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Identification of male-specific marker and development of rapid Loop-mediated isothermal amplification assay for sex identification in Indian major carp *Labeo rohita*

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Abstract

Freshwater aquaculture in India mainly relies on three Indian major carps, with rohu (*Labeo rohita*) being the most important species in polyculture systems. Quality hatchery seed production plays a vital role in enhancing aquaculture production. However, unorganised and poorly managed broodstock in hatcheries affect the seed quality leading to economic losses for farmers. These challenges can be tackled through the establishment of scientifically managed brooders. Developing an optimal female-to-male ratio for brooders requires maintenance of a large number of individuals for an entire year, as sex can only be determined at the maturity stage only. To address this issue, the current study developed a molecular marker and a Loop Mediated Isothermal Amplification (LAMP) assay for early gender determination, enabling the identification of the gender of rohu, even at the fingerling stage. This marker would enable the hatchery professionals and farmers to maintain an optimal male-to-female ratio from the early stage, leading to better broodstock management, reduced inbreeding and enhanced seed quality and quantity, which would ultimately benefit the aquaculture industry and promote sustainable fish farming practices in India.

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Introduction

The fisheries sector in India is one of the most critical and fastest growing segments of the agriculture and allied sectors. With 8% contribution to global fish production, India stands third globally and ranks second in aquaculture production (https://pib.gov.in/PressReleaseIframePage.aspx?PRID=1986155 dated 14 December 2023). About 88% of the total freshwater aquaculture production comes from Indian major carps (IMCs), comprising rohu (*Labeo rohita*), catla (*Labeo catla*) and mrigal (*Cirrhinus mrigala*) (FAO, 2023), under polyculture system (Nandeesha *et al*., 2013). Hatcheries are the major source of stocking material and under hatchery conditions, all the three major carps mature after two years of age and the two genders can be identified only at the sex-maturity stage (Holden and Raitt, 1979).

In aquaculture, identification of the sex of fish is crucial for effective breeding and broodstock management. For developing a breeding stock, maintaining an equal sex ratio is required to maximise the effective population size (N_e) and minimise the rate of inbreeding (ΔF) (Ojeda-Marín *et al.*, 2021). Since gender cannot be determined at the fingerling stage, segregation of males and females for broodstock development is not possible and the farmers end up in rearing fish for extra one year. To save space, farmers often maintain a higher stocking density in brooder ponds than recommended, which results in poor maturation during the breeding season which in turn affects seed production. Repeated use of the same males for breeding across multiple generations increases the risk of inbreeding depression leading to reduced productivity, and thereby leading to loss of fish farmers' income. Gender determination at the fingerling stage would be highly beneficial for milt cryo-banking, where rearing a maximum number of males is essential. Moreover,

lack of research on sex-specific markers for native Indian species highlights the urgent need for this study to fill existing knowledge gap.

Sex determination in fishes is a complex and diverse process, influenced by sex chromosomes, autosomes and environmental factors (Volff and Schartl, 2002). Sex determination systems have been described in a few species, which are closely related or belong to the same genus (Takehana *et al.,* 2007). In most fish species with sex chromosomes, the male may exhibit a simple heteromorphic XX/XY system (XY for males and XX for females); or a ZW/ZZ system, where the female is heteromorphic (ZW) and the male is homogametic (ZZ) (Kitano and Peichel, 2012). A multiple sex chromosome system can evolve through the fusion of an autosome with a sex chromosome, leading to complex variations in sex determination (Uyeno and Miller, 1971).

Recent advancements in molecular genetics have led to the development of sex-specific markers for various cultivated fish species, *viz.* common carp (Chen *et al.,* 2009), yellow catfish (Dan *et al.,* 2013), bighead carp (Liu *et al.,* 2018), Nile Tilapia (Taslima *et al.*, 2020), Mandarin fish (Han *et al.*, 2020), spinyhead croaker (Xiao *et al.*, 2020) and long snout catfish (Luo *et al.*, 2022). However, the application of these markers is limited by the inherent diversity in sex determination mechanisms between species, making cross-priming of markers impractical. In *L. rohita*, the karyotype analysis could not identify sex chromosomes (Bhatnagar *et al.,* 2014). However, through chromosome mapping studies, Arick *et al.* (2023) identified the number of regions in the genome of *L. rohita,* which have significant differences between female and male mapping read counts. It has been suggested that *L. rohita* follows a heterogametic (XX/XY) sex determination system, with only a small Y-specific region on the chromosome representing uniquely male regions (Arick *et al.,* 2023).

Identifying fish sex based on gel band patterns requires molecular biology laboratory facilities, specialised equipment and skilled technicians, The process may take approximately 4 h, encompassing reaction preparation, incubation and result detection (excluding DNA extraction) (Xu *et al.,* 2013). Alternatively, Loop-Mediated Isothermal Amplification (LAMP), is a widely adopted method, that offers rapid and precise detection, with applications in species identification (Hsu *et al.,* 2011; Centeno-Cuadros *et al.,* 2017). LAMP employs inner and outer primers, along with optional loop primers, operating isothermally at 60-65°C for 30 to 90 min (Zhao *et al.,* 2015).

This study aims to identify sex-specific markers in *L. rohita* and validation in larger sample size, enabling early gender determination and optimised broodstock management. The study anticipates to contribute to the growth and viability of the aquaculture sector in India, thereby benefiting both hatchery professionals and farmers.

Materials and methods

Sample collection

Farmed rohu, *Labeo rohita,* were procured from the Live Gene Bank of ICAR-National Bureau of Fish Genetic Resources (ICAR-NBFGR), Lucknow, during 2022 and 2023. These animals (F_1) were obtained after breeding of riverine stocks collected from the river Ganga, Allahabad, Uttar Pradesh in 2019. Fishes were maintained under standard recommended feeding regime and water quality. Gamete collection was done during the breeding season (June-July) in 2022 and left pelvic fin clips from every individual was collected and stored in 95% ethanol, to ensure known males and females. Sexually mature brooders (n=60) having weight 1500-2500 g, were anaesthetised using 2-Phenoxyethanol (0.4 ml l^{-1}) at the time of gamete collection.

Development of sex-specific marker

Sequence from chromosome 25 of the rohu genome available in NCBI under accession JACTAM000000000 (Arick *et al.,* 2023), showing significant mapping differences between males and females, was identified for development of sex-specific marker. Pimer3 v.0.4.0 (Untergasser *et al.,* 2012) was used to design ten primer pairs. A control primer pair was designed from the Beta-actin gene, *Danio rerio* actin, beta 2 (actb2), mRNA (NCBI Accession No. GU338376.1). The designed primers were screened using 4 pairs of known male and female samples. Genomic DNA was extracted from fin tissue samples using a modified Proteinase K digestion method (Sambrook *et al.,* 1989). PCR amplification conditions used were: initial denaturation at 94^{0}C for 5 min followed by 30 cycles of denaturation at 94^{0}C for 30 s; annealing at 60^{0}C for 60 s; extension at 72^{0}C for 90 s, with a final extension at 72^{0}C for 10 min. The PCR products were visualised using 2% agarose gel electrophoresis, using 100 bp standard marker panel.

Validation of sex-specific marker

After the first round of screening based on 4 female and 4 male samples, two-primer pair sets were selected for validation on a total of 60 mature rohu fish (30 phenotypic males and 30 phenotypic females), from different populations, including farm population. The PCR conditions remained same as previously described.

Screening of sex ratio in natural population

For screening of sex-specific markers, 50 specimens from the riverine stock of the Ghaghra River, Sitapur, Uttar Pradesh, weighing 30-70 g, were randomly sampled from the live gene bank of ICAR-NBFGR, Lucknow. Left pelvic fins were clipped using sterile scissors and forceps and preserved in 95% ethanol. These specimens were then screened using the developed marker for sex identification and to assess sex ratio in the natural population.

Reaction setup for LAMP assay

The LAMP primer sets were designed based on conserved gene sequences from GenBank (refer to section B), of chromosome 25 using the NEB LAMP primer design tools (https://lamp.neb.com/#!/). The LAMP assay was performed in a 25 µl reaction mixture comprising 1 µl template genomic DNA, 2.5 µl of Primer Master Mix (0.2 µM each of LR-F3 and LR-B3, 0.8 µM each of LR-FIP and LR-BIP, 1.6 µM each of LR-FIP and LR-BIP) and 12.5 µl of WarmStart® Colorimetric LAMP 2X Master Mix (Cat. No. M1804S, New England Biolabs, Massachusetts, USA) and ultrapure water to make up the final volume to 25 µl. To optimise the LAMP reaction conditions, evaluations were conducted at different amplification

temperatures (60-65°C) and reaction times (15-60 min). All reactions were incubated in a water bath and terminated by heating at 95°C for 5 min. The LAMP reaction products were detected by visual colour changes, observable by the naked eye. A positive amplification was indicated by a colour change from pink to yellow, while a negative result remained pink.

Results

Development and validation of sex-specific markers

After screening ten primer pairs, two-primer pairs (Primer pairs 1 and 2) successfully amplified single bands of 783 and 820 bp, respectively, in males, with no amplification in females. The control primer pair amplified 505 bp fragment in both males and females. Therefore, when primer 1 and primer 2 were used in combination with control primer, the male samples produced two PCR bands while female samples showed only one PCR band (Fig. 1).

Upon validation of one of the marker combinations (primer 1 and control), 100% accurate identification of males and females was achieved (Fig. 1). The PCR product patterns were completely consistent with the phenotypic sex of the tested individuals. The 30 phenotypic male individuals showed two distinct bands, whereas the 30 phenotypic female individuals showed a singele band. Moreover, this marker accurately identified sex in 49 out of 50 individuals from the riverine stock of the Ghaghra River, with one sample showing no amplification. A 1:1 sex ratio was observed in the population.

LAMP assay for early-stage male detection from DNA

In the present study, LAMP was chosen to evaluate its suitability, with primer sequences given in Table 2. Optimisation of reaction conditions was conducted by varying amplification temperatures (61-65^{0}C) and reaction time (15-60 min). Results revealed a visible colour change within 30 min at 65°C. Positive reactions in males exhibited a distinct shift from pink to yellow, which was visually detectable by the naked eye, while negative controls (females) retained their original pink hue (Fig. 2).

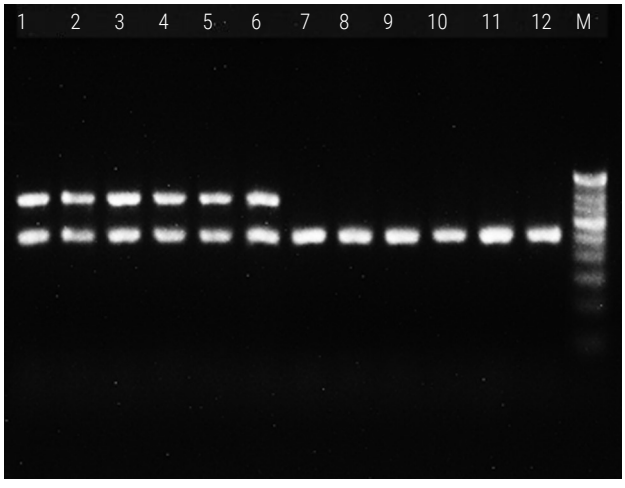

Fig. 1. PCR amplification in males and females of *L. rohita* using primer 1 and control primers. Samples 1-6: Males; 7-12: Females; M: 100 bp ladder

Table 1. Successful primer sets for development and validation of sex-specific markers

Primer pair	Primer name	Primer sequence 5' to 3'	Size of PCR product
1	LSMd1_F	5'GGTCTGGTATCCCATGATGC3'	783 bp
	LSMd1_R	5'GATGAGTGGTCGCTCCAAAT3'	
2	LSMfg2_F	5'CTCGGGGTGAACGTAAAGAA3'	820 bp
	LSMfg2_R	5'TATAGCGGTGA TGGCAACTG3'	
Control	Rbact1F	5'TGCGGAATATCATCTGCTTG3'	505 bp
	Rbact1R	5'GCACAAAGAACTTCCCATGC3'	

Table 2. Primer sets used forLAMP assay

Name of primers	Primer sequence 5' to 3'
F3	CGTTATCAGTACTATAGATGTCG
B3	CACCAAAGATGTAGCCCT
FIP	GTGGTAGAGAGATGGAACATGGACTGCTGTCGAATAAATGGTCA
BIP	CACCCACGAACACAACACAACTCTCTCTCTCTCTCCGC
LF	AGAAGGGTTCGGGATCGTGG
LB	CGGTGCTAGA GTCGAATAGACAC

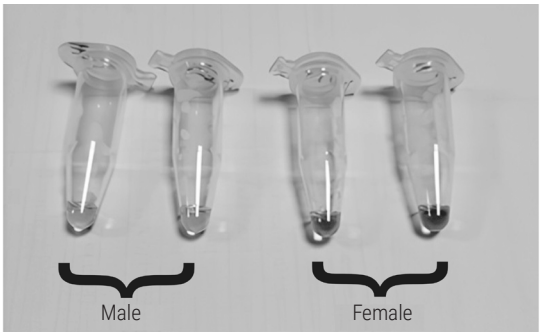

Fig. 2. LAMP assay for identification of *L. rohita* males and females

Discussion

The development and validation of sex-specific markers, in the present study, represent a significant advancement in early-stage sex identification of rohu. The testing of two sex-specific markers demonstrated their accuracy and reliability in effectively distinguishing male and female individuals. The consistent amplification patterns observed in phenotypic males and females, along with the complete concordance between genetic and phenotypic sex identification, emphasises the effectiveness of the markers in accurately predicting sex.

Furthermore, the application of these markers to assess sex ratios in natural populations has provided valuable insights into the reproductive dynamics of rohu fish. The identification of skewed sex ratios, particularly in farmed populations, highlights the importance of genetic tools for monitoring and managing breeding programs (Tave, 1999). Understanding and addressing imbalanced sex ratios are crucial for maintaining population sustainability and genetic diversity in aquaculture settings.

In addition to sex-specific markers, this study standardised LAMP assay for early detection of male rohu. By optimising reaction conditions, including temperature and reaction time, the LAMP assay demonstrated quick and sensitive detection of taget DNA sequences with high accuracy. Further, the LAMP assay developed for early-stage male DNA detection in farm settings offers ease of use by visual colour change detection in reaction tubes. The ability to observe the colour change with naked eye under natural light, without requiring a PCR machine further enhances its practicality and efficacy for field applications.

In conclusion, the development and validation of sex-specific markers along with the standardisation of the LAMP assay mark significant contributions to rohu fish genetics and aquaculture research. These tools offer practical solutions for sex identification, sex ratio estimation and early male DNA detection, enhancing the ability to monitor and manage rohu fish populations effectively. Moving forward, the integration of these genetic tools into breeding programs and conservation efforts will be essential for promoting sustainable aquaculture practices and preserving genetic diversity in rohu fish populations.

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