Molecular Fish Sexing on Taisho Sanshoku Koi (*Cyprinus carpio*) Based on ArS.9-15 Gene Amplification using PCR Method

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Abstract

Taisho Sanshoku is a variant of Koi fish (*Cyprinus carpio*) that has high demand due to its high economic value and relatively expensive price. This study aimed to determine the sex of the Taisho Sanshoku Koi fish by molecular sexing using the PCR method to amplify the ArS.9-15 gene. This study was initiated by rearing a 4–6 month-old of 10 Taisho Sanshoku Koi fish in a fish tank with a filter and oxygen aeration. The fish were fed with fish pellets for 1–3 days. The Koi fishes were then anesthetized using Koi anesthesia containing β-hydroxyethyl phenyl ether. Each fish's peripheral blood was collected as much as 0.5 mL per fish and then stored in tubes containing Ca-EDTA anticoagulant. The genomic DNA was extracted from peripheral blood samples and used as a template DNA for PCR amplification targeting ArS.9-15 gene. Agarose with 1.5% concentration and CybrSafe staining was used in electrophoresis for visualization of the PCR results then visualized in a dark chamber using a UV transilluminator. The Taisho Sanshoku Koi fish's sex was determined using descriptive analysis based on the electrophoresis results. According to the PCR results, the female Taisho Sanshoku Koi fish only produced one 800 bp DNA band, whereas the male fish produced two 800 bp and 1,100 bp DNA bands. The outcome of molecular fish sexing of the 10 Taisho Sanshoku Koi fish reported that 60% were male and 40% were female.

Keywords: ArS.9-15 gene, molecular fish sexing, Taisho Sanshoku Koi fish

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INTRODUCTION

One of the most popular varieties of ornamental fish that people in Indonesia like is koi fish, which is a highly valuable fishery product. Koi fish are popular because of their body's pattern and colors, as well as the folk belief that owning a koi fish will bring good fortune to its owner (Andrian *et al*., 2023). Koi fish is one of the freshwater ornamental fish commodities that has a potential trade volume on the global fish market (Monticini, 2010). Sanke or Taisho Sanshoku Koi fish is a Koi fish type that has three colors, namely white as a base color with a combination of red and black. Generally, the Taisho Sanshoku Koi fish has a red pattern on the head with a wide black stripe on the chest (de Kock and Gomelsky, 2015). Morphologically, between male and female Koi fishes can only be distinguished after reaching adulthood. After eight months, male koi fish reach gonad maturity, measuring 22 cm in length, whereas female koi fish reach gonad maturity after eighteen months, measuring 35 cm in length (Jaya and Iqbal, 2009).

In ornamental fish farming, the ability to determine and control the sex is an important factor for the efficient commercialization and maintenance of fish species because of its effect on reproductive performance, growth, and product quality (Budd *et al*., 2015). Fish that have XX/XY sex chromosomes can be distinguished between males and females based on the presence of the Y chromosome (Martínez *et al*., 2014; Mei and Gui, 2015). Previous molecular sexing on fish study already conducted in *Cyprinus carpio* (Aysi *et al*., 2022), *Cynoglossus semilaevis* (Nie *et al*.,

2021), *Protosalanx hyalocranius* (Xing *et al*., 2021), *Oplegnathus punctatus* (Li *et al*., 2020), *Hypophthalmichthys nobilis* and *Hypophthalmichthys molitrix* (Liu *et al*., 2018). There remains a notable lack of exploration into molecular techniques, such as molecular sexing, which offer precise and efficient methods for sex determination in koi fish. Despite the widespread popularity of koi fish in aquaculture and ornamental purposes, there is a noticeable gap in studies regarding molecular approaches for sex determination. Specifically, there is limited literature addressing the application and efficacy of molecular sexing techniques in koi fish. This gap presents a significant opportunity to investigate and enhance our understanding of koi fish biology and breeding practices. Sex determination of Koi fish is important because generally, female Koi fish have a less sharp color and less attractive shape when compared to male koi fish. It also explained that following gonadal differentiation, female koi fish grew noticeably quicker than male koi fish, at two years old the average weight of the females was almost 30% greater than that of the males at the same age (Chen *et al*., 2009).

Early sex determination offers several advantages for efficient commercialization, especially considering the faster growth of male koi fish at an early age and their development of more vibrant colors. By identifying the sex of koi fish at an early stage, breeders can selectively rear male individuals, which typically exhibit accelerated growth rates compared to females. This targeted approach allows for the optimization of resources and space in aquaculture facilities, thereby maximizing production efficiency (Falaye *et al*., 2024). Moreover, early sex determination facilitates the establishment of mono-sex populations, reducing unwanted breeding and enhancing uniformity in size and quality among stocked fish. Overall, the ability to identify the sex of koi fish early on streamlines management practices and enhances profitability in commercial operations. Hence, relying solely on the traditional method of observing physical characteristics to determine the sex of Koi fish, which can only be done after maturation, leads to increased production and maintenance costs for the fish. As a result, a more efficient, accurate, and timely approach to sex determination of Koi fish is required. Utilizing selection based on molecular markers emerges as a crucial tool in conserving time and resources in aquaculture, particularly if it can be implemented at the earliest stages of fish development (Yu *et al*., 2022).

Molecular sex determination of fish has been developed by Liu *et al*. (2018) using the polymerase chain reaction or PCR method based on SCAR for the first time to amplify male-specific DNA fragments in bighead carp (*Hypophthalmichthys nobilis*) fish and silver carp (*Hypophthalmichthys molitrix*) fish. These carp fishes have an XX or XY genetic sex determination system. Based on the phylogenetic relationships of carp fish, the entire family Cyprinidae is predicted to have an XX or XY sex-determination system. The male-specific DNA fragment was amplified using the PCR method, using primer pair ArS-9-15-Forward and primer ArS-9-15-Reverse. These primers were obtained from the genome using Next Generation Sequencing in *Hypophthalmichthys nobilis*, demonstrating a more specific male band (Liu *et al*., 2018). Male-specific DNA fragment amplification in male fish resulted in a DNA fragment of the Y chromosome in size 908 base pairs. The purpose of this study was to use PCR technology to do a quick, precise, and accurate sex detection of the Taisho Sanshoku Koi fish.

MATERIALS AND METHODS

Ethical Approval

This study was carried out with ethical approval from the laboratory animals use research ethics committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia with certificate No.043/EC-FKH/Int/2022.

Study Period and Location

This study was conducted during June–September 2021. This study was performed at the Biochemistry Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada to collect

blood samples, isolate DNA, and PCR for molecular sexing.

Fish Selection

The initial phase of the study involved the selection of suitable Koi fish sourced from a Koi farmer in Sleman, Yogyakarta. The Koi fish belonged to the Taisho Sanshoku variant, characterized by black, red, and white patterns. Ten Koi fish were selected from the same pond and shared the same parental lineage, with sizes ranging from 10–15 cm and ages approximately 4–6 months old. For exploratory studies aiming to uncover the genetic profile, it has been determined that analyzing ten samples is adequate to show the genetic differences (Foster *et al*., 2021; Tahir *et al*., 2024). Koi fish were randomly given sample codes, i.e. SK-1, SK-2, SK-3, SK-4, SK-5, SK-6, SK-7, SK-8, SK-9, and SK-10.

Fish Adaptation

The Koi fish were placed inside a plastic bag and then transferred to an aquarium measuring 40 cm x 50 cm x 60 cm. They were allowed to swim for 15 minutes to adjust to the temperature difference between the water in the plastic bag and the water in the aquarium. Furthermore, the 10 Koi fishes were disinfected with Potassium Permanganate (PK) solution at a concentration of 0.1% for 1 minute. After that, the 10 Koi fishes were moved into an aquarium containing fresh water with 1.5% NaCl.

Rearing Period

The 10 Taisho Sanshoku Koi fishes were adapted in the aquarium for three days by ad libitum feeding and O_2 aeration. Water changes, filter cleaning, and controlling the O_2 aeration system were also carried out to maintain the cleanliness of the aquarium, water quality, and O_2 saturation in the water.

Peripheral Blood Sample Collection

The peripheral blood was collected from 10 Taisho Sanshoku Koi fishes. Peripheral blood sample collection was performed by anesthetizing Koi fish in a measuring Chamber using Koi Anesthesia containing β-hydroxyethyl phenyl ether at a dose of 0.4 mL in 100 mL water. The anesthesia was administered by placing the koi fish inside the chamber for 5 minutes until there was a noticeable disorientation in the fish's swimming behavior. The Koi fish's body length was also measured during the anaesthesia procedure using a dedicated measuring chamber designed for Koi fish. Using a 1 mL syringe, blood samples were taken from the caudal vein along the lateral line of Koi fish. Peripheral blood samples were collected in a blood collecting tube which was contained with Ca-EDTA anticoagulant. Peripheral blood samples were kept in the refrigerator at 4°C for DNA extraction. After the blood collection, the fish is returned to the aquarium where it was previously adapted and cultivated, allowing it to recover and regain its vitality, as indicated by normal swimming and behavior.

DNA Extraction

Peripheral blood with a total volume of 10 µL was collected from 10 Taisho Sanshoku Koi fish for DNA extraction. The Geneaid gSYNC™ DNA Extraction Kit Quick was used to extract DNA from peripheral blood according to the standard protocol.

Amplification of ArS.9-15 Gene using PCR

The extracted DNA was used as a template for amplification by the PCR method. The DNA template was amplified by ArS.9-15 gene as an amplification target in sex chromosomes using ArS-9-15-Forward and ArS-9-15-Reverse primer pairs according to Liu *et al*. (2018). The nucleotide sequences of the ArS-9-15-Forward and ArS-9-15-Reverse primer pairs are presented in Table 1.

PCR amplification reactions were done in a total amount of 25 µL per sample, using the MyTaq™ kit protocol (Bioline UK). This amplification reaction was performed in a Thermocycler (Cleaver Scientific GTC96S) under optimal PCR amplification conditions, which included pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 35 seconds, annealing at 54°C for 35 seconds, extension at 72°C for 40 seconds, and final extension at 72°C

for 10 minutes. The denaturation, annealing, and extension steps were repeated 40 times.

Agarose Gel Electrophoresis and Visualisation of PCR Products

The PCR product was then electrophoresed on 1.5% agarose gel and visualized in a dark room with a UV Transilluminator at a wavelength of 260 nm using CybrSafe dye. For female Koi fish, the PCR product was a single DNA fragment of 800 bp, while male Koi fish products were double DNA fragments of 800 bp and 1,100 bp.

RESULTS AND DISCUSSION

The 10 Taisho Sanshoku Koi fish have been adapted and kept in the research aerator aquarium for 3 days. Photos of the 10 Taisho Sanshoku Koi fish are presented in Figure 1. Before collecting the peripheral blood samples, the 10 Taisho Sanshoku Koi fish were anesthetized by Koi Anesthesia containing β-hydroxyethyl phenyl ether at a dose of 0.4 mL in 100 mL H_2O. After the Koi fish was completely anesthetized, then the body length of Koi fish was measured in the measurement chamber. The data of body length measuring from each Taisho Sanshoku Koi fish were presented in Table 2. On average, the body length of male koi fish was recorded at 12.92 cm, while that of female koi fish was 12.63 cm. This indicates that male koi fish tend to exhibit faster growth compared to female koi fish of the same age and from the same parentage at an early age. This observation further supports the alignment between traditional size-based selection and molecular sexing outcomes in this study. Similar sex dimorphism is noted in other fish species like tilapia (*Oreochromis* spp.), where mono-sex cultures primarily consist of male fish owing to their enhanced growth potential (Lind *et al*., 2015). This pattern is also observable in koi fish cultivation, as revealed by the findings of this study, wherein male koi fish exhibit a longer length compared to females in the early stages before maturation. However, after gonadal maturation at 18 months, as mentioned by Chen *et al*., 2009, females tend to develop a larger body size. The faster growth of male fish in the early stage can be linked to their faster maturation at 8 months of age (Jaya and Iqbal, 2009).

DNA extraction from peripheral blood samples is an important initial step in molecular study to determine the sex in Koi fish. The quality of extracted DNA will determine the next stage of molecular study. Therefore, a method of DNA extraction that is properly carried out will be able to obtain the best quality of DNA templates from various sources of sample (Ariyanti and Sianturi, 2019). Minimum-invasive sample collection of various animals including fish can be performed from peripheral blood, skin, scales, and muscle tissue that did not cause major damage and trauma to the animal, so that can be obtained as many DNA template as possible (Wasko *et al*., 2003). In this study, peripheral blood samples were collected from the caudal vein down the side of the Koi fish body. Because fish erythrocytes are nucleated, using peripheral blood samples yields more DNA.

The agarose gel electrophoresis of genomic DNA isolated from 10 peripheral blood samples of Taisho Sanshoku Koi fish was analyzed using a UV transilluminator at 260 nm. The agarose gel electrophoresis results are shown in Figure 2. The extracted genomic DNA in all samples appeared thick and clear in all wells from code samples SK-1 to SK-10, indicating an excellent electrophoresis result (Lee *et al*., 2012). Using the primer pair ArS.9-15 forward and reverse, the isolated genomic DNA was subsequently utilized as a template to amplify the ArS.9-15 target gene by PCR amplification. Using a thermocycler, the ArS.9-15 gene was amplified by PCR for 40 cycles at the ideal annealing temperature of 54°C. The PCR products were then electrophoresed on a 1.5% agarose gel and displayed in Figure 3. Table 3 provided an interpretation of the ArS.9-gene PCR amplification products.

This study used molecular amplification for fish sexing, which involved amplifying the Ars.9–15 gene via PCR to determine the sex of ten Taisho Sanshoku Koi fish. The PCR method is a way to replicate DNA molecules in vitro on specific target regions that are restricted by pairs of oligonucleotide primers. The male Taisho Sanshoku Koi fish produced two DNA fragments,

each measuring 800 bp and 1,100 bp, from the amplification of the Ars.9-15 gene, but the female fish produced a single 800 bp DNA fragment. This information is consistent with the findings of a related study done on Kohaku Koi fish (Aysi *et al*., 2022). In a previous investigation into Kohaku koi fish sexing using the ArS.9-15 primer, it was demonstrated that the sex of koi fish can be differentiated between male and female individuals, with male koi fish exhibiting two bands. This suggests that these primers can be reliably used in both variants of koi fish, consistently producing accurate results.

Table 1. Nucleotide sequence of Ar-9-15-Forward and Reverse primer pairs

Primer	Nucleotide Sequence	Σ Base	Reference
ArS-9-15-Forward	5'-AGCAACTTTTGTCTGGTG-3'	18	(Liu *et al*., 2018)
ArS-9-15-Reverse	5'-AATGAATGGTGAATAGGG-3'	18	(Liu *et al*., 2018)

Table 2. Data of body length measuring of Taisho Sanshoku Koi fish 4–6 months age with sample code SK-1, SK-2, SK-3, SK-4, SK-5, SK-6, SK-7, SK-8, SK-9, and SK-10

No	Sample Code	Body Length (cm)
1	SK-1	15
2	SK-2	12
3	SK-3	12.5
4	SK-4	12.5
5	SK-5	12
6	SK-6	11.5
7	SK-7	13
8	SK-8	15
9	SK-9	13
10	SK-10	11.5

Table 3. Interpretation results of sex determination for Taisho Sanshoku Koi fish (*Cyprinus carpio*) based on PCR amplification products of Ar.S.9-15 gene

No	Sample Code	PCR Products	Interpretation
1	SK-1	Double bands, 800 bp dan 1,100 bp	♂ male
2	SK-2	Double bands, 800 bp dan 1,100 bp	♂ male
3	SK-3	Single band, 800 bp	♀ female
4	SK-4	Double bands, 800 bp dan 1,100 bp	♂ male
5	SK-5	Double bands, 800 bp dan 1,100 bp	♂ male
6	SK-6	Single band, 800 bp	♀ female
7	SK-7	Double bands, 800 bp dan 1,100 bp	♂ male
8	SK-8	Single band, 800 bp	♀ female
9	SK-9	Double bands, 800 bp dan 1,100 bp	♂ male
10	SK-10	Single band, 800 bp	♀ female

The ArS.9-15 gene in this study can be utilized to distinguish between the sex of Taisho Sanshoku Koi fish (*Cyprinus carpio*). In the other study, the ArS.9-15 gene can be used to determine the sex of two fish species that belong to the Cyprinidae family which are bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*). All male Cyprinidae fish samples containing primer pairs ArS.9–15 produced 908 bp DNA fragments, which are known as male-specific DNA fragments amplified, however, no DNA fragments were detected in female Cyprinidae fish samples (Liu *et al*., 2018; Fikri *et al*., 2022). Koi fish also belong to Cyprinidae family but our finding using ArS.9–15 gene on females produced a single 800 bp DNA fragment and male koi fish produced two DNA fragments which are 800 bp and 1,100 bp bands. Differences in results obtained when using the same primer may occur due to differences in species used even from the same family. Variations in species used can also

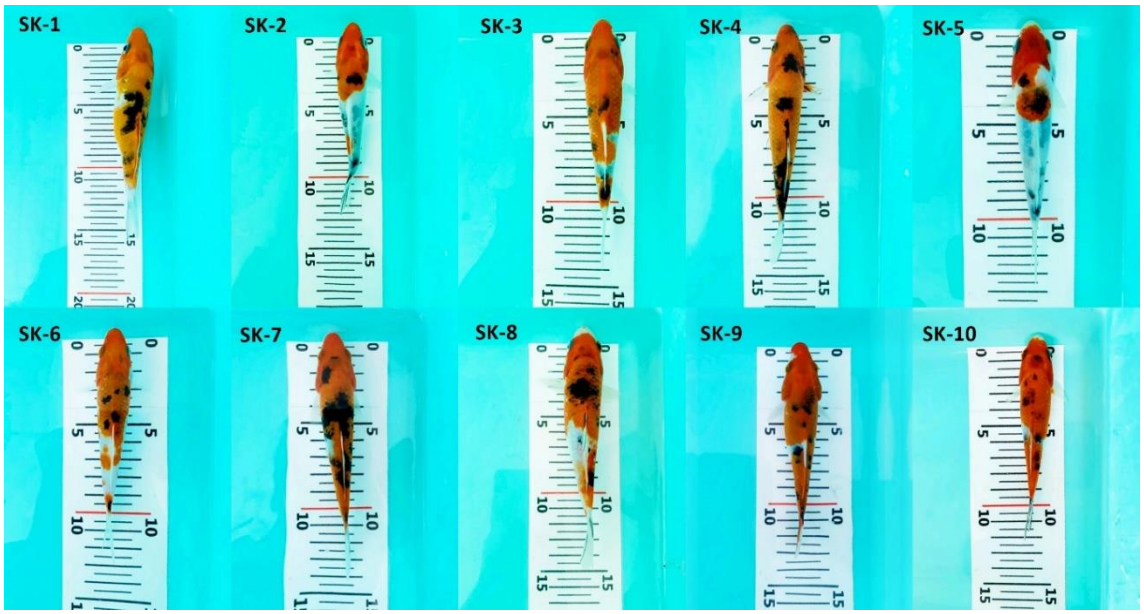

Figure 1. The study included samples of Taisho Sanshoku Koi fish, aged 4–6 months, with a body length of 11–16 cm, sample code SK-1, SK-2, SK-3, SK-4, SK-5, SK-6, SK-7, SK-8, SK-9, and SK-10.

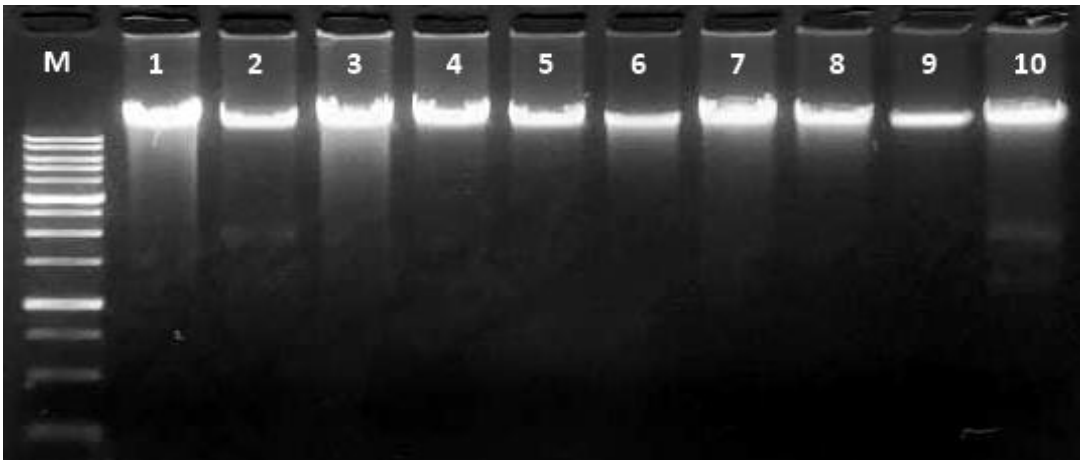

Figure 2. Extracted DNA Genome from peripheral blood of Taisho Sanshoku Koi fish (*Cyprinus carpio*). M= marker DNA ladder 100 bp, 1–10 were DNA samples with sample code SK-1, SK-2, SK-3, SK-4, SK-5, SK-6, SK-7, SK-8, SK-9, and SK-10.

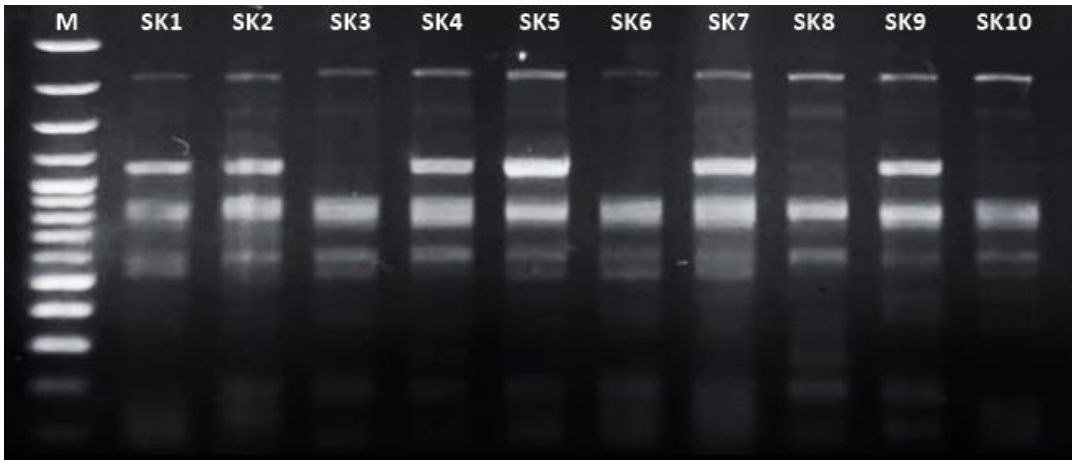

Figure 3. Agarose gel electrophoresis of PCR products of Ars.9-15 gene Taisho Sanshoku Koi fish. M= marker DNA ladder 100 bp (1–10 were with sample code SK-1 to SK-10).

lead to variations in outcomes even with the same primer. Results of molecular fish sexing in 10 Taisho Sanshoku Koi fish showed that 60% were male and 40% were female. Traditional sexing methods relying on secondary traits such as coloration and average size also confirm these findings. These findings demonstrate that the primer can identify the sex of Taisho Sanshoku Koi fish at a juvenile age.

These findings underscore the importance of early sex determination in koi fish cultivation, particularly for optimizing breeding strategies and resource allocation. By identifying the sex of koi fish at an early age, breeders can selectively rear male individuals, taking advantage of their faster growth rates and vibrant colors to potentially enhance overall productivity. Additionally, the observed sex dimorphism in growth patterns highlights the significance of considering gender-specific traits in aquaculture management practices. Moreover, the results emphasize the potential utility of molecular sexing techniques as efficient tools for enhancing commercial production and improving the profitability of koi fish farming operations. Further investigation of underlying genetic mechanisms driving sex dimorphism in koi fish growth patterns is needed. Understanding the molecular basis of these differences could lead to the development of targeted breeding programs aimed at maximizing growth potential and coloration traits in commercial koi populations. Additionally, exploring the application of advanced molecular techniques, such as genomic sequencing and gene editing technologies, may offer new options for enhancing the efficiency and precision of sex determination in koi fish. Furthermore, integrating multidisciplinary approaches that combine genetics, physiology, and aquaculture management could provide valuable insights into optimizing production systems and mitigating environmental impacts. Further study in these areas has the potential to revolutionize koi fish farming practices and contribute to the sustainable growth of the aquaculture industry.

CONCLUSION

It was determined that PCR amplification of the Ars.9-15 gene may be used for quick, accurate, and precise molecular fish sexing on Taisho Sanshoku Koi fish. The Taisho Sanshoku Koi fish that were studied had a 60% male to 40% female ratio.

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AUTHORS' CONTRIBUTIONS

BBMN and IAN: research conducting, drafting, and writing; KNA: writing, editing, and visualization: AH: reviewing, supervision, and funding acquisition. All authors have read, reviewed, and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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