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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

Table 1. Species and number of samples analyzed using P8 and P2 primers

Family	Scientific name	Total samples	Not amplified	Note
Psittacidae	*Psittaculirostris desmarestii*	1	1	
	Psittaculirostris edwardsii	1	1	
	Chalcopsitta duivenbodei	2	0	
	Trichoglossus h. haematodus	3	0	
	Pseudeos fuscata	4	1	
	Eos bornea (bornea)	2	1	
	Trichoglossus h. caeruleiceps	2	1	
	Trichoglossus euteles	2	0	
	Loriculus galgulus	3	1	
	Cacatua goffini	6	0	1-Thick band
	Alisterus chloropterus	5	1	
	Cacatua moluccensis	4	1	1-Thick band
	Cacatua sulphurea sulphurea	5	0	
	Probosciger aterrimus	2	1	
	Cacatua galerita	2	1	
	Lorius lory lory	2	0	
	Lorius garrulus	2	0	
	Cacatua sulphurea	2	0	2-Thick band
	Cacatua s. citrinocristata	4	1	
	Cacatua alba	5	0	
	Eclectus roratus	3	0	
	Chalcopsitta atra atra	1	0	
	Trichoglossus haematodus	2	0	
	Psittacula alexandri	1	0	
	Cacatua galerita triton	1	0	
	Loriculus pusillus	4	1	
	Cacatua s. parvula	2	0	
	Cyclopsitta diopthalma	1	1	
	Aprosmictus erythropterus	1	0	
	Charmosyna placentis	1	0	
Accipitridae	*Mivus migrans*	1	0	
	Spilornis cheela	1	0	
	Accipiter virgatus	1	0	
	Spizaetus bartelsi	1	0	
Cuculidae	*Centropus bengalensis*	1	0	
	Phaenicophaerus curvirostris	1	0	
Falconidae	*Falco tinnunculus*	1	0	
Strigidae	*Strix seloputo*	1	0	
	Otus lempijji	2	0	
	Ketupa ketupu	2	0	
	Bubo sumatranus	1	0	
Corvidae	*Corvus macrorhynchos*	1	0	1-Thick band
	Corvus enca	1	0	
Phasianidae	*Lophura ignita rufa*	1	0	1-Thick band
	Gallus varius	2	0	
	Pavo muticus	2	0	
	Argusianus argus	1	0	
Columbidae	*Goura cristata*	1	0	
Megapodiidae	*Macrocephalon maleo*	2	0	
Bucerotidae	*Rhyticeros undulates*	1	0	
	Anthracoceros malayanus	1	1	
Ardeidae	*Ardea cinerea*	1	0	
Irenidae	*Irena puella*	2	0	
Paradisaeidae	*Cicinnurus regius*	1	0	
	Paradisaea minor	1	0	
Alcedinidae	*Pelargopsis capensis* (*Halcyon capensis*)	3	0	
Total	56 species	110 identified samples	14 not amplified samples	6 thick band samples

determined correctly in breeding captivity. Before sexing of the 229 individual samples, a trial experiment for the 2550F/2718R primers was conducted with small number samples. We used fiveindividuals of known bird sex as a control result for molecular sexing technique. The 5-known bird sex of Hanging Parrots were used as control in this study (1. *Loriculus pusillus* ♀, 2. *Loriculus pusillus* ♀, 3. *Loriculus pusillus* ♀, 4. *Loriculus galgulus* ♂, 5. *Loriculus pusillus* ♀). By using morphological characters, sex of the 5-birds can be determined easily, i.e.: male Hanging parrot (*Loriculus* sp.) at the top of his chest there is a red circle-shaped, while the female Hanging parrot (*Loriculus* sp.) yellowish green color. Color difference at the top of the chest is what allows people to determine whether it is male or female Hanging parrot.

The thermal cycling conditions used for P8/P8 primer pairs were initial denaturation at 94 °C for 5 minutes, then 30 cycles of denaturation for 30 seconds at 94 °C,

Table 2. List of species birds used for sex identification with 2550F and 2718R primers

Family	Scientific name	Indonesian names	Samples type	Total
Megapodiidae	*Macrocephalon maleo*	Maleo	Blood	8
Sturnidae	*Leucopsar rothschildi*	Curik Bali	Blood	133
Psittacidae	*Probosciger aterrimus*	Kakatua Raja	Blood	4
Accipitridae	*Spizaetus bartelsi*	Elang Jawa	Blood	6
	Haliastur Indus	Elang Bondol	Blood	22
	Spizaetus cirrhatus	Elang Brontok	Blood	7
	Haliaeetus leucogaster	Elang Laut	Blood	2
	Spilornis cheela	Elang Bido	Blood	3
Sturnidae	*Gracula religiosa*	Beo	Plucked feather	17
Turdidae	*Zoothera citrina*	Anis merah	Plucked feather	27
Total samples				229

annealing for 45 seconds at 50 °C, and extension for 45 seconds at 72 °C. A final run of 48 °C for 1 minute and 72 °C completed the program for 5 minutes. PCR products were separated by electrophoresis in 3% agarose gel (FMC Bioproducts, SeaKem GTG Agarose). While the condition of PCR used for 2550F/2718R primer pairs were initial denaturation on the temperature of 94 °C for 5 minutes, then 30 cycles of denaturation for for 45 seconds at 94 °C, annealing for 45 seconds at 46 °C and extension for 90 seconds at 72 °C. At the end of the cycle was followed by the temperature of 72 °C during 10 minutes. PCR products were separated by gel electrophoresis through a 2% standard agarose gel (Agarose LE, Analytical Grade, Promega).

The gels were run in standard TBE buffer and stained with ethidium bromide. A commercial O'Range Ruler 100 bp DNA Ladder (Fermentas) was used as size marker in order to judge whether Z and W-bands were obtained. After electrophoresis at 100V for approximately 45 minutes, gels were examined and photographed by digital camera under UV light. A typical banding pattern was revealed sex of the birds examined, one band the male and two bands in the female.

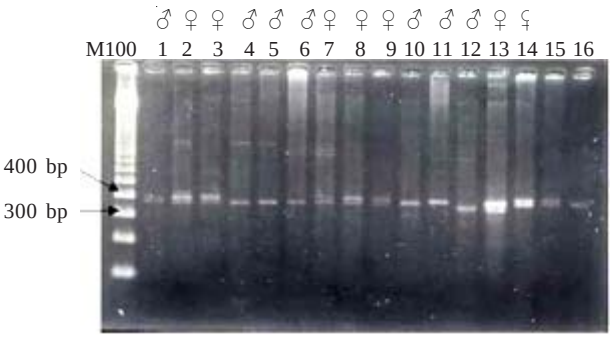

Figure 1. Identification of sex in birds with P8/P2 set of primers. The sixteen samples showed in the are: 1. *Cacatua moluccensis* (Male), 2. *Lorius lory lory* (Female), 3. *Lorius garrulus* (Female), 4. *Chalcopsitta duivenbodei* (Male), 5. *Pseudeos fuscata* (Male), 6. *Cacatua suphurea sulphurea* (Male), 7. *Probosciger aterrimus* (Female), 8. *Trichoglossus euteles* (Female), 9. *Lorius lory lory* (Female), 10. *Cacatua galerita* (Male), 11. *Spizaetus bartelsi* (Male), 12. *Cicinnurus regius* (Male), 13. *Pavo muticus* (Female), 14. *Cacatua goffini* (Female), 15. *Cacatua goffini* (Female), 16. *Cacatua alba* (Male). ♂ =Male and ♀ =Female; M = DNA ladder (100 bp).

RESULTS

Based on the visualization of PCR products, sex of birds can be determined. The PCR product indicated those birds with two bands are females and those with one band are male. The P8 and P2 sexing results of 30 species of Psittacidae (Table 1) showed three species failed to be identified (*Psittaculirostris desmarestii*, *Psittaculirostris edwardsii*, and *Cyclopsitta diopthalma*) and three species of psittacidae (*Cacatua goffini*, *Cacatua moluccensis*, and *Cacatua sulphurea*) produced thick single band.

We also found thick single band in C*orvus macrorhynchos* of corvidae and *Lophura ignita rufa* of phasianidae. The *Anthracoceros malayanus* sample of bucerotidae could also not be determined successfully. The results in total (Table 1) showed fourteen samples used in this study were failed to be amplified. The 14-unamplified samples were *Psittaculirostris desmarestii*, *Psittaculirostris edwardsii*, *Pseudeos fuscata*, *Eos bornea* (*bornea*), *Trichoglossus h. caeruleiceps*, *Loriculus galgulus*, *Alisterus chloropterus*, *Cacatua moluccensis*, *Probosciger aterrimus*, *Anthracoceros malayanus*, *Cyclopsitta diopthalma*, *Loriculus pusillus*, *Cacatua galerita*, and *Cacatua s. citrinocristata*. It is also noticed in this study that not all samples from the same species give amplified products. For example, only three out of 4 samples of *Cacatua moluccensis* showed amplification.

Sixteen individual samples of PCR product were demonstrated molecular sexing technique using P8/P2 primers. One hundreds base pairs (bp) of DNA ladder is indicated at the first line (Figure 1). The size of PCR products were between 300 to 400 bp and the size differences between the two amplified bands (CHD-W and CHD-Z) were short. Eight out of sixteen samples (Numbers 2, 3, 7, 8, 9, 13, 14, 15) were female birds and the remaining eight samples (Numbers 1, 4, 5, 6, 10, 11, 12, 16) were male birds (Figure 1). The products of PCR seem different appearance between species; for example, the two bands in the *Cacatua goffini* (line no.14) are larger than the two in the *Pavo muticus* (line no.13), also the single band in *Spizaetus bartelsi* (line 11) and *Cicinnurus regius* (line 12) showed different length. The different appearance on agarose gel occurred between *Cacatua goffini* in line 14 and 15, showing less bright in line 15 than in line 14 (Figure 1).

Sex determination results of 110 individual samples using P8/P2 primers, was only 81.8% sex correctly determined. While the remaining, the P2 and P8 primer combination used in this study was failed to assign their sex due to amplification failure (12.7%) and yielding a thick band (5.5%). The primer sets of 2550F/2718R was applied to solve the problem, particularly for thick band problem. They developed a technique that using PCR primers flanking introns which vary in size between CHD1W and CHD1Z, males being recognized in agarose electrophoresis as displaying a single PCR product (from CHD1Z) while females show two different products (CHD1W and CHD1Z).

Sex of ten *Haliastur indus* samples which previously identified using P8 and P2 primers, were retested using a set of 2550F and 2718R primers. Two set of primers used to identify 10-*Haliastur indus* showed different sexing results. Sex of the ten samples show numbers 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 were all males (Figure 2). The P8/P2 primer set failed to distinguish between male and female in species of *Haliastur indus*. In contrast to a set of 2550F/2718R primers in Figure 3, showed that 6 samples (numbers 1, 4, 6, 9, 10, 11) were females and 4 samples (numbers 2, 3, 5, 7) were males. False negative was obtained in when using P8/P2 primers.

Two hundreds and twenty nine samples were identified using a pair of 2550F and 2718R primers (Table 2). The results showed that sex of the 229-samples could be accurately determined (100%). The band patterns of all 229 samples from 10 species were in agreement with patterns expected from known sex of the samples. The results also showed that a male (♂) birds was represented by a single band fragment (CHD-Z) visualized at approximately 650 bp, where a female (♀) were represented by two amplified bands in the size of 400 and 650 bp respectively (Figure 4).

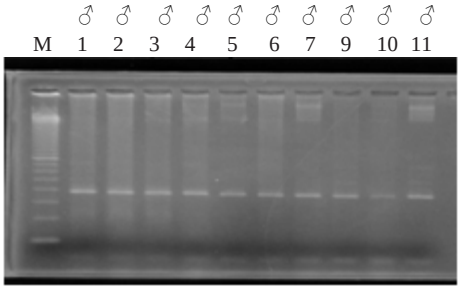

Figure 2. Sex identification in ten individuals of *Haliastur indus* with P8/P2 set of primers. ♂ =Male, M=DNA Ladder (100 bp).

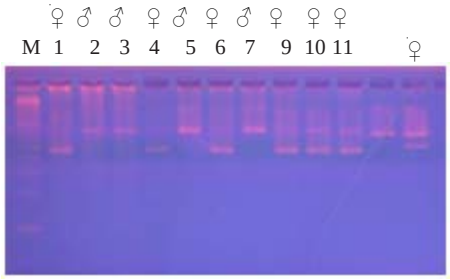

Figure 3. Sex identification in ten individuals of *Haliastur indus* with 2550F/2718R set of primers. ♂ = Male, ♀ = Female, M = DNA Ladder (100 bp).

DISSCUSSION

Sex of the parrots (Psittacidae) and Bali starling (Sturnidae) were the most widely identified in this study (Tables 1 & 2). This bird is one of the most in demand by collectors and bird fancier, because of the beautiful, elegant appearance and economic value. Sexing molecular technique was applied to those birds, because it is difficult to discriminate birds between male and female using morphological characteristic, on the other hand, early sex determination for captive breeding of birds must be known. Besides parrot and Bali starling (Bali mynah) birds, other pet birds which are very popular as cage-bird on Java, including *Gracula religiosa* (Beo) and *Zoothera citrina* (Orange-headed Thrush or Anis). The species of *Gracula religiosa* birds were preferred because of their versatility speaking people and *Zoothera citrina* birds were preferred because of having good and melodious voice. Recently, captive birds of Beo and Anis also become a lucrative business opportunity, captive breeding of the birds scattered across Indonesia.

Breeding of the 2-species birds need to know the information of sex correctly. Of the 229 samples tested (Table 2), forty four (44) extracted DNA which consisted of 17 samples of *Gracula religiosa* and 27 samples of *Zoothera citrina* were derived from plucked feather samples. DNA is extracted from the cells from the basal tip of the calamus (Morin *et al*. 1994) or from the blood clot embedded in the shaft (Segelbacher 2002; Horvath *et al*. 2005). According to Taberlet *et al*. (1999) a potential concern with feather-based DNA sampling is that the small number of cells present on within the feather could result in inadequate DNA yields for molecular analysis. The result of this study approved that bird sex identification of the 44 feather samples were determined successfully.

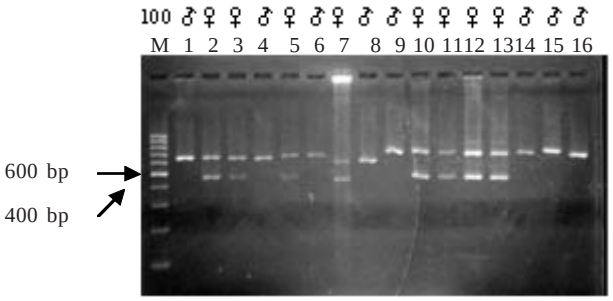

Figure 4. Identification of sex in birds with 2550F/2718R set of primers. 1. Control , 2. Control ♀, 3. *Probosciger aterrimus* (♀), 4. *Probosciger aterrimus* (♂), 5.*Leucopsar rothschildi* (♀), 6. *Leucopsar rothschildi* (♂), 7. *Macrocephalon maleo* (♀), 8. *Macrocephalon maleo* (♂), 9. *Haliastur indus* (♂), 10.*Haliastur indus* (♀), 11. *Spizaetus cirrhatus* (♀), 12. *Spizaetus cirrhatus* (♀), 13. *Spizaetus bartelsi* (♀), 14. *Spilornis cheela* (♂), 15. *Haliaeetus leucogaster* (♂), 16. *Spizaetus cirrhatus* (♂). ♂ = Male, ♀ = Female, M = DNA Ladder (100 bp).

The results showed different amplification such as one band was less bright than the other bands on an agarose gel (Figure 1; Line 14 & 15). DNA sample of *Cacatua goffini* (Line 14) was extracted from liver, while DNA sample of *Cacatua goffini* (Line 15) was extracted from feather. DNA quantity of plucked feather is lower concentration than DNA quantity of liver. That result likely occurred because prior to amplification, the DNA concentration of each sample was not equated in this study. One reason from Griffiths *et al.* (1998) stated that primer competition occurred during the amplification process, i.e. the primers may match one CHD gene slightly less well that the other. Harvey *et al.* (2006) supported our result give evidence that feathers can provide sufficient DNA for molecular sexing reactions. This low cost, speed, and ease of collection, storage, and transport of feather samples are the major advantages (Duan & Fuerst 2001).

The results indicated that only one out of 4 samples of *Cacatua moluccensis* was failed to be identified (Table 1). This problem of not all samples succeed to be amplified was due to unknown reasons. It might be caused by different quality of DNA sample. There may be too little DNA present in the isolated DNA sample to amplify. DNA concentration varied among samples. As described above that yield of DNA was dependent on sources of DNA material. In general, Sambrook *et al*. (1989) stated that isolation DNA aim to separate DNA present in the nucleus of the cell from other cellular components. In birds, because the nucleated erythrocytes of birds make avian blood an unusually rich source of nuclear DNA. DNA in plucked feather samples is typically present in much lower copy number than DNA from blood or tissue samples.

The results obtained six-amplified samples were shown thick single band using P8/P2 primers (Table 1). We found thick band in this study because agarose gel electrophoresis of P8/P2 products, however this bands can not be used for the differentiation of the sexes. Griffiths *et al*. (1998) explained that the PCR did produce CHD-W and CHD-Z bands but the introns were so similar in size that they could not be distinguished on a 3% agarose gel, and they also suggested to use an 8% denaturing acrylamide gel whose resolution is easily sufficient to discriminate the two products. Thus, sexing primers designed by Griffiths *et al*. (1998) seem not always possible to separate by standard agarose electrophoresis. Ellegren (1996) further suggested to use of single-strand conformation polymorphism (SSCP) analysis or Griffiths *et al*. (1996) suggested to differentially cut the PCR products with enzymes such as *Hae*III or *Mae*II to allow their separation on agarose gel. Other methods were developed to overcome the limited difference in the length of intron for CHD-Z and CHD-W by using PCR-RFLP (Sacchi *et al*. 2004; Reddy *et al*. 2007), RAPD (Wu *et al*. 2007), and AFLP (Huang *et al*. 2007) fingerprintings.

Naim *et al*. (2011) also reported that the P2 and P8 primer combination failed to assign sex for all individuals of white-bellied sea eagle. Increasing evidence (Fridolfsson & Ellegren 1999; Sacchi *et al.* 2004; De volo *et al*. 2005; Huynen *et al*. 2006; Reddy *et al*. 2007) shows that the gender of some avian species cannot be identified by the P2/P8 PCR-based protocol (Griffiths *et al*. 1998). By using P8/P2 set of primers a negative outcome of test was obtained from this study. Furthermore, the results were confirmed that apparently among those of 10 individuals produce offspring. This result gave an approval that the PCR method using a primer set of 2550F/2718R was able to correctly identify the sex of 10 individual samples of *Haliastur indus*. Based on Griffiths *et al.* (1998), this problem is always associated with systems based on the sole detection of W-linked sequences: a negative outcome of a test can result both from the sample being male and a technical failure.

Position of the amplified bands, CHD-W and CHD-Z was in between 300-400 bp (Figures 1 & 2). The P8/P2 primers amplified two alternative PCR fragments, but the size difference between two fragments were too short. Because the length in P8/P2-amplified Z- and W-fragments, which are extremely short, making it hard to resolve them on agarose gels (Chang *et al*. 2008). According to Fridolfsson and Ellegren (1999), the difference in size between Z-and W-spesific fragments amplified with the P8/P2 primers, ranging 10-80 bp. Furthermore Ito *et al*. (2001) discovered that the size difference between CHD1Z and CHD1W differ between species 2-8 bp in Accipitridae. That is why several PCR products tried using P8/P2 primers in Accipitridae (Figure 2) and visualized either using standard agarose or poly acrylamide gel agarose, showed female and male are still often indistinguishable. Dawson *et al*. (2001) reported that the fragments amplified with the P8/P2 primers cannot be distinguished on agarose gel in the auklets. Moreover, the assignment of sex on the basis of the P8/P2 primers may be in some species difficult because of a polymorphism in the Z chromosome (Dawson *et al*. 2001; Dubeic & Zagalska-Neubauer 2006).

Things to consider for this sexing study, if high concentration DNA in the isolated sample was directly used to amplify, amplification always failed. The high concentration DNA will inhibit the PCR reaction. In that case, the samples might be amplified, but both sexes yielding a single amplification product of identical size. They failed to produce a double band for female birds, therefore female bird was identified being male. To test this theory, information of DNA concentration on each sample was needed. The isolated DNA samples were measured using spectrophotometer. DNA concentration of several materials varied. From measurement of DNA concentration, the results indicated that blood has higher DNA concentration than feather. After DNA concentration was known, several dilutions of the isolated DNA samples were generated in this study. We found the best PCR product in gel electrophoresis derived from DNA concentration between 50-100 ng/µl, and finally this was used as a default DNA template in the PCR reaction.

Even though we identified sex of a limited number of representative species (Table 1 & 2), it is likely that molecular determination of a male from a female using 2550F/2718R was more effective way than using P8/P2. A problem of thick band was never found with 2550F/2718R

primers, so incorrect identification of female to male bird was avoided. The results of amplified bands (CHD-W and CHD-Z) produced in this study were the same as previous results obtained by Ong and Vellayan (2008); Vucicevic *et al*. (2012b), i.e. in female birds two amplified bands is visualized around 400 and 650 bp (CHD-W and CHD-Z) and in male birds only one band is visualized at approximately 650 bp (CHD-Z). Dawson *et al*. (2001) stated that 2550F/2718R primers amplified only two alternative PCR fragments, differing in size by 230 bp, while Fridolfsson and Ellegren (1999). stated that one such pair of primers (2550F and 2718F) designed to amplify both gene copies, i.e. located in region conserved between two genes, revealed a size difference of 150-250 bp in amplification of chicken CHD1W fragments (between 400 and 450 b p) and CHD1Z fragments (between 600 and 650 bp). They also confirmed the results using sequence analysis, i.e. the difference was due solely to a corresponding difference in intron size, i.e. the amplification products of both males and females, consistently confirming that the product seen in males represented CHD1Z and the products seen in females were CHD1W and CHD1Z. Due to the conservation of the CHD gene this second method has potential to be expanded to cover most bird species (Vucicevic *et al*. 2012a), including protected and endangered, which should be a subject of further research. Since the sexing primers were established by Griffith *et al*. (1998); Fridolfsson and Ellegren (1999), many other primers have been developed for sex identification. Based on a sexing report of Wang and Zhang (2009), they designed a primer pair sex1/sex2 from CHD genes of the Brown-eared Pheasant (*Crossoptilon mantchuricum*) and the primers can be used to sex many other pheasants accurately as well as some Passeriform species. Kasuga *et al*. (2012) said that the W- and Z-linked EE0.6 sequences used for molecular sexing of captive Japanese crested ibis on Sado island. Zhang *et al*. (2012) illustrated sex identification of four Penguin species using Locus-Soecific PCR.

Based on the results obtained from this study, it was suggested that the best effective way to distinguish a male from a female of birds used a set of 2550F/2718R primers. It is simple, rapid, and universal system for sex identification, because PCR products could be easily resolved on 2% agarose gels. The molecular sexing technique offer advantage of non-invasive sexing method and do not require anesthesia. Through the knowledge of sex identification genes, bird captive breeding programmes can be applied more successfully in Indonesia.

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