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 chromodomian 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 chromodomian 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 (Dobrova 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 (Ciorga 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 pair of adult Local breed chicken (*Gallus gallus domesticus*, family: Phasianidae), Domesticated Muscovy duck (*Anas platyrhynchos domesticus*, family: Anatidae), Lesser shortwing (*Brachypteryx leucophris*, 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 60°C for 5 min. Blood cells will completely be lysed using 200 μl of GSB buffer after incubated at 60°C 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 containants was washed away using 400 μl of W1 Buffer and 600 μl of Wash Buffer by centrifuged at 14.000 x g for 1 minute, 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 -20°C 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 DDH2O (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). DDH2O 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 (Omipure, Merck, USA), and FluorSafe 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 markers 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>
<thead>
<tr>
<th>Primer name</th>
<th>Oligonucleotide sequence</th>
<th>Amplicon length</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2/P8</td>
<td>P2 (5'-TCGATAGCATTTGAAATGTCGTTTCT-3')</td>
<td>300-400 bp, with a difference of 3' and 5' between 10-80 bp</td>
<td>(Griffiths et al., 1998)</td>
</tr>
<tr>
<td>1237L</td>
<td>1237L (5'-GAGAAACGTGCTTATGACAGAG-3')</td>
<td>250-290 bp</td>
<td>(Khan et al., 1998)</td>
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<tr>
<td>1272H</td>
<td>1272H (5'-TCGATAGCATTTGAAATGTCGTTTCT-3')</td>
<td>260-310 &amp; 290-290 bp</td>
<td>(Fridolfsson and Ellegren, 1999)</td>
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<td>2550F</td>
<td>2550F (5'-GTAAGTCAGTCGAAGAAGATGGGCCTG-3')</td>
<td>600-650 bp</td>
<td>(Lee et al., 2010)</td>
</tr>
<tr>
<td>2718R</td>
<td>2718R (5'-ATGAAAGATGGCAGCTGCTGCTG-3')</td>
<td>400-450 bp</td>
<td>(Li et al., 2019)</td>
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</table>
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 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>
<thead>
<tr>
<th>Step</th>
<th>P2/P8</th>
<th>1237L/1272H</th>
<th>2550F/2718R</th>
<th>CHD1F/CHD1R</th>
<th>CHD1LF/CHD1LR</th>
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<tbody>
<tr>
<td></td>
<td>Temp (°C)</td>
<td>Time (s)</td>
<td>Temp (°C)</td>
<td>Time (s)</td>
<td>Temp (°C)</td>
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<td>600</td>
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</table>
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 that the CHD gene of bird sex can be considered. 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 that the species to be tested. The CHD gene of bird sex can be considered.

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 was 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 that the CHD gene of bird sex can be considered. 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 that the CHD gene of bird sex can be considered. 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 that the CHD gene of bird sex can be considered.

References


