Sex determination of peach-faced lovebird (*Agapornis roseicollis*) using polymerase chain reaction (PCR) techniques

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Abstract. Molecular sex determination is an effective solution to determine sex because it can be done early in the growing phase of a bird and the results are more accurate. Molecular sex determination is carried out based on the chromodomain helicase DNA binding (CHD) gene by using NP, P2, and MP primers. The purpose of this study was to determine the sex of peach-faced lovebird (*Agapornis roseicollis*) by detecting the intron size of the CHD gene on the Z chromosome and W chromosome by polymerase chain reaction (PCR). DNA samples were isolated from feathers of 14 lovebirds belonging to bird owners, which was sent to the Biochemical Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Amplification of the CHD gene using PCR techniques with NP, P2, MP primers electrophoresed with agarose gel 2.5%. Visualization under UV Transilluminator with a wavelength of 280 nm produce an amplicon as long as about 300-400 bp with males showing a single DNA band (ZZ) and females showing a double DNA band (ZW). Based on the electrophoresis results, it showed eight females and six males in 14 samples of lovebird used.

1. Introduction

Lovebird is mostly preserved as an ornamental bird because of the beauty of its dazzling feather color. The difference in feather color will distinguish the species as well so that lovebird is called a unique bird. The color variations that can be produced by cross-breeding makes lovebird more attractive. Lovebird in Indonesia is not only kept as ornamental bird, but also for its chirping because of its long chirping sound. Lovebird is bred to get its distinctive and beautiful sounds so that it can be included in the birdsong competition. The interest of bird enthusiast towards lovebird and high selling market prices makes breeding lovebird a profitable business opportunity [5]. One species of lovebird that is now beginning to be in great demand and breed is peach-faced lovebird (*Agapornis roseicollis*). This species belongs to the group of monomorphic birds that males and females have the same physical characteristics making it difficult to distinguish. This limitation can cause feed losses during breeding due to mating without producing the expected new individuals. Time efficiency is also an important factor when producing new individuals with attractive feather color variants makes determining the sex of lovebird important [2]. Several methods of determining sex have been found such as vent sexing,
laparoscopy, sexing steroids, and karyotyping. These methods are often used to determine the sex of a monomorphic bird, but this method requires a long time and it is expensive time [3]. Some of these methods can cause pain and even lead to death in birds, making other safer methods are explored.

Molecular sexing is the right and accurate method for determining the sex of monomorphic birds. This method is the simplest deoxyribonucleic acid (DNA) based test mainly for determining sex by analyzing sex chromosomes. Molecular sexing is based on the detection of differences in the intron size of the chromodomain helicase DNA binding (CHD) gene on the Z chromosome and the W chromosome [9]. Chromodomain helicase DNA binding (CHD) genes are amplified by polymerase chain reaction (PCR) techniques using primers that work in most birds [10].

2. Metodology
This research was conducted at the Biochemistry Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada in October 2018 until February 2019. The research samples were feathers from 14 peach-faced lovebird (Agapornis roseicollis) from bird owners and are labelled with AR1 to AR14. Deoxyribonucleic acid (DNA) was isolated from samples based on the Geneaid Gsync DNA Extraction Kit protocol, but the incubation time was extended to overnight aimed at making DNA well extracted so that the DNA bands produced when exposed to UV light became clear.

The results of DNA isolation were used as DNA templates in the process of amplification using the PCR method. DNA fragments were amplified by targeting the CHD gene on the sex chromosome using P2, NP, and MP primers. Primary base arrangements, annealing temperature, and melting temperature (Tm) are presented in Table 1. A mixture of 25 µL of PCR reagents for bird DNA in one reaction consist of MyTaq™ DNA Polymerase, forward primer [10 pmol], reverse primer [10 pmol], and isolated DNA. The mixture was put into the PCR machine with controlled temperature and duration of the PCR reaction, beginning with predenaturation of 94°C for 2 minutes, denaturation of 94°C for 20 seconds, annealing 46°C for 30 seconds, elongation of 72°C for 40 seconds, and ends with post elongation at 72°C for 10 minutes. The denaturation, annealing and elongation stages were repeated as many as 40 cycles.

The results of DNA and PCR isolation can be identified by DNA electrophoresis. Fourteen DNA samples mixed with loading dye were compared with 100 bp hyperladder markers. Electrophoresis is done with a 2.5% agarose gel at a voltage of 100 volts and an electric current of 75 amperes for 45 minutes. DNA bands can be observed using UV Transilluminator wavelength of 280 nm.

3. Result and Discussion
Samples that can be used other than feathers to determine the sex of a bird are blood. Harvey et al [6] reported that blood samples contain more DNA than feathers, but need more energy to handle the birds when collecting blood samples. Birds have small blood vessels so extraction of DNA from blood is difficult. Improper handling of birds can also stressed birds, causing them to die.

Feathers as a sample in determining the sex of birds has advantages, the sampling process is easier and faster when compared to blood. Reduced handling time when collection of samples can reduce stress on birds [8]. The use of feather can also avoid pain in birds and reduce the risk of contamination so that the costs needed are lower [1]. The amount of DNA that can be isolated from feather samples is not as much as of DNA from blood samples, but with consideration of speed, convenience and minimal risk, feathers are chosen as samples to molecularly determine bird sex [6].
Hickman et al [7] stated that the source of DNA in feathers was obtained from the base of the feather (calamus) which contains many epithelial cells and contained inhibitors, namely keratin, making the extraction process quite difficult. The results of DNA extraction using a kit produce better DNA quality, but by using kits will increase costs. The kit used for DNA extraction in this study is Geneaid Gsync DNA Extraction Kit but the incubation time is extended to overnight so that DNA can be extracted properly. Electrophoresis results of DNA isolation (Figure 1) visualized under 280 nm UV light with a 100 bp hyperladder marker produced a luminous DNA fragment due to the presence of SYBRSafe in the sample. DNA bands are clearly seen in samples number seven, eight, nine, eleven, and twelve. Samples that are empty and do not display DNA bands show that the DNA in the sample does not exist or is so thin that it cannot be visualized.

**Figure 1.** Electrophoresis results from total DNA isolation. M= marker (hyperladder 100 bp), 1-14= sample of lovebird

Amplification of the CHD gene with primers of NP, P2, and MP visualized under UV light with a wavelength of 280 nm will produce one band for males and two bands for females. This is because Aves has a sex chromosome that is different from mammals. Heterogametic properties in birds are owned by females (ZW) while males are homogeneous (ZZ) (Ellergren, 1996). The CHD gene can show differences in Z and W alleles in females due to the linkage between the position of the CHD gene and the sex chromosome. The visualization results under UV Transluminator compared to 100 bp hyperladder resulted in a 400 bp PCR product on the Z chromosome and 300 bp on the W chromosome.

**Figure 2.** Electrophoresis of CHD gene amplification samples of lovebird. M= marker (hyperladder 100 bp), 1-14= lovebird samples, C♀= female control, C♂= male control.

| No. | Sample Code | Electrophoresis result | Interpretation |
|-----|-------------|------------------------|----------------|
| 1.  | AR1         | Two bands              | Female         |
| 2.  | AR2         | One band               | Male           |
| 3.  | AR3         | Two bands              | Female         |
| 4.  | AR4         | Two bands              | Female         |
| 5.  | AR5         | Two bands              | Female         |
| 6.  | AR6         | One band               | Male           |
| 7.  | AR7         | One band               | Male           |
4. Conclusion

Electrophoresis of PCR products with primers of NP, P2, and MP in peach-faced lovebird produces introns measuring around 400 bp on the Z chromosome and around 300 bp on W chromosome. Electrophoresis results of 14 lovebird samples showed a total eight females and six males lovebird.

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