new alleles of Marker-5, and (c) evaluate the possibility of establishing GMT in the Hainan strain. Our results suggested that the larger band amplified by Marker-5 should not be considered a female-specific fragment in all strains of Nile tilapia. In addition, XX male and XY female fish in the Hainan strain were identified by Marker-5. The results of this study will help to control the sex-genotype of farmed Nile tilapia using the reported sex-linked marker. Our research will help to use GMT technology in different Nile tilapia strains.

2 MATERIAL AND METHODS

2.1 Experimental fish

In this study, we used two different Nile tilapia strains, one from Japan and one from China. The Nile tilapia from Japan (laboratory of Prof. Yoshitaka Nagahama from the National Institute of Basic Biology, Okazaki) was first introduced from Egypt and procured by Prof. De-Shou Wang (School of Life Sciences, Southwest University, Chongqing, China). Fin clips were collected from female and male individuals of this strain. The genotypes corresponding to XX females, XY males, and YY males, were confirmed by a progeny test to validate the sex-linked markers used in this study. The other commercial breeding strain was established in Hainan, China. The Hainan strain was derived from 10 strains. Some of the original strains were directly related to Genetically Improved Farmed Tilapia (GIFT) from the Philippines. Detailed information for the 10 strains is given in Table S1. One male and one female fish each from the 10 different strains were kept in the same tank (300 L) with flowing water and were allowed to self-reproduce. To avoid attacks, a black net was placed in the middle of the tank without reaching the tank bottom to reduce contact between the fish. In 2012, 31 families considered G1 of the Hainan strain were established by crossing fish from the 10 strains. The fingerlings were tagged with an injectable ID chip (Muyu Information Technology Co., Ltd., Guangzhou, China) at around 50 days after hatching. The fish were weighed after 100 days of culture and candidate broodstock were selected. The selected broodstock was randomly paired and inbreeding was avoided. From 2012 to 2020, one generation was established per year, labeled G1-9. In the present study, 50, 118, and 130 fish were sampled from G7, G8, and G9, respectively. Tail fins of the Hainan strain of Nile tilapia were sampled and stored at 

2.2 Genotyping of the Hainan strain using sex-linked markers

A nucleic acid purification kit (N1173, Dongsheng Biotecon, Guangzhou, China) was used to extract DNA from the tail fin, following the manufacturer's instructions. In the Nile tilapia from Japan, the sex determining gene amhy is a tandem duplicate of the autosomal amh gene, which was labeled as amhx. The original amh on the Y chromosome was truncated through multiple insertions/deletions in its coding region and was labeled as amhΔy (Figure 1). Several sex-linked markers were developed from this sex-determination region based on the insertions/deletions to distinguish the XX, XY, and YY genotypes (Li et al., 2015). Marker-5 could specifically amplify fragments of approximately 1,422 and 982 bp of the X and Y chromosomes, respectively (Sun et al., 2014). The presence of the amhΔy and amhy genes was ascertained using Marker-amhΔy-233 and Marker-amhy-5608, respectively. Marker-amhΔy-233 amplifies
an amhx and amhy fragment containing approximately 1,000 bp, along with an amhΔy-specific fragment containing 767 bp corresponding to a 233-bp deletion. Marker-amhy-5608 amplifies an amhx fragment containing approximately 8,022 bp, along with an amhy-specific fragment containing approximately 2,414 bp corresponding to a 5,608-bp deletion (Li et al., 2015). Table 1 lists the primers used in this study. Polymerase chain reaction (PCR) amplification was carried out using a Takara Ex Taq Kit (RR001A; Dalian, China) following the manufacturer’s instructions. The PCR products were analyzed on a 1–2.5% agarose gel stained with ethidium bromide.

2.3 | Cloning and sequence analysis

We cloned and sequenced the PCR products amplified by Marker-5 in the Hainan strain. In brief, the amplified bands were excised from the gel, purified, and cloned into the p-Easy-T3 vector (TransGen Biotech, Beijing, China), following the manufacturer’s instructions. Next, the positive clones were cultured for plasmid extraction and sequenced by the Sanger method. Alignments of homologous sequences were generated using MegAlign (DNASTAR).

2.4 | T7 Endonuclease I assay

T7 Endonuclease I can recognize and cleave heteroduplex DNA; thus, it was used to confirm the existence of a heteroduplex in the DNA samples. The PCR products were purified using the Gel Extraction Kit (N1073; Dongsheng Biotech, Guangzhou, China). Next, we added the purified PCR product (1 μg) to 10× NEBuffer (2 μL; M0302S, NEB, Ipswich, MA), and the volume was adjusted to 20 μL using ddH2O. The mixture was heated to 95°C for 5 min and then gradually cooled to room temperature. Then, 1 μL (10 units) of T7 Endonuclease I, 0.5 μL of 10× NEBuffer, and 3.5 μL of ddH2O were mixed and incubated at 37°C for 15 min. The digested PCR products were analyzed on a 2% agarose gel stained with ethidium bromide.

2.5 | Progeny testing

To confirm the inheritance pattern corresponding to the bands amplified by Marker-5, a progeny test was carried out using the Hainan strain. G9 established in 2020 had 187 families, among which 65 families were randomly
**TABLE 1** Sex-linked or sex-specific markers used for genotyping along with their polymorphism characteristics, sequences, predicated size of the amplified product, the chromosomal location, and the corresponding references

| Markers ID   | Polymorphism detected                        | Primers sequences 5'-3'                      | Amplified fragments (bp) | Specificity for chromosome X or Y (amh gene) | Product name and references                   |
|--------------|----------------------------------------------|----------------------------------------------|--------------------------|-----------------------------------------------|-----------------------------------------------|
| Marker-5     | Deletion/insertion in the amhx promoter region | F1:ATGGCTCCGAGACCTTGACTG R1:CAGAAATGTAGACGCCCAGGTAT | 1,422 and 1,688          | X (amhx)                                    | Sun et al. (2014) and present study           |
|              |                                              |                                              | 982                      | Y (amhΔy and amhy)                           |                                               |
| Marker-amhΔy-233 | 233 bp deletion in the amhΔy exon 6         | F2:CGGTCCCAGTGACCTATGAG R2:AAGTACACGTGGTGTATTGTAATTGA | 1,000                    | X&Y (amhx and amhy)                          | Eshel et al. (2014)                           |
| Marker-amhy-5608 | 5,608 bp deletion in the amhy promoter region | F3:GAAAGGGGTGTTTGGTGCTGGC R3:ACCCAGGAAGGCTTCATCTC | 8,022                    | X (amhx)                                    | Li et al. (2015)                              |
|              |                                              |                                              | 2,414                    | Y (amhy)                                    |                                               |
chosen and screened by parental genotyping with sex-linked markers. Then, progeny from the families of G9 fish whose parents had the required genotypes were randomly sampled.

3 | RESULTS

3.1 | Sex genotyping of Nile tilapia from Hainan strain using sex-linked markers

First, farmed Nile tilapia from G7 of the Hainan strain (20 females and 30 males) was genotyped using Marker-5. Here, Marker-5 amplified a single X-specific band in a large proportion of female fish (15/20), consistent with the size in XX female fish from Japan (Figure 2). Therefore, these fish were labeled as XX females. However, apart from the X-specific band, Marker-5 amplified another relatively larger band in five female fish (Nos. 1, 3, 4, 18, and 19 of G7). We found a similar amplification pattern in one male fish (No. 16 of G7). Also, Marker-5 amplified both X- and Y-specific bands in a large proportion of the male fish (20/30), and only a single X-specific band in eight male fish (Nos. 1, 5, 9, 10, 18, 19, 21, and 24 of G7). These fish were labeled as XY or XX male fish. Interestingly, we found three amplified bands: one Y-specific band, one band exceeding the size of the normal X-specific band, and an unknown middle band, in a male fish (No. 8) of G7 (Figure 2).

Next, we tested the genotypes of G7 of the Hainan strain using two pairs of sex-linked markers, Marker-\(amh\Delta y\)-233 and Marker-\(amhy\)-5608 (Figures 3 and 4). In the control Japanese strain, Marker-\(amh\Delta y\)-233 amplified one \(amhx\)-specific band in the XX female fish. In the XY and YY male fish, it detected one band for \(amhx\) and/or \(amhy\), and another shorter band for \(amh\Delta y\). In G7 of the Hainan strain, Marker-\(amh\Delta y\)-233 detected no \(amh\Delta y\)-specific band in the XX female and male fish, while most male (21 out of 30) fish showed both the \(amh\Delta y\)-specific band and the Y-
specific fragment amplified by Marker-5, including fish No. 8 (Figure 3). In the Japanese strain, Marker-amh-DΔy-233 amplified approximately 8,022-bp amhx-specific bands and approximately 2,414-bp amhy-specific bands in the XX female and YY male fish, respectively. In the XY male fish, it typically amplified the 2,414-bp amhy-specific band and not the 8,022-bp amhx fragment because of the difference in amplification efficiency between the longer and shorter fragments. In all female fish of G7 of the Hainan strain, Marker-amh-DΔy-233 detected an 8,022-bp amhx-specific band; however, no 2414-bp amhy-specific bands were detected. Also, Marker-amh-DΔy-233 amplified the amhy-specific bands only in the male fish of G7 of the Hainan strain, which possessed the Marker-5-amplified Y-specific fragment (Figure 4).

### 3.2 | Sequencing of the bands amplified with Marker-5

The sequence of the X-specific bands from a normal XX female (No. 5) and normal XY male (Nos. 4 and 6) from G7 of the Hainan strain showed a high similarity (99.7%) to that of the Japanese strain (Figure S1). The sequence of the Y-specific bands from normal XY male (Nos. 4 and 6) and the unusual male (No. 8) from G7 of the Hainan strain showed a high similarity (>99.5%) to that of the Japanese strain (Figure S2). In female fish No. 1 from G7 of the Hainan strain, the smaller band was 1,422 bp and showed a high similarity (99.7%) to that of the Japanese strain, while the larger band was 1,688 bp and showed 99.1% and 99.0% similarity to those of 1,422-bp X-specific and 982-bp Y-specific bands of the Japanese strain, respectively (Figures S1 and 5). An alignment of the sequences revealed that the 1,422-bp X-specific band has two deletions of 36 and 234 bp at Positions 1 and 3, respectively, and an insertion.

**FIGURE 3** Sex genotyping of the Nile tilapia from G7 of the Hainan strain using a sex-linked marker (Marker-amh-DΔy-233). Controls are genetic XX, XY, and YY Nile tilapia from the Japanese strain confirmed by a progeny test. Female 1, 3, 4, 18, and 19 are XX, identified by Marker-5, while the other female are XX. One XX, male (No. 16), one X,Y male (No. 8) and eight XX male (Nos. 1, 5, 9, 10, 18, 19, 21, and 24) were identified by Marker-5, while the other male fish are XY. In G7 of the Hainan strain, Marker-amh-DΔy-233 detected no amh-DΔy-specific band in the XX/XXL fish, while XY/X,Y male fish showed both the amh-DΔy-specific and amhx/amhy specific bands. M, DNA Marker
of 4 bp at Position 2, compared with the 1,688-bp band (Figure 5). The 1,688-bp band was classified as an allele of the normal X- and Y-specific bands based on the high sequence similarity. In five female individuals (Nos. 1, 3, 4, 18, and 19) in G7 of the Hainan strain, 1,422-bp X-specific and 1,688-bp bands were amplified by Marker-5, and amhy was not found. Thus, the 1,688-bp fragment might not be linked to amhy. We deduced that it was female sex linked and labeled it XL. Thus, the genotype of female Nos. 1, 3, 4, 18, and 19 of G7 along with male No. 16 of G7 was labeled as XXL. The largest band from male fish No. 8 of G7 was also 1,688 bp and was 99.5% similar to XL of female fish No. 1 (Figure S3). Thus, the genotype of male No. 8 of G7 was labeled as XLY. The NCBI accession number of 1,688 bp XL is MT385057. Next, we cloned and sequenced the middle band of male fish No. 8 and found that the clones from the middle band were either 1,688 bp XL or a 982 bp Y fragment, indicating that this band could be a heteroduplex of XL and Y.
3.3 Mimic the special amplification in X_{L}Y male fish

The plasmids containing 1,688 bp X_{L}, 1,422 bp X, or 982 bp Y-specific fragment, either alone or combined, were amplified using Marker-5. The results showed that Marker-5 amplified a single band in plasmids containing X_{L}-, X-, or Y-specific fragment alone. As expected, the mixed plasmids of X and Y produced two bands; however, the mixed plasmids of X_{L} and Y produced an additional band between the predicted X_{L}- and Y-specific bands. This special band was similar in size to that of the X_{L}Y male fish (No. 8 of G7). Thus, we deduced that the special band in the X_{L}Y

FIGURE 5  Sequence alignments of Nile tilapia genomic DNA, amplified with specific primers for Marker-5. Each fish genotype was sequenced at least five times. The image was created using DNASTAR and GeneDoc. X 1688: cloned from female fish No. 1 of the Hainan population; X 1422: the Japanese strain X-specific fragment; Y 982: the Japanese strain Y-specific fragment. P1: X-specific 36-bp deletion; P2: X-specific 4-bp insertion; P3: X-specific 266-bp deletion
fish, which was amplified by Marker-5, does not exist in the genome, and was produced by the mixture of the Xₐ and Y fragments during PCR (Figure 6).

Next, we mixed the PCR products of the plasmids containing Xₐ- and Y-specific fragments directly without purification to investigate the generation of this special band during PCR amplification with Marker-5. The mixed PCR products were directly tested using agarose gel electrophoresis, both before and after treatment at different temperatures. The mixture of PCR products without heating produced only Xₐ- and Y-specific bands. However, if the mixture of PCR products was heated to 94°C for 30 s, we observed three bands in the agarose gel, including the special band (Figure S4). Next, we purified the PCR products of Xₐ- and Y-specific fragments and mixed them with the primers, PCR buffer, and Taq enzyme, either alone or combined, to explore the critical factors in the PCR reaction, which resulted in the formation of the special band after heating. The results showed that only the PCR buffer was necessary for the formation of the special band. We observed that excluding the addition of the buffer after heating resulted in DNA damage, which was evident based on the observation of multiple bands in the agarose gel (Figure S5A,B). We amplified the DNA from the XₐY fish using Marker-5 and analyzed the PCR products by gradient agarose gel electrophoresis. We observed that as the gel concentration increased from 1.5 to 2.5%, the special band moved closer to the Xₐ band (Figure S6). Thus, the T7 Endonuclease I assay was performed to confirm the presence of a heteroduplex in the special band. The purified Xₐ- and Y-specific fragments were mixed with the NEBuffer and heated. We observed that the special band of the NEBuffer group was detectable but was weaker than that of the PCR buffer group. T7 Endonuclease I completely removed the special band, thus increasing the smear signal, while the Xₐ- and Y-specific bands were unaffected (Figure S7). The single nucleotide polymorphisms (SNPs) between Xₐ and Y might have been responsible for the smear-like nature of the digested heteroduplex. The results of this assay indicated that the special band was a heteroduplex of the Xₐ- and Y-specific fragments.

3.4 Mendel inheritance of X-, Xₐ-, and Y-specific fragments

In the Hainan strain, 61 female and 53 male fish from G8 were used to establish 65 families of G9. Marker-5 identified 8 female and 6 male XXₐ fish among the 114 fish from G8, while no XₐY fish were identified, reflecting the low allele frequency of Xₐ in the Hainan strain (Figure S8). Of course, more samples should be tested to confirm the allele frequency of Xₐ in the Hainan strain. In addition, five XY fish were found in 65 G8 female fish.
Two families were randomly selected to determine whether X_L is an allele of X- and Y-specific fragments amplified by Marker-5. In family 200604 of G9, an XX_L female (family ID 190531 of G8) crossed with an XX male (family ID 190516 of G8) yielded 16 female and 14 male fish, suggesting that the sex ratio does not deviate from 1:1 ($\chi^2 = 0.133, 0.5 < p < .75$) in this family. Marker-5 identified 16 XX ($9\varphi, 7\delta$) and 14 XX_L ($7\varphi, 7\delta$) fish, suggesting that the X and XL segregation ratio is similar at 1:1 ($\chi^2 = 0.133, 0.5 < p < .75$) (Figure 7). In family 200629 of G9, female XX_L (family ID 190522 of G8) were crossed with male XY (family ID 190208 of G8), and XX, XX_L, XY, and X_LY offspring were identified, with a segregation ratio of 1:1:1:1 ($\chi^2 = 5.4, 0.1 < p < .25$). All X_LY offspring showed the special heteroduplex band, as expected. In this family, all XX and XX_L fish were female, while balanced sex ratios were found in XY ($\chi^2 = 0, p > .99$) and X_LY ($\chi^2 = 0.44, 0.25 < p < .5$) fish (Figure 7). In the offspring and parents of families

![Figure 7](image-url)
200604 and 200629, amhΔy and amhy specific bands were only found in XY/XLY fish and were not found in XX/XXL fish (Figures S9 and S10).

3.5 | Polymorphism of the Y-specific fragment in one family from the Hainan strain

Consistent with the G7 fish, Marker-amhΔy-233 detected no amhΔy-specific band in the XX and XXL female and male fish from the G8 fish (Figure S11). Also, consistent with the G7 fish, Marker-amhy-5608 detected an 8,022-bp...
amhx-specific band in female XX and XXL fish from G8, while no 2414-bp amhy-specific bands were present (Figure S12). The XY female and male fish from G8 also showed the same genotype to the XY male in G7 identified by the sex-linked markers (Figures S8, S11, and S12). Unexpectedly, in three XXL males (23, 27, and 28) from G8, Marker-5 identified 17 XX and 13 XXL individuals, which suggests that the X and XL segregation ratio is balanced. P indicates the parent. M, DNA Marker. Black and red numbers indicate female and male fish, respectively.

In family 200516 of G9, for the cross between female XX (family ID 190221 of G8) and male XXL (family ID 190234 of G8), Marker-5 identified 17 XX and 13 XXL individuals, which suggests that the X and XL segregation ratio is balanced. P indicates the parent. M, DNA Marker. Black and red numbers indicate female and male fish, respectively.

amhx-specific band in female XX and XXL fish from G8, while no 2414-bp amhy-specific bands were present (Figure S12). The XY female and male fish from G8 also showed the same genotype to the XY male in G7 identified by the sex-linked markers (Figures S8, S11, and S12). Unexpectedly, in three XXL males (23, 27, and 28) from G8, Marker-amhΔy-233 and Marker-amhy-5608 detected the amhΔy- and amhy-specific bands, respectively (Figure S8, S11, and S12). These three unusual XXL males belonged to the same family (family ID 190234). This implies that either X or XL amplified by Marker-5 in those fish is linked to amhΔy and amhy. Thereafter, two families with male parents from family 190234 were evaluated by a progeny test.

In family 200516 of G9, for the cross between female XX (family ID 190221 of G8) and male XXL (family ID 190234 of G8), Marker-5 identified 17 XX and 13 XXL individuals, which suggests that the X and XL segregation ratio is balanced. P indicates the parent. M, DNA Marker. Black and red numbers indicate female and male fish, respectively.

FIGURE 9 Sex genotyping of offspring from family 200539 of the Hainan strain using sex-linked markers (Marker-5, Marker-amhΔy-233, and Marker-amhy-5608). The dam was a sex-reversed XY female fish. The sire was the rare XXL fish genotyped by Marker-5, as amhΔy- and amhy-specific bands were detected. The boxed offspring are XXL fish in which amhΔy- and amhy-specific bands were not found, while amhΔy- and amhy-specific bands were found in all offspring identified as XX, XY, and X1Y by Marker-5. These results suggest that amhΔy and amhy are linked to the fragment with a same size to the X-specific band amplified by Marker-5 in the sire, but are not linked to X1. The XX, XY, and X1Y offspring are all male, while the sex ratio of the XXL offspring is balanced. P indicates the parent. M, DNA Marker. Black and red numbers indicate female and male fish, respectively.
was nearly 1:1 ($\chi^2 = 0.53, 0.25 < p < .5$) (Figure 8). All XX progeny were male, while a female-skewed sex ratio (84.6% female, $n = 13$) was found in XXL progeny. In the progeny of family 200516, the amhΔy-specific band and amhy-specific band were detected in XX offspring by Marker-amhΔy-233 and Marker-amhy-5608, respectively, unlike in the XXL offspring (Figure 8). In family 200539 of G9, for the cross between female XY (family ID 190603 of G8) and male XXL (family ID 190234 of G8), Marker-5 identified XX, XXL, XY, and XLY progeny, with a segregation ratio of approximately 1:1:1:1 ($\chi^2 = 1.47, 0.5 < p < .75$). In this family, a balanced sex ratio was only found in the XXL progeny ($\chi^2 = 0.07, 0.75 < p < .9$), while the other offspring were all male. In the progeny of family 200539, except for XXL offspring, Marker-amhΔy-233 and Marker-amhy-5608 only showed the amhΔy-specific band and amhy-specific band (Figure 9). Sequences of the X fragment amplified by Marker-5 from the male parents of families 200516 and 200539 were the same (1,422 bp), as determined by Sanger sequencing, with 99.9% similarity with the 1,422 X-specific band from the Japanese strain, with only one SNP (Figure S13). Combined with the progeny testing results for families 200516 and 200539, the 1,422-bp fragment, but not XL amplified by Marker-5 was linked to amhΔy and amhy, and the 1,422-bp fragment was located on the Y chromosome. To distinguish the 1,422-bp X-specific and 982-bp Y-specific fragment, the 1,422 bp fragment linked to amhΔy and amhy from family 190234 was marked YL. XX and XXL offspring from family 200516 were designated XYL and XXL, respectively. In family 200539, XX, XXL, XY, and XLY offspring were marked XYL, XXL, YYL, and XLY, respectively (Figure 10). The progeny test showed that X, XYL, Y, and YLYL fragments amplified by Marker-5 are alleles. The X- and YLYL-specific fragments were both 1,422 bp. The utilization of other sex-linked markers can help to distinguish among XX, XXL, and YLYL fish based on Marker-5 amplification in Nile tilapia.

4 | DISCUSSION

All-male sex farming is the industry standard for the tilapia species. All-male tilapia fingerlings are mostly obtained by androgen hormone treatment. Nile tilapia is a worldwide aquacultured species and thus billions of all-male fry are produced as a result of hormonal treatments. It is difficult to predict the impact of hormone use on the environment and human health. GMT is an ideal substitute for hormonal treatment and has been investigated extensively (J. Chen, Fan, Tan, Jiang, & Wang, 2018, 2019; Gennotte et al., 2015; Mair et al., 1995; Salirrosas et al., 2017; Sultana et al., 2020; Sun et al., 2014). Sex-specific or sex-linked markers accelerated the development of this technology in multiple strains of Nile tilapia. The present study presents the first analysis of polymorphism in Marker-5, one of the
most widely used sex-linked markers, which will be helpful for the application in GMT technology to different strains of Nile tilapia. There is no report of testing polymorphism of Marker-5 in other strains of Nile tilapia.

Sequence analysis indicated that $X_L$ amplified by Marker-5 was similar to, but longer than $X$-specific fragment; and $Y_L$ amplified by Marker-5 was also similar to, but longer than $Y$-specific fragment. In families 200516 and 200629, the sex ratios of $XX_L$ fish were female-sex skewed and all-female, respectively, suggesting that $X_L$ might be linked to the female genotype in tilapia. In addition, $amhx$ was linked to either 1,688-bp $X_L$-specific fragment or 1,422-bp $X$-specific fragment suggesting that $X_L$ was an allele of the 1,422 bp $X$-specific fragment and was located on the $X$ chromosome. On the other hand, the progeny test revealed that the 1,422-bp $Y_L$-specific fragment was linked to $amhy$ in the family 190234. To our knowledge, this is the first evidence report that the 1,422-bp fragment amplified by Marker-5 is also located on the $Y$ chromosome in Nile tilapia. Therefore, the 1,422-bp fragment amplified by Marker-5 could be located on either the $X$ or $Y$ chromosome in Nile tilapia and should not be considered a female-specific fragment in all strains. As Marker-5 is very close to $amh$, $amhΔy$, and $amhy$ on the sex chromosomes, we deduced that the co-existence of the 1,422-bp $Y_L$ fragment amplified by Marker-5 and $amhΔy/amhy$ is not caused by recombination near the sex determination region. In contrast, the duplication and pseudogenization of $amh$, producing $amhy$ and $amhΔy$, have already happened on the $Y$ chromosome. In the future, it is necessary to determine if the 1,688-bp $X_L$ fragment amplified by Marker-5 is co-located with $amhy$ on the $Y$ chromosome in some strains of Nile tilapia. In summary, in a Hainan strain of Nile tilapia, $X_L$ and $Y_L$ fragments amplified by Marker-5 are female- and male sex-linked, respectively.

$XX$ fish, detected by Marker-5, was 100% female in the Japanese strain, while $XX$ male fish were found in small proportion of some tilapia strains in China (Sun et al., 2014). Two explanations have been proposed for the existence of $XX$ males identified by $amhy$-linked markers in Nile tilapia (Sissao et al., 2019). First, there might be other major male-determining loci in these $XX$ male fish, such as the sex-determination locus on LG1 of Nile tilapia, which could explain the balanced sex ratio in the progeny produced by the cross between $XX$ males with $XX$ females (Palaiokostas et al., 2013). The major male sex-determination locus on LG1 was probably introduced by closely related species, such as blue tilapia, via hybridization (Lee et al., 2005). In this study, balanced sex ratios were found in both $XX$ and $XX_L$ fish from the family 200604, suggesting that another male determination locus exists in this family. There might be some female sex determination alleles that could be also introduced into the Hainan strain, such as the female sex determination allele on W chromosome (LG3) from blue tilapia which is epistasis to male sex determination allele on LG1 (J. Chen et al., 2018; Wu et al., 2021). The different $XX/XX_L$ male ratios observed in the present study might be due the presence or absence of the major female sex determination alleles on W chromosome from blue tilapia. Thus, future studies using positional cloning aimed at the identification of the major sex-determination gene on LG1 and LG3 in different species of tilapia will help us interpret the unusual $XX/XX_L$ male fish in the present study. Another explanation for $XX$ male fish is sex reversal. It is possible that the sex of the $XX/XX_L$ fish was reversed by environmental factors, such as temperature (Baroiller, D’Cotta, & Saillant, 2009; Zhao et al., 2020). When the temperature increased from 25 to 35°C, the proportion of male Nile tilapia fry (4 days after hatching) increased from 52.5% to 75.9% after one week of rearing (Khater, Ali, & Mohamed, 2017). In the present study, the offspring from families of G9 of the Hainan strain were cultured at 30 ± 1°C. A relatively low proportion of males (2 of 13, 15.4%) was found in $XX_L$ offspring from family 200516 of G9. The sex-reversed $XX_L$ offspring from this family might be caused by water temperature. In family 200539, progeny testing showed that $XY_L$ offspring are all male, suggesting that $Y_L$-linked $amhy$ can control the male sex. In contrast, agarose gel electrophoresis could not distinguish between $XX$ and $XY_L$ fish. The $XY_L$ fish identified by Marker-5 could be mistakenly identified as $XX$ fish. Thus, marker polymorphism is the third explanation for the existence of $XX$ male fish. This implies that Marker-5 could not be used alone to genotype Nile tilapia of different strains. Taken together, three explanations were proposed for the identification of $XX$ male fish by Marker-5. Marker polymorphism, other major male determination loci, and sex reversal should explain for all male, balanced, and female-skewed sex ratios, respectively, in $XY_L/XX/XX_L$ fish identified by Marker-5 in Nile tilapia.
Herein, Marker-5 also identified XY/X_L Y female fish in Hainan strain. Unlike the female-to-male sex reversal of XX fish, which might be caused by high temperatures, there is no report of sex reversal in XY Nile tilapia because of water temperature. Of course, male-to-female sex reversal in fish could be induced by other environmental factors, such as environmental endocrine-disrupting chemicals (Dong et al., 2014). There were no factories around the fish farm where the Hainan strain was cultured in this study, but we could not exclude the possibility that fish sex was influenced by chemicals from agriculture or aquaculture farms. Mutations in the major sex-determination gene \( \text{amhy} \) result in sex reversal in Japanese medaka (Matsuda & Sakaizumi, 2016). A wild population of Nile tilapia with a high proportion of females possessed \( \text{amhx} \) and \( \text{amhy} \) and lacked \( \text{amh} \Delta y \) (Sissao et al., 2019); according to the authors, \( \text{amhy} \) alone, without \( \text{amh} \Delta y \), could not reach the threshold necessary to induce male sex differentiation (Sissao et al., 2019). However, the XY fish with \( \text{amh} \Delta y \) mutant alleles displayed no sex reversal indicating that \( \text{amh} \Delta y \) should be not critical for sex determination in Japanese Nile tilapia strain (Li et al., 2015). In the present study, balanced sex ratios were found in both XY and X_L Y fish possessing both \( \text{amh} \Delta y \) and \( \text{amhy} \) from family 200629. The high proportion of XY/X_L Y females suggests that a mutation in \( \text{amhy} \) could weaken its ability to control the sex-type. Natural XY female Nile tilapia from a fish farm in China were crossed with an XY male fish from the Japanese strain; their F1 YY male fish was crossed with an F1 XX female fish to produce the F2 generation (D. Jiang et al., 2020). In the F2 generation, two naturally sex-reversed XY female fish (2%, \( N = 97 \)) were found, indicating that there might be some female sex-determination alleles present, which could influence the ability of \( \text{amhy} \) to control the male sex type.

While evidence of the presence of female sex-determination alleles is still lacking, female sex-linked marker might be identified in the XY/X_L Y fish in our future study. In summary, female XY fish identified by Marker-5 could also be attributed to environmental factors, mutations related to \( \text{amhy} \), and other female sex-determination alleles. In addition, XX_L fish, which showed two bands by Marker-5, could be empirically mistaken as XY. Marker-5 also identified XY female fish in farmed Nile tilapia from China (Sun et al., 2014). Some of which were a result of wrong genotyping because of polymorphism. Thus, the results of the present study should contribute to more accurate identification of the sex genotype of Nile tilapia. These studies also imply that the strains with natural sex-reversed XY female fish, identified by Marker-5, should not be used directly to develop GMT technology. A female ratio of progeny of YY males of <2% is still acceptable, as the male ratio is usually controlled at over 98% by methyltestosterone in large-scale production in fish farms. However, a higher proportion of XY females from YY males should be the main constraint against the use of GMT. In families 200516 and 200539, all fish possessing \( \text{amhy} \) were male, indicating that \( \text{amhy} \) is a strong male determination locus in these families. In addition, the XY/XY/XY_L male ratio reached 89.1% (\( N = 55 \)) in randomly sampled fish from G7 and G8 of the Hainan strain. These results suggested that \( \text{amhy} \) can control the male sex type in some families in the Hainan strain, providing a basis for the development of GMT with \( \text{amhy} \)-linked markers. Future studies should investigate the mechanisms underlying the formation of naturally sex-reversed XY/X_L Y female Nile tilapia. Continuous studies of sex determination in tilapia will help to resolve these issues, which limited the utilization of GMT.

Nile tilapia can hybridize with closely related species, such as blue tilapia and Mozambique tilapia. A single band similar to that of the 1,422-bp X-specific fragment was amplified by Marker-5 in both male and female blue tilapia (D. Jiang, 2016). Therefore, in the Hainan strain, the 1,688-bp X_L allele was not introduced from blue tilapia. The most farmed GIFT tilapia population shows introgression from Mozambique tilapia genomic DNA (C. H. Chen, Li, Gu, Lin, & Xia, 2019; Taslima et al., 2020). Thus, X_L, amplified using Marker-5, might have been introduced from Mozambique tilapia. However, it is possible that the 1,688-bp X_L allele existed in the natural Nile tilapia population. \( Y_L \) amplified by Marker-5 is linked to \( \text{amhy} \) and \( \text{amh} \Delta y \), while this is only found in Hainan strain of Nile tilapia. There is the need for further study to clarify whether \( Y_L \) exists in other Nile tilapia strains or tilapia species. The Hainan strain used in this study was not a pure strain, but a mixed strains of 10 Chinese strains, the genetic background of some strains were unknown. It is also important to analyze the polymorphism of Marker-5 in Nile tilapia pure strain in the future study.

Interestingly, when the PCR products of X_L Y fish, amplified by Marker-5, were tested using agarose gel electrophoresis, another band, apart from the X_L or Y fragment, was observed. Cloning and Sanger sequencing of this
special band from X\(_{L}\)Y fish showed that its sequence was highly similar to the X\(_{L}\) or Y sequences. The sequence analysis of X\(_{L}\) and Y showed that they shared high similarity in their 5' and 3' regions. X\(_{L}\) and Y could also form heteroduplex during annealing after the denaturation at 94°C. T7 Endonuclease I assay confirmed that the special band detected by the agarose gel contained heteroduplex. On the contrary, we did not detect an additional band in the PCR products of the normal XY fish whose DNA was amplified by Marker-5. This could probably be attributed to the length difference between 1,422-bp X fragment and 982-bp Y fragment, which is relatively small, and thus their heteroduplex might have had migration rates similar to that of the normal X fragment. Sanger sequencing revealed that part of the clones from the normal X-specific band of the XY fish was consistent with the 982-bp Y fragment (data not shown). When heteroduplex were tested by polyacrylamide gel electrophoresis, the migration rate of the heteroduplex was much slower than that of homodimers (D. N. Jiang et al., 2016). The difference between polyacrylamide gel and agarose gel is based on the gel characteristics, such as the gel density and hole diameter. Thus, our results showed a special electrophoresis pattern of the sex-linked DNA Marker-5 in Nile tilapia, which might have universal implications for other DNA markers in other aquaculture species.

5 | CONCLUSION

Here, we studied polymorphism in a sex-linked DNA marker, Marker-5. The 1,688-bp X\(_{L}\) and 1,422-bp Y\(_{L}\) alleles, amplified using Marker-5, were first analyzed in a Nile tilapia strain from Hainan. X\(_{L}\) and Y\(_{L}\) were linked to amhx and amhy, respectively. Both X\(_{L}\) and Y\(_{L}\) sequences were similar to the sequences of the normal X and Y amplified by Marker-5. X\(_{L}\) might have originally been introduced from other tilapia species via hybridization. PCR analyses of X\(_{L}\)Y fish using Marker-5 produced an additional band between the X\(_{L}\)- and Y-specific bands, which was a heteroduplex. Marker-5 could not distinguish between XX and X\(_{L}\)-Y\(_{L}\) fish or between XX\(_{L}\) and X\(_{L}\)-Y\(_{L}\) fish alone. The results of molecular sexual identification by the Marker-5 in Hainan strain are complex, and it is inappropriate as one strain for GMT development at present. X\(_{L}\) and Y\(_{L}\) are rare cases in some strains of Nile tilapia. In most cases, Marker-5 is applicable in aquaculture as reported by Sun et al., 2014. The results of the present study can improve the utilization of Marker-5 for the development of GMT technology in farmed Nile tilapia strains.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Dong-Neng Jiang and Zi-Ying Kuang carried out the majority of the experiments, performed the statistical analyses and drafted the manuscript. Kong-Song Yang provided the fish from Hainan. Yuan-Qing Huang, Umar Farouk Mustapha, Xiang-Zhao Guo, and De-Feng Zheng performed the experiments. Dong-Neng Jiang, Hong-Juan Shi, and Zhong-Dian Dong designed and supervised the experiments, analyzed data, and critically edited the manuscript. All authors read and approved the final manuscript.

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