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Appropriate Primer Selection Improves Molecular Bird Sexing Accuracy

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ABSTRACT

Birds sexing utilize the Polymerase Chain Reaction (PCR) technique is increasingly being used by researchers and breeders. The PCR technique has high sensitivity, but its success is influenced by the specificity of the DNA template with the oligo primer used. This study aimed to evaluate 5 types of PCR primers P2/P8, 2550F/2718R, CHD1F/CHD1R, 1237L/1272H, and CHD1LF/CHD1LR to determine the sex of Phasianidae, Anatidae, Muscicapidae, and Psittacidae families. This research was conducted by tested primers mentioned above to amplify the target gene chromodomain helicase DNA binding 1 (CHD1) on DNA samples of each pair of males and females from four bird families, respectively. The results indicated that CHD1LF/CHD1LR PCR primer gave the best results and was recommended to determine the sex of four families tested. Some of other primers tested in this study failed to amplify targeted gene correctly, it is important to use appropriate primer to increase bird sexing accuracy.

Keywords: Bird, PCR sexing, Primer, Selection

Introduction

Birds sexing using the Polymerase Chain Reaction (PCR) technique is increasingly being used by researchers and breeders for breeding and sustainable conservation efforts. The PCR technique has high sensitivity, can be performed at all ages from juvenile to adults, and even could identify the sexes using the eggshell membrane as a DNA sample (Begović *et al*., 2017; Akrom *et al*., 2020; Yuda and Saputra, 2021; Turcu *et al*., 2023). PCR technique has advantages over conventional methods such as external morphological observation, vent sexing, laparoscopy, karyotyping, faecal sexing steroids, and ultrasound for oviduct identification (Khaerunnisa *et al*., 2013; Casana *et al*., 2019; Turcu *et al*., 2020; Hidayat *et al*., 2021). Those conventional methods above tend to have weaknesses, such as requiring a long time, experience, special expertise and invasive (Volodin *et al*., 2015; Purwaningrum *et al*., 2019; Elnomrosy *et al*., 2022). Disastra (2021) and Fitriana *et al*. (2023) has been demonstrated the error of bird sellers in determining sexes in various families, based on external morphological observations.

The PCR technique in birds sexing is based on the detection of the target gene chromodomain helicase DNA binding 1 (CHD1) on the Z and W chromosomes. It is known the sex chromosomes of male birds are composed of Z homogametes, whereas in female birds ZW heterogametes (Dobreva *et al*., 2021; England *et al*., 2021; Kroczak *et al*., 2022). Although the PCR method provides good results in determining the sex of birds, one of its successes is greatly influenced by the compatibility between the primers used and the DNA template (van der Velde *et al*., 2017). The CHD-W and CHD-Z genes in avian species are known to have varied nucleotide base sequences (Ciorpac *et al*., 2016; Kroczak *et al*., 2021; Kulibaba and Liashenko, 2021). Variations in nucleotide sequence can cause annealing process of primer PCR not run perfectly to the DNA template, which implicate the success of PCR amplification (Morinha *et al*., 2015; Green and Sambrook, 2019).

Four primers set P2/P8 (Griffiths *et al*., 1998), 1237L/1272H (Khan *et al*., 1998), 2550F/2718R (Fridolfsson and Ellegren, 1999), and CHD1F/CHD1R (Lee *et al*., 2010) are a popular primer used in molecular sexing worldwide. However, some studies have shown those primers to be unusable in certain species (Gebhardt and Waits, 2008; Vucicevic *et al*., 2013). On the other hand, new primer sets have been published, one of which is CHD1LF/CHD1LR (Liang *et al*., 2019). Previous research showed that CHD1LF/CHD1LR primers showed good potency in the Columbidae bird family (Disastra, 2021; Fitriana *et al*., 2022).

Exploring potential uses of P2/P8, 1237L/1272H, 2550F/2718R, CHD1F/CHD1R, and CHD1LF/CHD1LR primers for further research needs to be done to determine its specificity of various bird families in Indonesia especially the

species used in this study, so that it can provide information on primary choices that can be used in molecular sexing. This study aimed to evaluate the use of the five primers above in Phasianidae, Anatidae, Muscicapidae, and Psittacidae families. Information the performance of five primer types will be useful for other researchers and the users of molecular sexing in choosing the right primers, especially for the bird families tested.

Materials and Methods

Materials

The birds used in this study were a pairs of adult Local breed chicken (*Gallus gallus domesticus*, family: Phasianidae), Domesticated Muscovy duck (*Anas platyrhynchos domesticus*, family: Anatidae), Lesser shortwing (*Brachypteryx leucophrys*, family: Muscicapidae), and Lovebird (*Agapornis fischeri*, family: Psittacidae). Lesser shortwing and Lovebird were purchased from traditional bird markets in the Yogyakarta area, while Local breed chicken and Domesticated Muscovy duck were obtained from traditional slaughterhouses. The research and animals used has been approved by ethical committee of Veterinary Medicine Faculty, Universitas Gadjah Mada, Yogyakarta, Indonesia, with ethical clearance numbers 00023/EC-FKH/Eks./2021.

Blood collection and DNA extraction

DNA was extracted using Blood/Cell DNA mini kit (Geneaid, Taiwan) as manufacturer protocol. A total of 5 µl of fresh blood was collected from the bird's feet using a disposable blood lancet (Onemed, Indonesia) and mixed with 195 µL of sterilized Phosphate Buffer Saline (Sigma, USA) in 1.5 mL Eppendorf tube. Twenty microliters of Proteinase K (25 mg/mL, Geneaid, Taiwan) were added, then incubated at 60ºC for 5 min. Blood cells will completely be lysed using 200 µL of GSB buffer after incubated at 60ºC for 5 min. Two hundred microliters of absolute ethanol (Merck, Germany) were added to the sample lysate, mixed immediately by shaking, then the DNA were bound to GS column by centrifuged at 14.000 x g for 1 minute. The contaminants were washed away using 400 µL of W1 Buffer and 600 µL of Wash Buffer by centrifuged at 14,000 x g for 1 min, respectively. The column was centrifuged again for 3 min at 14.000 x g to dry the column matrix. Finally, the DNA was eluted in 50 µL of pre-heated Elution Buffer and stored -20ºC before use.

CHD gene amplification

Twenty-five microliters of PCR mixture consisting of 5 µL mastermix (5X PCR Master Dye Mix, ExcelTaq, SMOBIO, Taiwan), 1 µL forward primer (10 pmol/µL), 1 µL reverse primer (10 pmol/µL), 16 µL DDH_2O (Ultrapure water, Sigma Aldrich, USA), and 2 µL DNA template. The sequence of the 5 PCR primers used in this study are listed in Table 1. All primers were purchased from IDT (Integrated DNA Technologies, Inc., Singapore). DDH_2O were used as negative control for PCR amplification.

The PCR mixture was then homogenized and centrifuged for a few seconds, then the PCR tube was inserted into the thermal cycler machine (SelectCycler II Thermal Cycler, Select BioProducts, USA) with the PCR program as shown in Table 2. The PCR products were electrophoresed or stored -20°C until used.

Electrophoresis and visualization of PCR products

PCR products were separated using 1.5% agarose gel (Bioron, Germany) in Tris-borate-EDTA (TBE) buffer (Omnipure, Merck, USA), and FloroSafe DNA staining (First BASE, Taiwan). PCR products were added to each well. The size of PCR product was determined using 100 bp DNA ladder (Geneaid, Taiwan). The gel was electrophoresed using a submarine electrophoresis system (Mupid-exU, Japan) with a voltage of 135 V for 20 min. Then the gel was visualized on Dual LED Blue Transilluminator (BIO-HELIX, Taiwan). The appeared PCR bands were compared to DNA ladder and interpreted the bird sexes using targeted molecular size from the references listed in Table 1.

Results and Discussion

The electrophoresed agarose gel showed that all primers tested produced band products (Figure 1). However, the CHD1LF/CHD1LR primers showed the clearest, easy to analyze, and consistent bands across all species and sexes. It is demonstrated that the male showed single band around 474 bp, while the female showed 2 bands 474 and 319 bp (Figure 1A). These results indicate the CHD1LF/CHD1LR has potential use in Phasianidae, Anatidae, Muscicapidae, and Psittacidae families.

Table 1. Primer sequence used for CHD gene amplification

Primer name	Oligonucleotide sequence	Amplicon length	Refferences
P2/P8	P2 (5'-TCTGCATCGCTAAATCCTTT-3') P8 (5'-CTCCCAAGGATGAGRAAYTG-3')	300-400 bp, with a difference of ♂ and ♀ between 10-80 bp	(Griffiths *et al.*, 1998)
1237L/ 1272H	1237L (5'-GAGAAACTGTGCAAAACAG-3') 1272H (5'-TCCAGAATATCTTCTGCTCC-3')	♂: 250-290 bp ♀: 260-310 & 250-290 bp	(Khan *et al.*, 1998)
2550F/ 2718R	2550F (5'-GTTACTGATTCGTCTACGAGA-3') 2718R (5'-ATTGAAATGATCCAGTGCTTG-3')	♂: 600-650 bp ♀: 600-650 & 400-450 bp	(Fridolfsson and Ellegren, 1999)
CHD1F/ CHD1R	CHD1F (5'-TATCGTCAGTTTCCTTTTCAGGT-3') CHD1R (5'-CCTTTTATTGATCCATCAAGCCT-3')	♂: 389-553 bp ♀: 389-553 & 316-497 bp	(Lee *et al.*, 2010)
CHD1LF/ CHD1LR	CHD1LF (5'-TTCTGAGGATGGAAATGAGT-3') CHD1LR (5'-AGCAATGGTTACAACACTTC-3')	♂: 474 bp, ♀: 474 & 319 bp	(Liang *et al.*, 2019)

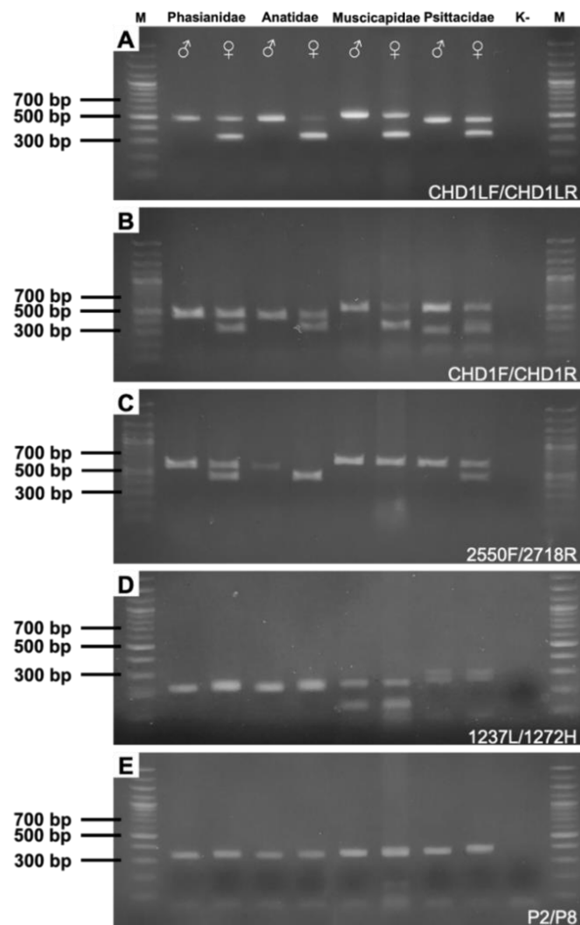

Figure 1. PCR products visualization of 5 primer sets in different bird families.

In contrast to CHD1LF/CHD1LR, the variations of PCR products are shown in visualization of P2/P8, 2550F/2718R, CHD1F/CHD1R, and 1237L/1272H. It is showed that the PCR products that appear in those 4 primers failed in some bird families tested. Figure 1B showed CHD1F/CHD1R could only differentiate the sexes in Phasianidae, Anatidae, and Muscicapidae families. It is demonstrated PCR products for male showed 1 band measuring around 500 bp, while for female 2 bands are around 500 and 320 bp. On the other hand, 2550F/2718R primer can be used for Phasianidae and Psittacidae only (Figure 1C). In contrast to CHD1LF/CHD1LR and CHD1F/CHD1R, as shown in figure 1D and 1E, primers P2/P8 and 1237L/1272H could not differentiate between the sexes of the 4 bird families tested. This study shows that the selection of primers is important in PCR sexing identification, not all primers can be used in various families, it is necessary to determine the right primer to get accurate results.

The success of PCR amplification is influenced by many factors, one of which is the suitability of the DNA template with the primer used. The nucleotide sequence in the primer that does not match the DNA template may not produce PCR products with the actual target size or even no amplification at all (Green and Sambrook, 2019). The failure of various primer sets in molecular sexing PCR has been widely reported (Vucicevic *et al.*, 2013; van der Velde *et al.*, 2017; Mazzoleni *et al.*, 2021; Pamulang and Haryanto, 2021). Gebhardt and Waits (2008) reported P2/P8, 1237L/1272H, and 2550F/2718R were failed in *Phasianus colchicus* and *Ara macao*. Moreover, Vucicevic *et al.* (2013) study reported that primers

Table 2. PCR program for amplification of CHD target genes

Step	P2/P8		1237L/1272H		2550F/2718R		CHD1F/CHD1R		CHD1LF/CHD1LR	
	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)
Initial denaturation	94	90	94	120	94	120	95	300	94	300
Denaturation	94	30	94	30	94	30	94	30	94	30
Annealing	48	45	56	60	50	30	48	30	53.5	30
Extension	72	45	72	120	72	30-40	72	60	72	30
Cycle	30		30		25-35		28		38	
Final extension	72	300	72	600	72	300	72	300	72	300

2550F/2718R and P2/P8 were unable to identify 8 bird species out of a total of 58 species tested.

The CHD-W and CHD-Z genes among avian species are known to have wide diversity (Ciorpac *et al.*, 2016; Kroczak *et al.*, 2021; Kulibaba and Liashenko, 2021). Huang *et al*. (2011) reported differences in the sequences of the CHD-W and CHD-Z genes among 3 Columbidae species (*C. livia, C. pulchricollis,* and *S. tranquebarica*). Lee *et al*. (2010) also demonstrated the CHD Z gene variations in 4 sequence samples of *Aegithalos concinnus*, from 476 bp it was found that there were 8 base substitutions and 1 deletion. In summary, it is suggested genetic diversity in bird CHD gene, the variation of nucleotide bases for each bird species is a major challenge in the success of molecular sexing, it is necessary to use primers that are appropriate to the species to be tested.

Conclusions

CHD1LF/CHD1LR PCR primer showed the best results and was recommended to determine the sex of Phasianidae, Anatidae, Muscicapidae, and Psittacidae families. P2/P8, 1237L/1272H, 2550F/2718R, CHD1F/CHD1R primers were failed to amplify targeted gene correctly in one or some of 4 families tested. Primer selection in bird sexing should be considered.

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