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Original Article

Evaluation of primers targeting chromo helicase DNA-binding gene (*CHD*) for molecular sexing identification in four bird families

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Abstract

Objective: Sex determination of birds is crucial role in breeding and conservation purposes. Genomic approaches by using basis of bird sex polymorphism are accepted to generate an accurate sexing procedure. The PCR method is commonly used and known to provide accurate test results in determining the sex of birds, but its successes is strongly influenced by the compatibility of the primers used with the DNA template. This study aimed to determine the potential use of CHD1LF/CHD1LR targeting CHD gene fragment in 4 species from Phasianidae, Psittacidae, Estrildidae, and Passeridae families.
Method: Blood samples (n= 30) from representatives of species in 4 families above were collected for DNA isolation, then PCR test was performed using CHD1LF/CHD1LR primers. The PCR results obtained were compared with positive controls, necropsy examination and information from bird sellers.
Result: The results showed that the primer CHD1LF/CHD1LR could be used in Phasianidae, Psittacidae, Estrildidae, and Passeridae families. The PCR product was confirmed to be same as well as the positive control and necropsy results. It is demonstrated that male birds showed a single band (474 bp), while double bands (474 and 319 bp) were observed in female birds. Different accuracies were observed during this study between molecular approach and manual sexing by bird seller i.e: sparrows (46%) and finches (50%), while the accuracy of parakeets was 75%, and quails 80%.
Conclusions: Primer CHD1LF/CHD1LR can be used to determine the sex of birds from the families Phasianidae, Psittacidae, Estrildidae, and Passeridae. Our study showed that molecular-based method is valuable and able to reduce error rate to 100% in bird sexing procedures.

Keywords: Birds; *Molecular sexing*; PCR; Sex determination

INTRODUCTION

Sex determination of birds is important for breeding and conservation purposes. There are several methods that are often used to determine the sex of birds, such as laparoscopy, karyotyping, and vent sexing [1,2]. The use of these methods tends to have disadvantages, such as requiring a long time, special expertise and invasive [3,4]. Compared to the conventional methods above, the Polymerase Chain Reaction (PCR) method shows more

accurate, efficient, and popular among researcher [1,5,6]. The PCR method is based on the detection of DNA variations in the chromodomain helicase DNA binding 1 (CHD1) target gene on the Z and W chromosomes [7-9]. Sex chromosomes in male bird is composed of Z homogametes, while in female birds are heterogametes ZW [10-12]. Four sets of primers commonly used in PCR amplification of molecular sexing are P2/P8 [13], 1237L/1272H [14], 2550F/2718R [15], and CHD1F/CHD1R [3]. Although PCR method gives good results in determining the sex of birds, one of its successes is strongly influenced by the suitability between the primers used and DNA template [16]. Variations the sequence of nucleotide bases in the PCR target gene may cause the primers annealing to the template DNA perfectly, which has implicate the success of PCR amplification [17]. CHD-W and CHD-Z genes in avian species are known to have varied nucleotide sequences [5,18,19]. Fitriana *et al.* [20] reported P2/P8, 1237L/1272H, 2550F/ 2718R, and CHD1F/ CHD1R primers gave unsatisfactory PCR results such as the size of PCR band did not match to the reference, extra bands or the absence of PCR products in determining the sex of 5 species of Columbidae family *Columba livia*, *Streptopelia chinensis*, *Treron griseicauda*, *Spilopelia chinensis*, and *Geopelia striata*.

In addition to the commonly used P2/P8, 1237L/1272H, 2550F/2718R, and CHD1F/ CHD1R primers, the CHD1LF/ CHD1LR primer [21] is a relatively new used in bird sexing, published in 2019. The research by Disastra [22] and Fitriana *et al.* [20] previously, showed CHD1LF/CHD1LR gave satisfactory and consistent results in 5 species of the Columbidae family. Information on the primary use of CHD1LF/CHD1LR is still very limited and needs to be explored more deeply, especially outside the Columbidae family. This study aimed to determine the potential use of CHD1LF/CHD1LR primers in 4 species from Phasianidae, Psittacidae, Estrildidae, and Passeridae families.

MATERIALS AND METHODS

Materials

The birds used in this study were 5 quails (*Coturnix coturnix*, family: Phasianidae), 4 parakeets (*Melopsittacus undulates*, family: Psittacidae), 8 finches (*Estrildid finches*, family: Estrildidae), and 13 sparrows (*Passer montanus*, family: Passeridae). Parakeets, finches, and sparrows were purchased from traditional bird markets in the Yogyakarta area, while quails were obtained from traditional slaughterhouses. Sex information were obtained from the sellers or slaughter master, then recorded for comparison with PCR validation. Necropsy of a pair of male and female quails were performed to confirm sex on blood samples taken shortly before slaughter. Meanwhile, parakeets, finches, and sparrows that have been blood drawn were then released into nature. The animals used and research has been approved by ethical committee of Veterinary Medicine Faculty, Universitas Gadjah Mada, Yogyakarta, Indonesia, with ethical clereance numbers 00023/EC-FKH/Eks./2021.

Blood collection and DNA extraction

Blood samples were taken from the bird's feet using a disposable blood lancet (Onemed, Indonesia). A total of 5 µl of fresh blood was collected using a micropipette (Eppendorf, Germany), then put into a 1.5 ml Eppendorf tube containing 195 µl of sterile Phosphate Buffer Saline (Sigma, USA). The mixture was then homogenized and resuspended using a micropipette for further DNA isolation using the spin-column based method according to the instructions of the isolation kit (*Blood/Cell DNA mini kit*, Geneaid, Taiwan).

DNA amplification

PCR sexing was performed by targeting the CHD gene, using primers CHD1LF/CHD1LR according to Liang *et al.* [21] which forward sequence is 5′ TTCTGAGGATGGAAATGAGT 3′ and reverse 5′ AGCAATGGTTACAACACTTC 3′. A total 25 µl of PCR mixture containing 5 µl master mix (5X PCR Master Dye Mix, ExcelTaq, SMOBIO, Taiwan), 1 µl primer of each forward and primer reverse (10 pmol/µl) (Integrated DNA Technologies, Inc., Singapura), 17 µl DDH_2O (Ultra Pure water PCR Grade, Sigma, USA) and 2 µl DNA template. DNA of pigeon (*Columba livia*) from Disastra [22] was used as positive control. PCR

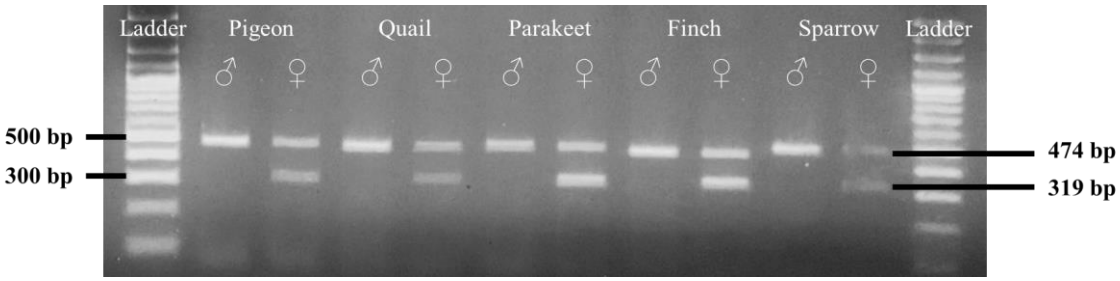

Figure 1. Results of PCR amplification of positive control and four birds families.

mixture was vortexed to homogenized (IKA, Germany) and spinned down (DLAB, China) before running on thermal cycler (SelectCycler II Thermal Cycler, Select BioProducts, Taiwan). PCR was performed with condition pre-denaturation 94°C (5 minutes), denaturation 94°C (30 second), annealing 53.5°C (30 second), extension 72°C (30 second) and post-extension 72°C (5 minutes). PCR amplification was completed in 38 cycles.

PCR product visualization

DNA staining (FluoroVue, SMOBIO, Taiwan) and 1.5% agarose gel (Bioron, Germany) in Tris-borate-EDTA buffer (Omnipure, Merck, USA) were used to separating the PCR products. PCR products and 100 bp DNA ladder (AccuBand, SMOBIO, Taiwan) in the gel was electrophoresed using a submarine electrophoresis system (Mupid-exU, Japan) with a voltage of 135 volts for 20 minutes. The presence of PCR products was visualized using Dual LED Blue Transilluminator (BIO-HELIX, Taiwan). The resulting PCR bands were then compared with the DNA ladder and documented with a camera.

RESULTS

The PCR electrophoresis in Figure 1 showed that all tested bird families had the same band size as the positive control (pigeon). In detail, the PCR product of male birds showed 1 band with 474 bp, while 2 bands in female birds with 474 and 319 bp (Figure 1). These results indicate that the primary use of CHD1LF/CHD1LR is not limited to pigeon and the Columbidae family as reported previously [20,22]. Primer CHD1LF/CHD1LR has potential use in Phasianidae, Psittacidae, Estrildidae, and Passeridae families.

We also confirmed PCR results with necropsy examinations on representative a pair of quail which were carried out shortly after blood sample collection. It showed that the PCR results are in accordance with the necropsy performed, indicated by the presence of the testes in the male quail (Figure 2a), and the ovaries in the female quail (Figure 2b). The PCR product for male quails showed 1 band with 474 bp, while in female quail showed 2 bands with 474 and 319 bp (Figure 2c).

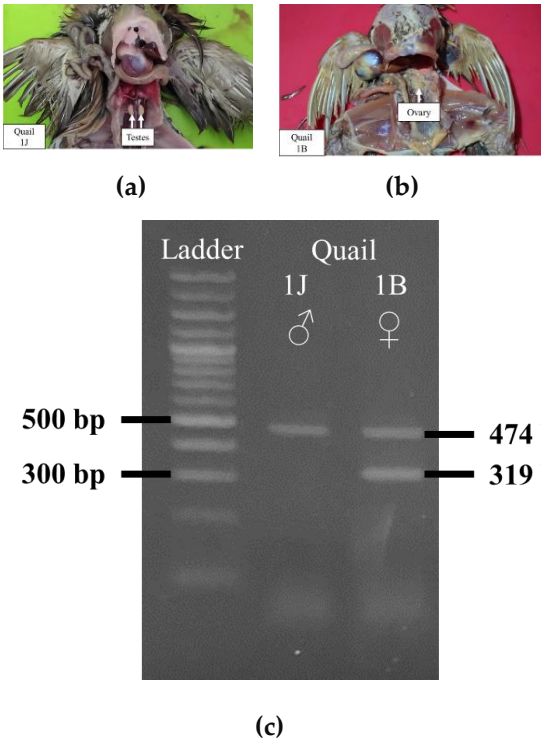

Figure 2. Confirmation of quail sexes by necropsies: a. Code 1J has testes; b. Code 1B has ovary; c. Electrophoresis results of PCR products samples 1J and 1B.

This study also confirms that neither seller nor slaughter master is completely precise in determining sex based on the external morphology of birds compared to PCR

Table 1. Comparison of seller or slaughter master information with PCR amplification results

No	Birds	Code	Identification		Discrepancy	Accuracy percentage
			External morphology	PCR		
1	Quail	1B	♀	♀	-	4/5 (80%)
2		2B	♀	♀	-	
3		3B	♀	♀	-	
4		1J	♂	♂	-	
5		4B	♀	♂	✓	
6	Parakeet	J1	♂	♂	-	3/4 (75%)
7		B1	♀	♂	✓	
8		Pa1	♂	♂	-	
9		Pa2	♀	♀	-	
10	Finch	J1	♂	♂	-	4/8 (50%)
11		B1	♀	♂	✓	
12		J2	♂	♂	-	
13		B2	♀	♂	✓	
14		EJ1	♂	♀	✓	
15		EB1	♀		-	
16		EJ2	♂	♂	-	
17		EB2	♀	♂	✓	
18	Sparrow	J1	♂	♀	✓	6/13 (46%)
19		B1	♀	♀	✓	
20		J2	♂	♀	✓	
21		B2	♀	♀	-	
22		Ger1	♂	♀	✓	
23		Ger2	♂	♀	✓	
24		Ger3	♂	♀	✓	
25		Ger4	♀	♀	-	
26		Ger5	♀	♀	-	
27		No1	♀	♀	-	
28		No2	♂	♂	-	
29		G1	♂	♀	✓	
30		G2	♀	♀	-	
Total						13/30

results (Table 1). There is a difference between the seller's or slaughter master information and the results of the PCR validation. It shown that they have a low accuracy rate on sparrows 46% (6/13) and finches 50% (4/8), while the accuracy of parakeets is 75% (3/4) and quails 80% (4/5). Similar to Disastra [22] finding on Columbidae family, this indicates that sex determination based on external observations has limitation, on the other hand the PCR method as in this study has better accuracy.

DISCUSSION

Approximately 1,777 different bird species have been recognized in Indonesia as of 2019, and they are widely dispersed throughout the country [23]. Interestingly, sex determination of bird species is a major problem for researchers, bird breeders, songbird lovers, or the community. Griffiths *et al.* [13], stated that more than 50% of bird species in the world have external morphological characters that are difficult to distinguish between males and females, which is commonly called "monomorphic".

This study also shows the inaccuracy of bird sellers in sex determination, it's known that the level of accuracy in finch and sparrow species is only around 50%. Disastra [22] previously also showed the seller was failed to determine the sex of the family Columbidae.

This indicates that sex determination based on external morphology is not accurate enough, even in many bird families. Molecular sexing is a method that is increasingly being used by researchers and the public, but still has limitations, there is no universal primer that can be used on all bird species [24,25].

Primer selection is important in molecular sexing [16,25,26]. The failure of many primer sets in PCR sexing has been reported previously. Gebhardt dan Waits [27] reported primers P8/P2 and 1237 L/1272 and 2550 F/2718R had a mean of PCR error about 1.9% to 24.2% in Ring-necked Pheasant (*Phasianus colchicus*) and Scarlet Macaw (*Ara macao*) species. In the other hand, Vucicevic *et al.* [24], reported that primers 2550F/2718R and P2/P8 failed to identify 8 bird species out of a total of 58 species tested. Moreover, Çakmak *et al.* [28] demonstrated the comparative use of three specific primers such as CHD1F/ CHD1R, 2550F/2718R, and P2/P8 in 77 different bird species. The results showed that the three primers still did not show it accuracy up to 100%. More detail, Huang *et al.* [29] showed differences in the nucleotide sequences of the CHD-W and CHD-Z genes in one family Columbidae tested, *C. livia, C. pulchricollis*, and *S. tranquebarica* species. These indicates that the failure of PCR method may cause by a mismatch of primers with the DNA template of the birds tested, suggesting the variations in the nucleotide sequences of each bird species become a major challenge in the success of molecular sexing.

The newly discovered alternative primers, need to be further studied to see their potential, especially in bird species that cannot be identified by the common primers used in molecular sexing earlier. This study shows that primers CHD1LF/CHD1LR are not only limited to the Columbidae family but can also be used on birds of the Phasianidae, Psittacidae, Estrildidae, and Passeridae families.

CONCLUSION

Primer CHD1LF/CHD1LR can be used to determine the sex of birds from the families Phasianidae, Psittacidae, Estrildidae, and Passeridae. Exploration of the potential use of CHD1LF/CHD1LR primers needs to be carried out to determine their specificity in other birds outside the bird family that has been tested, it's provided information on the choice of primers that can be used in molecular sexing method.

CONFLICT OF INTEREST

The authors declare no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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