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Application of Two Molecular Sexing Methods for Indonesian Bird Species: Implication for Captive Breeding Programs in Indonesia

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Visually identifying the sex of a bird can be difficult. It cannot be done in half the world's species when they are adults, and virtually none can be sexed as chicks. Despite this, the sex of a bird is vital for captive breeding. An increased number of birds are being sexed using DNA amplification techniques. In this approach, the CHD-W and CHD-Z are distinguished by the amplification of an intron present in both genes. PCR products on the gel electrophoresis vary in size revealing one band in males at the CHD-Z, and two bands in females corresponding to both the CHD-W and CHD-Z. Two independent sets of primer (P8/P2 and 2550F/2718R) were used to amplify the CHD gene region from both the Z and W chromosome. One hundred and ten (110) birds were sexed using first pair of primers: (P8/P2). Sexing results indicated that 81.8% were successfully determined, 12.7% failed to be amplified and 5.5% were not perfectly determined because the PCR products showed thick band. The thick band caused misidentified female to male birds. An alternative primer (2550F/2718R) was applied to solve the problem. Two hundreds and twenty-nine birds were sexed and the results showed 100% successfully determined. From this study, it is suggested to use a pair of 2550F and 2718R primers for distinguishing a male from a female bird.

Key words: sex identification, Indonesian birds, primer sexing, PCR, captive breeding

INTRODUCTION

Indonesia which is one of rich countries in biodiversity of birds has 1598 species and 372 species of them are endemic to Indonesia (Sukmantoro *et al*. 2007). On the other hand, the damaged natural habitat and uncontrolled exploitation of exotic species lead to Indonesia has the highest number of threatened birds in the world. A study reported by Baillie *et al*. (2004) that Indonesia recorded 118 (7.38%) bird species categorized as endangered species in 2004 IUCN Red List of threatened species. Efforts to protect those birds are conducted through government's act or through *in-situ* or *ex-situ* conservation.

Several efforts in *ex-situ* conservation has been successfully conducted in Indonesia, such as: *ex-situ* conservation in the ZOO; *ex-situ* conservation in captive breeding by community, collection and documentation of fauna specimen (Museum Zoologicum Bogoriense) and DNA genome as genetic resources in Division of Zoology, Research Center for Biology, the Indonesian Institute of Sciences (LIPI). Breeding in captivity can be an important factor as preservation measure for the species. Captive breeders in several location in Indonesia such as Indonesian Safari Park or Bird's park or ZOO or Bird's association required a technique allowing early sex determination of the birds. One of difficulties encountered in their captive breeding is that their genetic sexes are difficult to be identified from their external morphological characteristics at the time of pairing. It is believed that bird sexing is one of the important factors for successful ex-situ conservation program. If sex determination in the birds is well established, better conservation program will be optimistically achieved.

Sex is one of the most variable to distinguish individuals. Sex identification of birds can provide researchers with important information regarding the ecology and behavior of bird species (Helander *et al*. 2007), also provides valuable insights into their breeding strategies, conservation and management programs (Helander *et al*. 2007; Garcia *et al*. 2009; Naim *et al*. 2011), reproduction programs of threatened species (Ellegren & Sheldon 1997). In birds, the absence of juvenile sexual dimorphism often makes it difficult or even impossible to determine a chick's sex on the basis of external morphology. A similar problem exists for fully grown individuals of many birds species where adult sexual dimorphism is absent or at least not very pronounced. Efforts to determine sex in birds have been done from time to time. Up to now, there have been various approaches being used for sex identification other than molecular techniques for monomorphic birds including avian laparoscopy, biochemical analysis, and cytogenetic analysis (Richner 1989; Dubiec & Zagalska-neubauer 2006). However, these approaches are usually time consuming or invasive to individuals.

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With the development of molecular techniques, improved sexing techniques have been developed. Molecular sexing is attractive since it can be potentially provide an accurate and rapid means for sex identification if based on non-invasive techniques (Lessells & Mateman 1996; Ellegren & Sheldon 1997; Sheldon 1998). The chromosomal sex determination system of bird is different from that of mammals. In birds, female are heterogametic (ZW) while males are homogametic (ZZ), and sexing can thus be made by the detection of the W chromosome or W chromosome sequences in a sample of unknown sex. In 1995, Griffiths and Tiwari discovered the first and only avian W chromosome (analogue to Y chromosome in mammals) "Chromo-helicase-DNA-binding gene" (CHD-W). This gene is remarkably conserved and it has been shown that a single set of PCR primers can be used to sex birds throughout the class aves, with the exception of ratites (Griffiths & Tiwari 1996; Griffiths *et al*. 1996).

These sex-specific genetic markers simultaneously amplify homologous part of CHD-W and the related gene CHD-Z (referred to as CHD-NW but is actually Z linked (Griffiths & Korn 1997). Because CHD-Z occurs in both sexes it should always be amplified and this ensures that the PCR reaction has worked. Unfortunately, the two CHD products were the same size; therefore Griffiths *et al*. (1996) used a restriction enzyme to selectively cut a fragment from the CHD-Z version before gel electrophoresis. Female, therefore female had two bands and male had one band. More recently, Griffith *et al*. (1998) introduced new approach in which no restriction enzyme was needed. They employ two primers which anneal to conserved exonic region but then amplify across an intron in both CHD-W and CHD-Z. Because these introns are noncoding they are less conserved and their length usually differ between the genes. It leads to the PCR product vary in size. Therefore, the gel electrophoresis immediately reveals one band the male and two bands in the female.

In 1999 Fridolfsson and Ellegren also developed a simple and universal method for molecular sexing of non-ratite birds, which based on the detection of a constant size difference between CHD1W and CHD1Z introns. Using highly conserved primers flanking the intron, PCR amplification and agarose electrophoresis, females are characterized by displaying one (CHD1W) or two fragments (CHD1W and CHD1Z), while males only show one fragment (CHD1Z) clearly different in size from the female-specific CHS1W fragment.

It is known that sex identification of birds is essential part of *ex-situ* conservation breeding programmes. Although the CHD gene has been used successfully in many bird species (Griffiths *et al*. 1998; Miyaki *et al*. 1998; Ito *et al*. 2003; Sacchi *et al*. 2004; Lee *et al*. 2007, 2010), but we mainly discussed the merits of two such methods for the molecular sexing of captived birds in this study, the Griffith *et al*. (1998; P8/P2) and Fridolfsson and Ellegren (1999; 2550F/2718R). The aim of this work was to test the 2-molecular sexing method on bird species, particularly for birds kept in captivity in Indonesia.

MATERIALS AND METHODS

Sampling and DNA Extraction. Three hundreds and thirty-nine (339) material DNA samples of birds from across the class aves i.e. 110 samples consisted of 56 species and 229 samples consisted of 10 species were used in this study (Table 1 & 2). Only 8 samples of *Macrocephalon maleo* were collected from Sulawesi island, and the remaining samples were collected from bird captivities in Java and Bali islands, including Indonesian Safari Park (Bali), Indonesian Safari Park (Prigen) and Indonesian Safari Park (Cisarua), Gembiraloka Zoo (Yogyakarta), Surabaya Zoo (Surabaya), Taman Margasatwa Ragunan (Jakarta), Bali Bird Park (Bali), Bird Traders (West Java), and Pro Animalia. The material DNA samples used in this study were deposited at the DNA Bank of Indonesian Fauna, Division of Zoology, Research Center for Biology-LIPI. Material DNA (blood and plucked feathers) samples which precipitated with ethanol were extracted using phenol/chloroform procedures (Sambrook *et al*. 1989).

DNA Amplification. Molecular technique for sex identification in birds conducted in this study, based on polymerase chain reaction (PCR), in which sex-specific DNA is located by primers and then amplified. The two CHD-related primer sets (P8/P2 and 2550F/2718R primers) used in sex identification were designed to flank the fragment of the gene with the intron. This allows discrimination between the products from the Z and W chromosomes on a gel. One hundred and ten (110) samples (Table 1) were sexed using a set of P8/P2 primers (Griffiths *et al*. 1998). The first set of primer sequences were as follows: P8: 5'-CTCCCAAGGATGAGRAAYTG-3' and P2: 5'-TCTGCATCGCTAAATCCTTT-3'; 2550F (5'-GTTACTGATTCGTCTACGAGA-3'). If a set of P8/P2 primers could not differentiate between male to female, an alternative primer set (2550F/2718R) was applied to solve the problem (Fridolfsson & Ellegren 1999). Sum of 229 samples (Table 2) were sexed using a pair of 2550F/2718R primers (Fridolfsson & Ellegren 1999). The second set of primer sequences were as follows: 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (ATTGAAATGATCCAGTGCTTG-3').

PCR amplification for both primer pairs were carried out in a total volume of 15 µl. The final reaction condition were as follows: reaction containing 0.2 mM of each dNTP, 0.3 pmol of each primer, 2.5 mM $MgCl_2$, 0.5 Units of Taq DNA polymerase in 1x reaction buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl), and 0.3 mg/ml of BSA. Reactions of PCR for both primer pairs were made in the tube 0.2 ml and the reaction process of PCR were carried out on the thermocycler machine Gene Amp*PCR system 9700 (Applied Biosystem, USA).

As many as 229 samples from several captivities were identified using the 2550F/2718R primers. Molecular sexing was conducted because sex of the 229 birds could not be identified by morphological appearance. It is believed that bird sexing is one of the important factors for successful *ex-situ* conservation program. Female or male must be