Molecular fish sexing on Kohaku Koi (*Cyprinus carpio*) based on ArS.9-15 gene amplification by PCR method

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Abstract. This study aims to sex determine on Kohaku Koi fish rapidly, precisely, accurately, and as early as possible based on the amplification of the ArS.9-15 gene using the PCR method. This study began with the maintenance of 10 Kohaku Koi fish, obtained from Koi fish farmers in the Sleman area in Yogyakarta. The 10 Kohaku Koi were adapted in a filtered aquarium with O2 aeration and fed ad libitum by fish pellets for 1-3 days. Then, a collection of 0.5 ml peripheral blood samples per fish from the caudalis vein was performed under anesthesia conditions using special anesthetic preparation for Koi fish, namely Koi Anesthesia mixed with fresh water. Those samples were then collected in tubes containing the Ca-EDTA anticoagulant. The DNA then was extracted from peripheral blood samples and used as a template for PCR amplification by specific oligonucleotide primer pairs to amplify the ArS.9-15 gene. PCR products in the form of DNA fragments were visualized with 1.5% agarose gel electrophoresis and SybrSafe staining using Transilluminator-UV in a dark room. The DNA fragments were analyzed descriptively to sex determination between male and female Kohaku Koi. Electrophoresis results of PCR products showed two DNA bands of 850 bp and 1,100 bp in male Kohaku Koi, while in female Kohaku Koi showed only a band DNA in size of 850 bp. The results of the molecular fish sexing of the 10 Kohaku Koi showed that 70% are female and 30% are male.

Keywords: Kohaku Koi fish, molecular fish sexing, Ar.S.9-15 gene.

1. Introduction

The production of ornamental fish has increased significantly yearly in Indonesia because it is one of the potential fisheries commodities in generating foreign exchange for the country and can prosper the community of fish breeders [1]. Koi fish (*Cyprinus carpio*) is a freshwater fish that is still the main commodity in the international fisheries market [2]. Production of Koi fish in Indonesia is reported to increase every year. Koi fish is one of the ornamental fish that has a beautiful body shape and color pattern, so it has a high economic value [3, 4].

Kohaku is one type of koi fish that belongs to the Gosanke category, which is the most popular type of Koi fish and is kept by many Koi fish breeders. Kohaku Koi fish has a red/orange and white color pattern. A good Kohaku Koi fish has the color pattern as a thick red or orange color pattern with clear edges. Some of these patterns are broken and some are big or swept. A good Kohaku also has a color pattern that does not go down between the eyes and a balanced color pattern [5].
In the Koi fish cultivation process, the long-time growth of fish Koi is one of the obstacles in increasing the production of koi fish commodities. The high need for good quality feed can be a problem for koi fish farmers [6]. To obtain beautiful colors, ideal body shapes, and attractive movements, Koi breeders are performing a series of efforts. One of these efforts is to determine the sex of Koi rapidly, precisely, accurately and as early as possible [7]. Sex determining of koi fish before reaching adulthood, or before the Koi fish reaches a body length of 20-30 cm is difficult [8]. If this sex determination can be done as early as possible, the financial benefits will increase significantly. One way to reduce koi production costs is to do mono-sex culture (cultivating fish of certain sex only). This is because the color and shape of male Koi fish are usually better than female Koi fish.

Mono-sex culture is usually carried out after the age of koi reaches ± 5 months, and it is time-consuming by koi breeders. Besides, it is high cost during fish maintenance, staff, and consumption of a lot of fish feed, while the yield obtained is only about 20% of Koi fish kept for 5 months. Therefore, the cost of maintenance, staff, and feed consumption during this time is very high economically. So far, the sex-determination of Koi is done conventionally, so it takes a long time so that the physical characteristics of sex distinguishers can be seen clearly. While the production costs that have to be paid by fish breeders are increasing, therefore an earlier and better way of sex determination is needed [7]. Understanding the genetic-based on the sex determination to implement breeding processes, production management, and sex control [9] can add to the advantages of mono-sex culture in some fish species [10].

Several methods for sex-determining of fish have been developed by molecular fish sexing. Various species of intensively cultured fish have been identified by modern molecular-based sex identification, for example, Rainbow trout or Oncorhynchus mykiss by researchers [11], Medaka fish or Oryzias latipes by other researchers [12], Tiger pufferfish or Takifugu rubripes by other researchers [13], Half-smooth tongue sole or Cynoglossus semilaevis by other researchers [14] and Nile tilapia or Oreochromis niloticus by other researchers [15]. The purpose of this study was to sex-determine Kohaku Koi fish (Cyprinus carpio) rapidly, precisely, accurately, and as early as possible based on the PCR amplification of the ARS-9-15 gene by using the PCR method.

2. Materials and Methods

2.1. Koi fish preparation

The Kohaku Koi fish used in this study were 10 fish with body lengths ranging from 11-13.5 cm which were chosen randomly and they have a white color as the base color with red or orange color pattern on their body. The Kohaku Koi fish samples were then assigned a sample code: KH-1, KH-2, KH-3, KH-4, KH-5, KH-6, KH-7, KH-8, KH-9, and KH 10. The ten samples of Kohaku Koi fish are presented in Figure 1. The ten Kohaku Koi fish were obtained from a koi fish farmer in Sleman, Yogyakarta, with an estimated age of 4-6 months. At the age of 6 months, usually Koi fish will have a body length of around 13 cm. The length of the koi fish before reaching maturity, or before the body length of the koi fish reaches a size of 20-30 cm, is difficult for sex-determining [10].

2.2. Specimen collection

The study begins with peripheral blood samples collected from 10 Kohaku Koi fish taken from the caudalis vein. Koi fish were anesthetized first by using general anesthesia specifically for koi fish, namely Koi Anesthesia. 0.5-1 ml of Peripheral blood samples were taken using a 1 ml syringe for each koi fish. The collected blood is accommodated in a blood collecting tube that already contains the anticoagulant Ca-EDTA. Blood samples were stored in a refrigerator at a temperature of 4°C until the DNA extraction process.

2.3. DNA extraction

A total of 10 μl peripheral blood samples were used for the DNA extraction. This process was carried out following the standard procedure of the Geneaid gSYNC™ DNA Extraction Kit Quick Protocol. The extracted DNA was then used as a template for PCR amplification and visualized by 1% agarose gel electrophoresis with CybrSafe staining.

2.4. Amplification of ArS-9-15 gene by PCR

The amplification was employed using the ArS-9-15 primers (Table 1). The total volume of the PCR mix was 25 μl, which consisted of 12.5 μl Bioline Taq DNA Polymerase, 0.5 μl DNA template, 1 μl forward primer, 1 μl reverse primer, and 10 μl H2O. The mixture was put into a microtube and then homogenized by
a vortex mixer. It was put in a spindown apparatus, so that the solution went down into the bottom of the tube. The air bubbles then were removed from the tube. The PCR amplification process was carried out in a thermal cycler, with optimal temperature and time conditions: pre-denaturation 94°C for 5 minutes, denaturation 94°C for 35 seconds, annealing 54°C for 35 seconds, elongation 72°C for 10 minutes and post elongation. 72°C for 10 minutes. The denaturation, annealing, and elongation stages were carried out for 40 cycles. Furthermore, the PCR products were visualized by 1% agarose gel with an electric current of 80 amperes, a voltage of 100 volts for 45 minutes using CybrSafe staining.

3. Results and Discussion

In this study, peripheral blood was chosen as the research sample, because like other vertebrates, fish generally has oval erythrocytes and it has a nucleus with a diameter of 7-36 microns. The number of erythrocytes per 1 ml of blood ranged between 20,000-3,000,000 erythrocytes [17]. DNA was extracted from nuclear DNA or chromosomal DNA and it was not from mitochondrial DNA. Total DNA extraction from peripheral blood samples was then electrophoresed on 1% agarose gel with CyberSafe staining and analysis using a UV Transilluminator with a wavelength of 280 nm. The results of 1% agarose gel electrophoresis of genomic DNA were presented in Figure 2. It showed that the migration of extracted DNA in agarose gel electrophoresis was influenced by several factors, e.g., the size and conformation of the DNA molecule, agarose concentration, the strength of magnitude electric current, and temperature of the room. The total whole-genome DNA should be electrophoresed with a lower concentration of agarose, about 0.8%, whereas the amplified DNA with a higher concentration, around 1.5-2% [18].

Figure 1. Ten Kohaku Koi fishes were used in this work with sample code KH1, KH-2, KH-3, KH-4, KH-5, KH-6, KH-7, KH-8, KH-9, KH-10.

Genomic DNA bands from Kohaku koi fish with sample code of KH-1 to KH-10 showed clean and thick DNA. The extracted DNA was good as indicated by the appearance of clear and bright bands in all wells. The thick and bright DNA band indicates that the concentration of extracted DNA was high, while the thin DNA band indicated that the concentration of extracted DNA was low. The purity of DNA purity can be known qualitatively by the presence or absence of a smear DNA band [19]. In this study DNA extraction was well done, as seen from the DNA bands which were thick, clean, and without any smear band. The extracted DNA was then used as a template to amplify the ArS-9-15 gene on Y chromosome sex by using specific primer pairs on male-specific markers [16].

In contrast to mammals and birds which show significantly differences in sex chromosome, the variation of genomic region is rare in fish. Therefore, it is difficult to find specific sex-determining regions in the fish

| Primer     | Nucleotide Sequence               | Number Base | PCR Product |
|------------|-----------------------------------|-------------|-------------|
| ArS.9-15 F | 5’- AGC AAC TTT TGT CTG GTG -3’   | 18          | ♂ = 908 bp  |
| ArS.9-15 R | 5’- AAT GAA TGG TGA ATA GGG -3’   | 18          | ♂ = 908 bp  |

Table 1. The nucleotide sequences of forward and reverse primers amplify the ArS-9-15 gene [16].
According to researchers [21] fish species of C. carpio has XX homozygous chromosomes in females and XY heterozygotes in males.

**Figure 2.** Genomic DNA extracted from the peripheral blood of Kohaku Koi fish. M = marker DNA ladder 100 bp. No. 1 to 10 are samples with sample code KH-1 to KH-10.

In this study, the molecular fish sexing of ten Kohaku Koi fish were based on the results of PCR amplification of the Ar.S.9-15 gene. The PCR is a technique in molecular biology to amplify one or more DNA fragments according to their nitrogen base sequence, which resulting thousands to millions of copies of a specific DNA sequence [22]. The basic principle of this method is the amplification of DNA fragments by the DNA polymerase enzyme at high temperatures which is carried out repeated. PCR amplification requires short oligonucleotides (primer) which play a role in initiating the amplification process. The primer will anneal to the single strand of DNA, and when the temperature is raised there will be a separation of the double strand of DNA. PCR products can be visualized by using the agarose electrophoresis technique [23].

The amplification of the ArS.9-15 gene was carried out in a thermal cycler with an optimal annealing temperature of 54 °C for 40 cycles. The sex determination of the Kohaku Koi fish was confirmed using the design of the primers from researchers [16] and visualized using a UV Transilluminator with a wavelength of 280 nm. In the study of researchers [16], the results of PCR amplification of the ArS.9-15 gene in Bighead carp (Hypophthalmichthys nobilis) and Silver carp (Hypophthalmichthys molitrix) showed a single clear DNA band with a size of 908 bp in male fish.

**Figure 3.** PCR amplification products of Ar.S.9-15 in Kohaku Koi fish (KH-1 to KH-10) in 1% agarose gel electrophoresis.

The results of the PCR amplification of the ArS.9-15 gene from the code samples KH-1 to KH-10 can be seen in Figure 3, while the electrophoretic interpretation was presented in Table 2. It showed the presence of double DNA bands with a length of 850 bp and 1,100 bp in male Kohaku Koi fish, as seen in samples KH-2, KH-7, and KH-9. While in female Koi fish only seen single DNA bands in the size of 850, as seen in samples KH-1, KH-3, KH-4, KH-5, KH-6, KH-9, and KH-10. The amplification products of the ArS.9-15 gene in the ten samples of Kohaku Koi fish found a discrepancy in the size of the ArS.9-15 gene amplicon, which should be 908 bp, but from the seven samples, the amplicon of DNA band was 850 bp. Double DNA bands were seen in the sample with codes of KH-2, KH-7, and KH-8 within the size of 850 bp and 1,100 bp. It is probably caused by the type of Kohaku fish used in this study, which is a local type of Kohaku Koi fish that is genetically different from the imported Kohaku Koi fish. According to researcher [22], the presence of non-specific bands in the PCR results can be caused by the low annealing temperature. DNA amplification by PCR will be more efficient if it is carried out at a lower temperature, but this can cause an increase in mispriming, namely the anneal of oligonucleotide primers in the wrong site of the DNA template.
Table 2. Interpretation of molecular fish sexing of Kohaku Koi fish based on PCR amplification product of Ar.S.9-15 gene.

| No | Sample Code | PCR Products | Interpretation |
|----|-------------|--------------|----------------|
| 1  | KH-1        | Single-band, 850 bp | female ♀ |
| 2  | KH-2        | double bands, 850 bp, and 1,100 bp | male ♂ |
| 3  | KH-3        | Single-band, 850 bp | female ♀ |
| 4  | KH-4        | Single-band, 850 bp | female ♀ |
| 5  | KH-5        | Single-band, 850 bp | female ♀ |
| 6  | KH-6        | Single-band, 850 bp | female ♀ |
| 7  | KH-7        | double bands, 850 bp, and 1,100 bp | male ♂ |
| 8  | KH-8        | double bands, 850 bp, and 1,100 bp | male ♂ |
| 9  | KH-9        | Single-band, 850 bp | female ♀ |
| 10 | KH-10       | Single-band, 850 bp | female ♀ |

In Table 2, it showed that the interpretation of ten tested Kohaku Koi fish indicated that the PCR amplification of the ArS.9-15 gene showed a single DNA band of 850 bp in female Kohaku Koi fish, namely in the 7 samples (70%) with sample code of KH-1, KH-3, KH-4, KH-5, KH-6, KH-9, and KH-10. Meanwhile, the male Koi Kohaku showed double DNA bands in the size of 850 bp and 1,100 bp which were seen in 3 samples (30%) with sample codes KH-3, KH-7, KH-8. In the Koi fish species (Cyprinus carpio) including the Kohaku species, the sex chromosomes are homozygous XX in female fish, while in male fish the sex chromosomes are heterozygous XY [21].

Except for the Kohaku Koi fish, the Bighead carp (Hypophthalmichthys nobilis) and Silver carp (Hypophthalmichthys molitrix) are the most closely related to the family Cyprinidae. Cyogenetic analysis revealed that these fishes shared the same karyotype but lacked heteromorphic sex chromosomes [25, 26]. However, researchers [16] reported that there are sex-specific markers that confirm the XX/XY sex-determination system in Bighead carp and Silver carp. In addition researchers [16] succeeded in amplifying Y-specific fragments on these two carp fishes. The ArS-9-15 gene only appears in male Bighead Carp and Silver Carp and has a target length of 908 bp. PCR detection using the ArS.9-15 primer design revealed one consistent band measuring 908 bp in male fish [16]. In this study, we used 10 local Kohaku Koi fish from Sleman Yogyakarta for sex-determining based on the results of PCR amplification of the ArS.9-15 gene, which showed that 70% are female Kohaku Koi fish, while 30% are male Kohaku Koi fish.

4. Conclusion

It can be concluded that sex determination in Kohaku Koi fish could be performed quickly, precisely, accurately and as early as possible based on PCR amplification of the ArS.9-15 gene. In ten of tested Kohaku Koi fish in this study, 70% were female and 30% were male Kohaku Koi fishes.

References

[1] Bachtiar Y, and Lentera T 2002 Mencegah ikan hias mudah mati. (Bogor: Agromedia Pustaka).
[2] Kusrimi E, Cindelaras S, dan Prasetio AB 2015 Media Akuakultur 10(2) 71-78
[3] Effendie MI 1979 Metode biologi perikanan. (Bogor: Yayasan Dewi Sri) 112.
[4] Papilon UM and Effendi M. 2017 Ikan koi. (Jakarta: Penebar Swadaya).
[5] Yusuf SE 2010 Ragam jenis ikan air tawar populer (Jakarta: Putra Danayu Publisher).
[6] Sutiana, Erlangga and Zulfikar 2017 Acta Aquatica 4(2) 76-82
[7] Jaya I dan Ibqal M 2009 Ilmu-ilmu Perairan dan Perikanan Indonesia 16(1) 7-15
[8] Twingg D 2008 Buku pintar Koi. (Jakarta: PT. Gramedia Pustaka)106
[9] Martinez P, Vinas AM, Sanchez L. 2014 Front. Genetic 5 340
[10] Wang D, Mao HL, Chen HX, Liu HQ and Gui JF. 2009 Curr. Biol 22 1423–1428
[11] Yano A, Guyomard R, Nicol B, et al. 2012. Oncorhynchus mykiss. Curr. Biol. 22 1423–8
[12] Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, and Kobayashi T 2002 Nature 417 559–563
[13] Kamiya T, Wataru K, Satoshi T 2012 PLoS Genet. 8
[14] Chen SL, Zhang G, Shao C 2014 Nat. Genet 46 253
[15] Li MH, Yunlv S, Jiue Z 2015 PLoS Genet. 11
[16] Liu H, Pang M, Yu X, Zhou Y, Tong J and Fu B 2018 *DNA Res.* **25**(3) 257–26
[17] Burhanuddin AI 2012 *Ikhtiologi, ikan dan segala aspek kehidupan.* (Yogyakarta: Deepublish).
[18] Fachtiyah, Arumingtyas EL, Widyarti S, Rahayu S 2011 *Biologi molekuler.* (Jakarta: Erlangga).
[19] Hidayati, Saleh E, Aulawi T 2016 *Jurnal Peternakan* **13**(1) 1-12
[20] Piferrer F, Ribas and Diaz N 2012 *Mar. Biotechnol.* **14** 591–604
[21] Mei J and Gui JF 2015 *Sci China Life Sci.* **58**(2) 124–136
[22] Shafique S 2012 *Polymerase chain reaction.* (Morrisville: Lulu Press)
[23] Puspitanิงrum R, Adhiyanto C, Solihin and Attas SG 2018 *Genetika molekuler dan aplikasinya.* Cetakan pertama. (Yogyakarta: Deepublish).
[24] Yuwono T 2006 *Teori dan aplikasi polymerase chain reaction.* (Yogyakarta: Penerbit Andi)
[25] Su ZG, Xu KS, Chen SP and Bai GD 1984 *Zool. Res.* 15–20
[26] Kong QL, Li ZY, Fu ML, Wang Q and Wang HY. 2006. *Sichuan J. Zool.* **25** 64–7