Sex-Associated Microsatellite Marker for Neomale Identification and Histological Analysis of Gonadal Maturation in *Ompok bimaculatus*

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ABSTRACT

This study aimed to develop sex-associated microsatellite markers for identifying neomales in *Ompok bimaculatus* and to characterize their gonadal structures histologically. Newly hatched larvae were treated with 17α-methyltestosterone (MT), and MT-treated fish were sampled up to 180 days post-hatching. Gonadal tissues were examined histologically, and caudal fins from ten males, ten females, and ten MT-treated individuals were used for DNA extraction. MT-treated fish showed smaller, translucent testes with markedly reduced spermatozoa density. A novel sex-associated microsatellite marker was identified, characterized by a dinucleotide (CA)n repeat: $(CA)_{19}$ in males and $(CA)_{15}$ in females and neomales, indicating sex linkage. These findings support the molecular identification of neomales and provide a foundation for developing reliable markers for sex determination and controlled breeding in *O. bimaculatus*.

Keywords: DNA markers, Histological analysis, Neomale, *Ompok bimaculatus*

INTRODUCTION

The butter catfish (*Ompok bimaculatus*, Siluridae) is a commercially important freshwater species in South and Southeast Asia, valued for its delicate flavor, firm flesh, and high nutritional quality (Paul *et al*., 2018; Biswas *et al*., 2023). Wild populations have sharply declined due to overexploitation and habitat loss, leading to its near-threatened status (IUCN, 2014). Sustainable aquaculture and genetic improvement programs are therefore critical for both conservation and production. Sex control and selective breeding are key strategies for enhancing productivity in *O. bimaculatus*, as females exhibit superior growth, reaching up to twice the length and five times the body weight of males (Gupta, 2015). Producing all-female cohorts has been proposed to improve growth uniformity and yield, and this approach has been successfully applied in other aquaculture species via hormonal manipulation, chromosomal modification, or gynogenesis (Nagahama *et al*., 2021; Rahman *et al*., 2021). Androgen-induced masculinization of genetic females (XX) to generate neomales enables the production of all-female progeny when crossed with normal females in XX/XY or XX/XO systems (Brown *et al*., 2021; Colihueque and Parraguez, 2024). *O. bimaculatus* likely follows an XX/XY system, where neomales are phenotypically indistinguishable from XY males, which highlights the need for DNA-based sex markers. Traditional progeny testing is laborious and time-consuming; molecular markers allow early, non-lethal genotypic sex identification, streamlining broodstock selection.

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SOX family genes, particularly *SOX9*, are key regulators of testis development in vertebrates and have been associated with male sex in several teleosts, including *Oreochromis niloticus* (Wei *et al*., 2016), *Danio rerio* (Chiang *et al*., 2001), and *Clarias gariepinus* (Raghuveer and Senthilkumaran, 2010). The *SOX5* gene also contributes to early gonadal differentiation, though its role is less well defined. In *O. bimaculatus*, partial SOX sequences are available, but their association with phenotypic sex or hormone-induced sex reversal remains unexplored, making them promising targets for molecular marker development. Histological examination of gonads remains essential for confirming phenotypic sex and gonadal development. Combining molecular and histological analyses provides a comprehensive framework for validating sex reversal and enhancing monosex seed production. This study aimed to develop and validate sex-associated microsatellite markers for neomale identification in *O. bimaculatus* and to characterize gonadal structures via histology. The results are expected to support the development of molecular tools for sex-controlled breeding and sustainable aquaculture of this economically valuable species.

MATERIALS AND METHODS

Ethics statement

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Thaksin University (Approval No. TSU 2024-015) and conducted with established ethical guidelines for the care and use of animals in research.

Production and rearing of neomales

Neomales were produced through hormonal sex reversal using 17α-methyltestosterone (MT). Fertilized eggs were obtained from naturally spawned broodstock of known phenotypic sex, verified by gonadal examination. Spawning was induced by intramuscular injection of buserelin acetate (20 $\mu g \cdot kg^{-1}$) and domperidone (10 $mg \cdot kg^{-1}$) in females, while males received half of the dosage. Following hatching, larvae were immersed in an MT solution (200 $\mu g \cdot L^{-1}$) for 3 h. Subsequently, they were fed live *Daphnia* spp. enriched with MT (60 $\mu g \cdot g^{-1}$) for four weeks. After the hormone treatment period, fish were transitioned to a commercial pellet diet and reared under standard hatchery conditions until 180 days post-hatching.

Phenotypic sex and histological analysis

A total of 40 phenotypically male fish were selected for inclusion in the study, comprising 20 individuals treated with MT and 20 untreated controls. Phenotypic sex was confirmed based on the presence of rough, serrated pectoral fins, a characteristic indicative of male individuals. Prior to dissection, all specimens were anesthetized using clove oil at a concentration of 160 $mg \cdot L^{-1}$, in accordance with standard ethical guidelines to minimize animal distress. The testes were subsequently excised for further analysis. Initially, gross morphological characteristics of the testes were assessed, after which the tissues were preserved for histological evaluation. Fixation was performed by immersing the samples in 10% neutral buffered formalin for 24 h. The fixed tissues were then processed using an automated tissue processor, following standard histological protocols described by Humason (1979), with slight modifications. The samples were dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin wax. Serial transverse sections were prepared at a thickness of 5 µm using a rotary microtome. For each specimen, a minimum of 10 sections was selected and stained with hematoxylin and eosin (H&E) to evaluate testicular architecture. Microscopic examination was conducted using a Zeiss Axio Imager A2 light microscope equipped with an Axiocam 506 digital camera for photomicrographic documentation. Testicular structures and developmental stages were identified and classified according to the histological criteria established by Siddiqua *et al*. (2000).

PCR primer design for specific gene markers

Specific gene markers were designed based on genomic and transcriptomic data from *O. bimaculatus*, with a focus on sex determination-related regions (Dhar *et al*., 2019). Reference sequences for *O. bimaculatus SOX9* (*SRY-box 9a2*), *Dmrt1b* (double sex and mab-3 related transcription

factor 1b), and β-actin mRNA were retrieved from GenBank under accession numbers MN095653.1, MN095654.1, and MZ666132.1, respectively. To broaden marker identification, a comparative analysis was conducted using differentially expressed genes from *Silurus aristotelis*, a closely related species within the Siluridae family. The *S. aristotelis* transcriptome was assembled de novo using Trinity v2.2.0 (Grabherr *et al*., 2011) and used to identify orthologous sequences associated with sex differentiation. These included *SOX5* (a sex-determining region Y-related transcription factor 5), *NANOS1* and *NANOS3* (involved in germ cell development), and α-2 actin (a smooth muscle-specific housekeeping gene). Primers for target genes and internal controls were designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to ensure specificity and optimal amplification of candidate sequences. The selected primers (Table 1) were used for subsequent PCR amplification and molecular analysis.

Validation and identification of sex-associated markers

Genomic DNA was extracted from caudal fin tissues of *O. bimaculatus*, including 10 genetic females (XX), 10 genetic males (XY), and 10 MT-treated fish. DNA extraction was performed using the TIANamp Marine Animals DNA Kit (Tiangen, China), following the manufacturer's protocol. The quality and concentration of the extracted DNA were assessed using a NanoDrop spectrophotometer and confirmed by 1% agarose gel electrophoresis. All DNA samples were stored at -20 °C until further analysis.

Polymerase chain reaction (PCR) was carried out to amplify the target DNA regions. Annealing temperatures were optimized using gradient PCR. Each 25 µL reaction contained 5 µL of 5x HOT FIREPol® Blend Master Mix (Solis Biodyne, Tartu, Estonia), forward and reverse primers at a final concentration of 0.4 µM, 100 ng genomic DNA, and nuclease-free water to complete the volume. The thermal cycling protocol consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at the primer-specific temperature (Table 1) for 30 s, and extension at 72 °C for 30 s. A final extension was performed at 72 °C for 5 min. The amplified PCR products were separated on a 1.8% agarose gel and visualized to assess the presence of DNA banding patterns, which were used to evaluate the ability of candidate markers in distinguishing males and females.

Table 1. The primers for PCR amplification of specific genes.

Primer name	Sequence 5' to 3'	Tm (°C)	Amplicon size (bp)
SOX9	F: GCGTCCATTCGTAGAAGAGG	55	157
	R: TATGGCGTTGGTGGAGACAT		
SOX5	F: GTGAGCAAGGCCTACCAGG	55	300
	R: AAGTCCATCATGGCGTGTGT		
Dmrt1b	F: TCAGTGCCAGAAGTGCAAAC	55	100–1,500
	R: CACTGCAAAGCCGTAATCAA		
nanos3	F: ATACTCTTGGACTCGGGCCT	53	150–1,000
	R: ATCTGCTTTTGTGGGGGAGG		
nanos1	F: TCTGTTCGCTCCTTGCCATT	53	No band
	R: CCACCACCTCCACCGAAG		
β-actin	F: CACCCCGTCCTGCTTACTG	50	300, 470
	R: CACCGGAGTCCAGCACAATA		
α-2 actin	F: CGGCTCAGGACTGTGTAAGG	55	1,000
	R: GCCTCTGTCAGTAAGGTCGG		

Nucleotide sequence analysis of amplified DNA fragments

The PCR products were separated by electrophoresis on a 1.8% agarose gel, and the target bands were excised and purified using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, USA) according to the manufacturer's protocol. The quality and size of the purified PCR products were verified on a 1.8% agarose gel. Purified fragments were either directly sequenced or cloned the DNA fragments, which were subsequently sequenced. Sequencing was performed using specific primers and confirm by cloning the fragments into the pGEM-T Easy vector system (Promega, USA). All amplified DNA fragments were sent to Ward Medic Ltd., Thailand, for sequencing.

To evaluate sequence variations among DNA fragments and confirm sequence identity, the aligned sequences were compared against the NCBI database using the Basic Local Alignment Search Tool (BLAST), accessible at https://blast.ncbi.nlm.nih.gov/Blast.cgi. To assess DNA polymorphism, the MISA tool (http://pgrc.ipk-gatersleben.de/misa/) was employed (Beier *et al*., 2017). The obtained sequences were aligned and processed to produce interpretable results using QIAGEN CLC Main Workbench 21.0 from QIAGEN in Aarhus, Denmark.

RESULTS AND DISCUSSION

Both morphological and histological characteristics of the testes exhibited apparent differences between normal male fish and those treated with 17α-methyltestosterone (MT). In normal males, the testes were relatively large and opaque (Figure 1a), indicative of active spermatogenesis. Conversely, MT-treated fish displayed smaller and more translucent testes (Figure 1b). Histological examination further revealed that the testes of normal males exhibited complete spermatogenic progression, characterized by the presence of spermatogonia (SPG), spermatocytes (SPC), spermatids (SPT), and abundant mature spermatozoa (SPZ) within the seminiferous tubules (Figure 2a). In contrast, the testes of MT-treated males showed impaired spermatogenesis, with only a few spermatogonia (SPG), spermatocytes (SPC), spermatids (SPT), and markedly reduced numbers of spermatozoa (SPZ) (Figure 2b). These findings suggest that MT administration can suppress normal testicular development and hamper spermatogenic maturation. The observed morphological and histological abnormalities, characterized by testicular hypoplasia, degeneration of seminiferous tubules, and reduced germ cell populations, are indicative of compromised gonadal development and impaired reproductive function. Such adverse effects are likely attributable to supra-physiological androgen exposure, resulting from either excessive MT dosage or prolonged treatment duration, both of which are known to disrupt normal testicular differentiation and germ cell viability. Consistent with these findings, Shen *et al*. (2015) reported severe testicular degeneration in yellow catfish (*Pelteobagrus fulvidraco*) following oral administration of 100 $mg \cdot kg^{-1}$ MT from 10 to 59 days post-hatching (DPH). Their histological analyses showed extensive vacuolization of seminiferous tubules and complete depletion of male germ cells in some individuals. Similarly, Ankley *et al*. (2001) observed spermatocyte necrosis and testicular atrophy in fathead minnows (*Pimephales promelas*) exposed to MT concentrations above 100 $mg \cdot L^{-1}$. These studies support a dose-dependent gonadotoxic effect of MT.

Overall, the present results, together with previous reports, highlight the importance of optimizing MT treatment protocols tailored to species-specific responses. Careful management of hormone dosage and exposure duration is crucial to maximize masculinization efficiency while minimizing adverse effects on testicular structure and reproductive capacity. This optimization is crucial for producing reproductively viable neomales in commercial aquaculture settings.

Identification of specific DNA fragments

In the initial PCR screening using *O. bimaculatus* DNA template, five primers associated with sex-linked genes (*SOX9, SOX5, Dmrt1, NANOS1*, and *NANOS3*) and two primers for housekeeping genes (β-actin and α-2 actin) were used. PCR amplification results were analyzed via gel electrophoresis and compared among male,

female, and MT-induced phenotypic male fish. The SRY-like box gene family members *SOX9* and *SOX5* were successfully amplified in all sexes, including MT-induced phenotypic males, producing major bands at approximately 157 bp and 300 bp, respectively (Figure 3 and Figure 4a. *Dmrt1* showed a wide range of DNA fragment sizes, from 100 bp to 1,500 bp. *NANOS3* exhibited no distinct band, while *NANOS1* failed to amplify under the PCR conditions. *Dmrt1* and *NANOS* genes play essential roles in sexual differentiation, primarily associated with testicular development rather than ovarian or somatic tissues (Webster *et al*., 2017; Li *et al*., 2021). Notably, *Dmrt1* expression has been reported as gonad-specific (Yamaguchi *et al*., 2006; Xie *et al*., 2025), and both Dmrt1 and NANOS are suitable candidates for further tissue-specific expression studies.

Actin genes produce three major isoforms, α, β and γ which are found in the contractile filaments of smooth muscles and are associated with several actin-binding proteins (Ruan and Lai, 2007). Here, β-actin and α-2 actingenes served as internal controls: β-actin yielded two distinct fragments at approximately 300 bp and 470 bp (Figure 3). In contrast, α-2 actin amplified a single product of approximately 1,000 bp (Figure 4b).

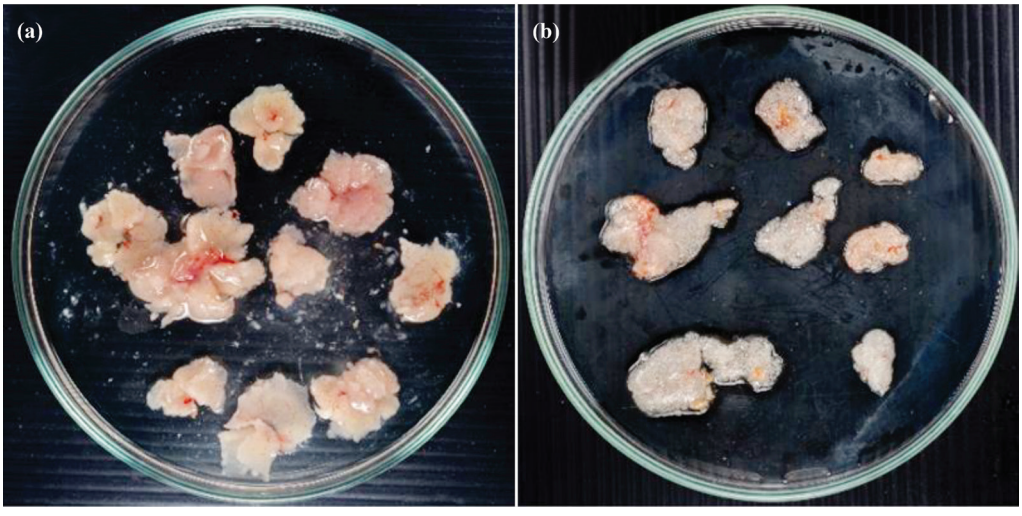

Figure 1. Testicular morphology of a normal male (a) and a MT-treated male (b).

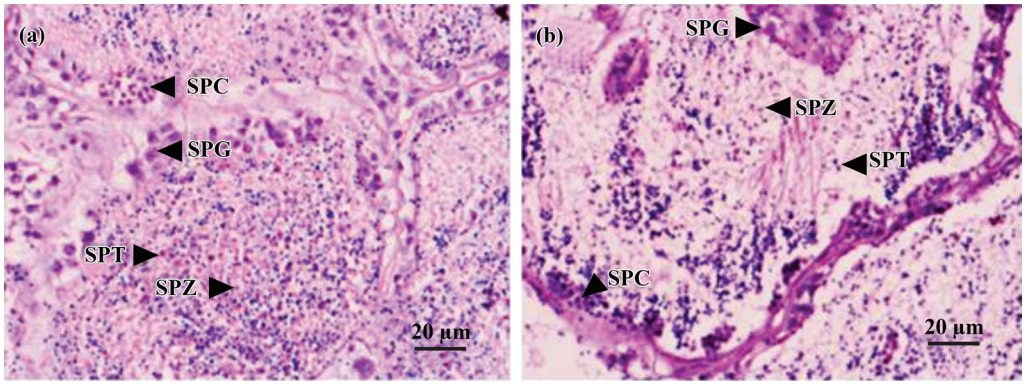

Figure 2. Testicular histology of normal male (a) and MT-treated male (b) stained with Haematoxylin and Eosin.
Note: SPG = spermatogonia; SPC = spermatocyte; SPT = spermatid; SPZ = spermatozoa

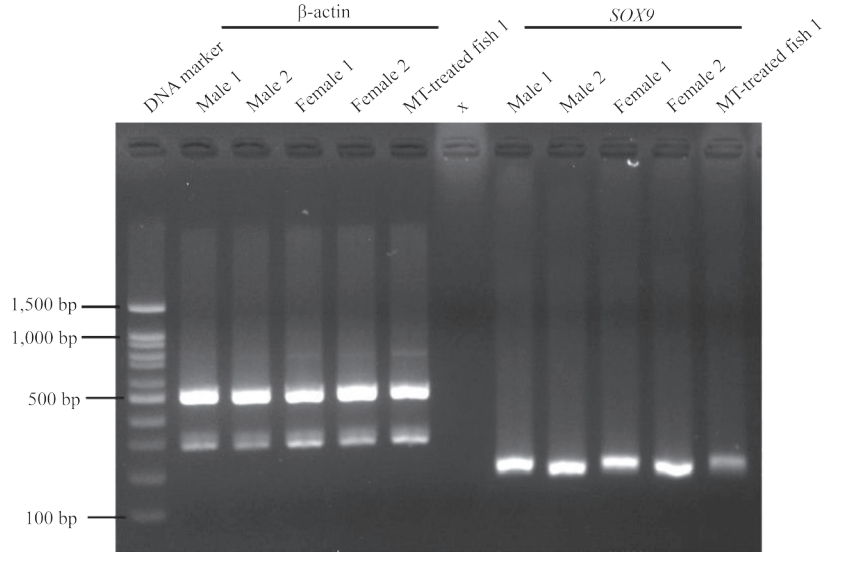

Figure 3. PCR amplification results of β-actin and *SOX9* genes in *Ompok bimaculatus* from different sexes.
Note: X = blank lane; DNA marker = 100 bp DNA ladder

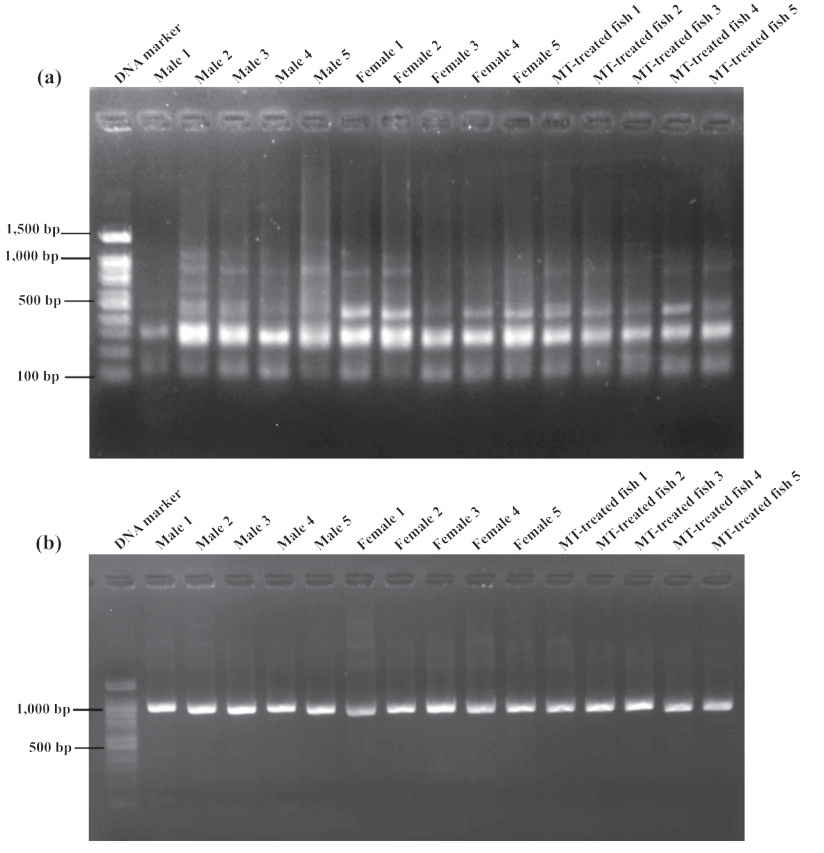

Figure 4. PCR amplification results of specific genes in *Ompok bimaculatus* across different sexes. (a) Amplification of *SOX5* and (b) Amplification of α-2 actin.
Note: DNA marker = 100 bp DNA ladder

Sequence analysis of sex-associated genetic markers

The amplified DNA fragments for each DNA region in an individual, including internal controls, were excised and purified for sequencing. Based on sequence alignment and BLAST analysis against the NCBI database, the *SOX9* gene was confirmed to be homologous with *SRY-box 9a2* of *O. bimaculatus*. BLASTN and BLASTX results showed 99% identity with the nucleotide sequence (NM_095653.1) and 100% identity with the protein sequence (QHS38716.1), respectively.

The *SOX5* gene sequence aligned with various genes from other teleosts. BLAST results showed 88% identity with a zinc finger protein of *Carassius gibelio* (XM_052552626.1), 86% with a tumor necrosis factor receptor superfamily member 14-like gene of *Brienomyrus brachyistius* (LOC125 745245), and 80% with a crystallin beta-gamma domain-containing gene of *Megalobrama amblycephala* (crybg1b, XM_048155236.1). Notably, sequence variation was observed in male individuals, suggesting putative sex-associated polymorphism.

Sequencing of β-actin fragments revealed two distinct products. The upper fragment aligned with the mRNA of β-actin in *Metynnis argenteus*, *Clarias magur*, and *Pelteobagrus fulvidraco* with 100%, 100%, and 80% identities, respectively. The lower fragment matched β-1 actin, skeletal muscle b (*acta1b*) in *Pangasianodon hypophthalmus*, *C. gariepinus*, and *Ictalurus furcatus*, all showing 97% identity.

In contrast, the putative α-2 actin fragment showed no significant similarity to sequences in the NCBI database, indicating that it may be a novel or uncharacterized sequence. Interestingly, a microsatellite region containing dinucleotide (CA)n repeats was identified. Males exhibited $(CA)_{19}$ repeats, while females and neomale displayed $(CA)_{15}$ repeats (Figure 5), suggesting that the α-2 actin sequence may be sex-associated.

These results indicate that the β-actin gene is highly conserved across different sexes and is suitable as an endogenous housekeeping gene. It can serve reliably as an internal reference marker. However, the variability observed at the putative α-2 actin sequence suggests potential functional differences between sexes. Ruan and Lai (2007) reported that β-actin expression can vary in response to biochemical stimuli during growth and differentiation. Further studies on actin gene functions under different physiological conditions may provide deeper insights into their roles beyond structural maintenance.

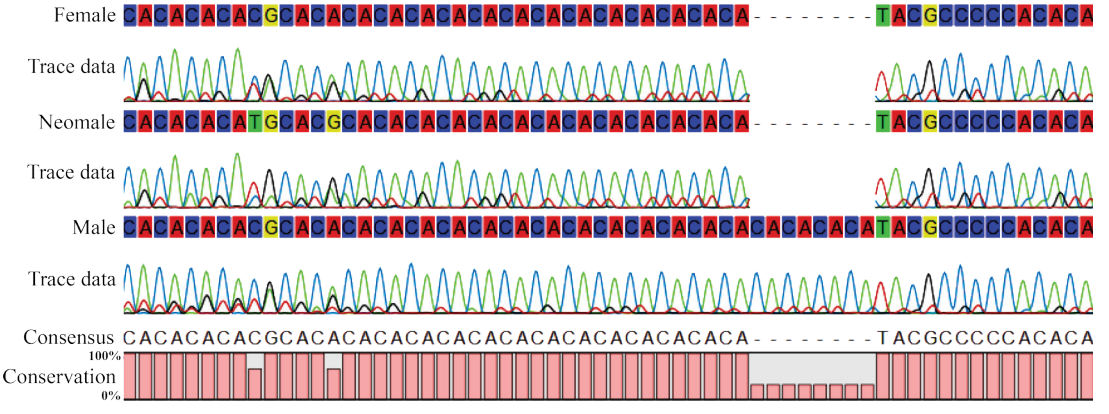

Figure 5. Sequence alignment of amplified α-actin DNA fragments from male, female, and neomale *Ompok bimaculatus*. A microsatellite region containing dinucleotide (CA)n repeats was identified, with male exhibiting $(CA)_{19}$ repeats, while female and neomale displayed $(CA)_{15}$ repeats.

In this study, no genetic differences were observed between females and MT-treated fish, except for differences in gonadal tissue development. This supports the idea that MT treatment disrupts the reproductive system, as noted by Liu *et al*. (2023). MT exposure may influence gene regulation in female fish, particularly those involved in cellular processes, metabolism, genetic information processing, and hormone biosynthesis pathways.

The distinct sequence characteristics of the α-2 actin gene, along with its sex-associated microsatellite polymorphisms at this locus, indicate its potential as a sex-linked marker candidate in *O. bimaculatus*, enabling sex genotype determination independently of phenotype. Sex-linked microsatellite markers have proven effective in other teleost species, such as *Oplegnathus fasciatus* (Xu *et al*., 2015) and *O. niloticus* (Sultana *et al*., 2020), facilitating rapid and reliable molecular sex identification. In this study, the α-2 actin locus is proposed as a novel sex-linked marker in *O. bimaculatus*, which may enable the determination of sex genotype independently of phenotypic characteristics. This capability is particularly valuable for verifying hormonally masculinized neomales prior to their incorporation into breeding programs aimed at generating all-female progeny through neomale × female crosses. By enabling accurate sex screening, the marker enhances the efficiency of sex control strategies and supports the development of hormone-free, all-female production systems. However, further validation is required to confirm the marker's tight linkage with the sex-determining region and to assess its reliability across diverse genetic backgrounds and larger population samples. Given the limited genomic resources currently available for *Ompok* species, continued molecular characterization and the development of comprehensive genomic datasets are essential to advance sex-associated marker-assisted selection and genetic management in this species.

CONCLUSIONS

The results of this study underscore the complementary roles of hormonal and molecular approaches in advancing sex control and sex identification strategies for *O. bimaculatus*. While masculinization using high-dose or prolonged exposure to 17α-methyltestosterone (MT) can induce phenotypic sex reversal, it may also adversely affect testicular development, as evidenced by reduced testis size, diminished spermatozoa abundance, and histopathological features indicative of testicular hypoplasia and reproductive dysfunction. These impairments raise concerns regarding the long-term reproductive viability and breeding value of hormonally induced neomales.

To support accurate sex identification, a novel DNA fragment derived from the α-2 actin gene was identified, revealing distinct allelic patterns between males and females/neomales. This fragment demonstrates strong potential as a sex-associated microsatellite marker candidate, providing a promising, non-invasive tool for preliminary identification and selection of neomales, although its effectiveness requires further validation across broader populations. Implementation of these markers and histological analysis of gonadal maturation could significantly improve the efficiency of monosex breeding programs, thereby supporting genetic enhancement and sustainable aquaculture development of *O. bimaculatus*.

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