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A NON-INVASIVE TECHNIQUE FOR SEX DETERMINATION OF MONOMORPHIC BIRDS

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ABSTRACT

We report on the unambiguous identification of monomorphic bird from a freshly plucked feather in Gunung Ledang, Taman Negara, Johor. Genetic analysis accurately recognizes gender by comparing the intron length between CHDZ and CHDW that is preserved within the avian Z and W sex chromosomes. The species under study were Stripe-throated Bulbul (*Pycnonotus finlaysoni)*, Olive-winged Bulbul *(Pycnonotus plumosus)*, White-throated Kingfisher (*Halcyon smyrnensis*) and Collared Scops Owl (*Otus lettia*). PCR amplification using P2/P8 primer pairs produced single band in male (ZZ) and two bands in female (ZW) birds that ranged between 300-400 base pairs in length. The PCR products were confirmed by the preliminary experiment of known sex of both species of polymorphic birds. Thus, this PCR-based test is fast, accurate and applicable in these species. Therefore, it could be used for many conservation studies notably in threatened or rare species.

Key words: CHD genes, non-invasive, molecular sexing, monomorphic bird, P2/P8

INTRODUCTION

About 50% of bird species worldwide are monomorphic (Griffiths *et al.,* 1998). To identify the bird species based on morphological traits alone is inadequate especially in nestlings. Without the appropriate technique of sex identification, bird conservation programs such as captive breeding for threatened and endangered species is futile. In fact, gender recognition is significant to veterinary, medical and ecological sciences and is advantageous in imposing legislation and resolving paternity conflict (Lee *et al.,* 2010). Problem arises in many conservation centers in Malaysia as they have difficulties in sexing monomorphic birds due to lack of noticeable morphological differences between sexes. It is difficult to distinguish the sex of these birds by looking at their morphological features as compared to species that are sexually dimorphic which it can be identified phenotypically and much easier after puberty (Cerit and Avanus, 2006). Therefore, it is difficult to establish the breeding strategies, conservation and management programs (Naim *et al.,* 2011). The development of Polymerase Chain Reaction (PCR) solved these difficulties due to the ability to amplify the CHD gene (Chromo Helicase DNA-binding gene) in both Z and W chromosome mostly in ratites birds (Ellergen and Sheldon, 1997; Griffths *et al.,* 1998; Bouetette *et al.,* 2002).

The CHD gene was firstly found on the W chromosome (Griffiths and Tiwari, 1995), and then was later discovered on a closely associated Z chromosome (Griffiths and Korn, 1997). The distinguished sex was highly influenced by the differences in the intron length between CHD-Z and CHD-W chromosomes. The universality of the CHD gene is not doubted and recorded a number of successful sex determinations in almost all bird species until this day. Female birds appear as heterogamete double band (ZW), whereas male birds show single band (ZZ) in gel electrophoresis (Norris-Caneda and Elliot, 1998). Genetic analysis accurately recognizes gender by comparing the intron length between these genes. Molecular sexing is found to be more reliable and accurate compared to the traditional method such as laparoscopy and karyotyping. It is a non-invasive approach to gather proper amount of tissue cells where the outside feather contains an old skin cells, while inside consists of old blood cells that is enclosed with a growing feather (Honkatukia *et al.,* 2003). This

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technique is performed mostly on threatened species due to the accessible sample collection and unnecessary interaction that would harm the birds. This study was conducted to test the efficacy of using the PCR-based technique alone in screening the intron length in CHD gene that is located in Z and W chromosome in these birds. The feathers from four species of monomorphic birds were chosen to be the source of genomic DNA as the availability of the CHD gene preserved in the Z and W chromosomes.

MATERIALS AND METHODS

Sample collection

Feathers were collected from four species of monomorphic birds; Stripe-throated Bulbul (*Pycnonotus finlaysoni)*, Olive-winged Bulbul *(Pycnonotus plumosus)*, White-throated Kingfisher (*Halcyon smyrnensis*) and Collared Scops Owl (*Otus lettia*). Two flight feathers were sampled by plucking from each bird around Gunung Ledang Taman Negara, Johor. Upon receipt, the samples were placed in clean plastic bags without touching their tip and were kept frozen at –20°C. Species identification was confirmed morphologically by referring to David and Yeap (2010) descriptions.

DNA extraction

An approximately 0.5-1.0 cm segment from two - three individual feathers was cut from the calamus end and placed in a 1.5 ml Eppendorf tube. Genomic DNA was extracted from the feather tips using the Invisorb Spin Tissue Mini Kit according to the manufacturers' instructions and lysis was performed at 52°C for 4 hours.

PCR amplification

A part of the CHD gene was amplified using P2/P8 primer pair (Griffiths *et al.,* 1998). PCR was performed in 20 µL final volume using 4 µL of Master Mix ready-to-load, 0.4 µL of forward and reverse primer, 10.2 µL distilled water and 5 µL genomic DNA. Positive and negative controls were included in order to rule out contamination of the samples with exogenous DNA or PCR product. The amplification process begin with an initial denaturing step at 95°C for 13 minutes followed by 35 cycles of denaturation (95°C for 20 seconds), annealing (40.2°C for 30 seconds) and extension (72°C for 3 minutes) then a final extension step at 72°C for 10 minutes. The PCR products were separated in 1.2% agarose gel at 68 V for approximately 45 minutes and photographed under UV light.

RESULTS AND DISCUSSION

So far, four species of monomorphic birds were used to validate the PCR-based molecular technique (Figure 1). Two replicates were done for each species and the results were consistent. The analysis of P2/P8 sexing method showed the Stripe-throated Bulbul as male (ZZ) by the existence of a single band of 400 bp. The Olive-winged Bulbul, White-throated Kingfisher and Collared Scops Owl appeared to be heterogametic female by the occurrence of the second fragment length between 300-400 bp (Figure 1). Molecular technique by utilizing feathers as DNA source is more convincing than other conventional sexing method as this procedure would accurately discriminate between avian sexes and moreover, it is a more ethical way to handle live specimens. Similar study using the same primer pair was proven to be effective in distinguishing sex in *Nymphicus hollandicus* (Cerit and Avanus, 2006). The current study involving amplified CHD gene as a molecular marker using P2/P8 was successful in sexing 50 bird species (Vucicevic *et al.,* 2013).

Most ornithologists utilize feather sampling as DNA source over blood collection as this method is quite easy with no specialized training and the storage of collected sample is uncomplicated (Bello *et al.,* 2001; Smith *et al.,* 2003; Harvey *et al.,* 2006). In addition, it exhibits the most typical sample for DNA isolation as it causes minimal pain if plucked or no pain at all if collected after molting (Seki, 2006; Leekaew *et al.,* 2008). Among all the feather types, flight feathers which are larger, are most adequate for sampling as more DNA can be extracted (Segelbacher, 2002; Harvey *et al.,* 2006). Sampling of feathers should be taken into consideration the birds' flight performance as it may disturb its mobility and take-off trajectories notably when escaping from predators (Thompson *et al.,* 2010).

The broad use of CHD gene has made it a useful molecular marker that contributes to the sexing in most monomorphic bird species. The use of the P2/P8 primer pair contains intron length differences in CHD-W and CHD-Z where the primers incorporated made the sexing successful. The primer pair has been shown to amplify the target regions in a great number of non-ratites (Fridolfsson and Ellergen, 1999). However, this primer pair showed difficulty as it amplifies the smaller Z chromosome more than the larger W chromosome, and this leads to the misidentification of females as males (Dubiec and Neubauer, 2006). Some bird species failed to sex due to very small variation in the intronic region or none at all. The Griffiths' method is not suitable in Stork species due to the difficulty of isolating two

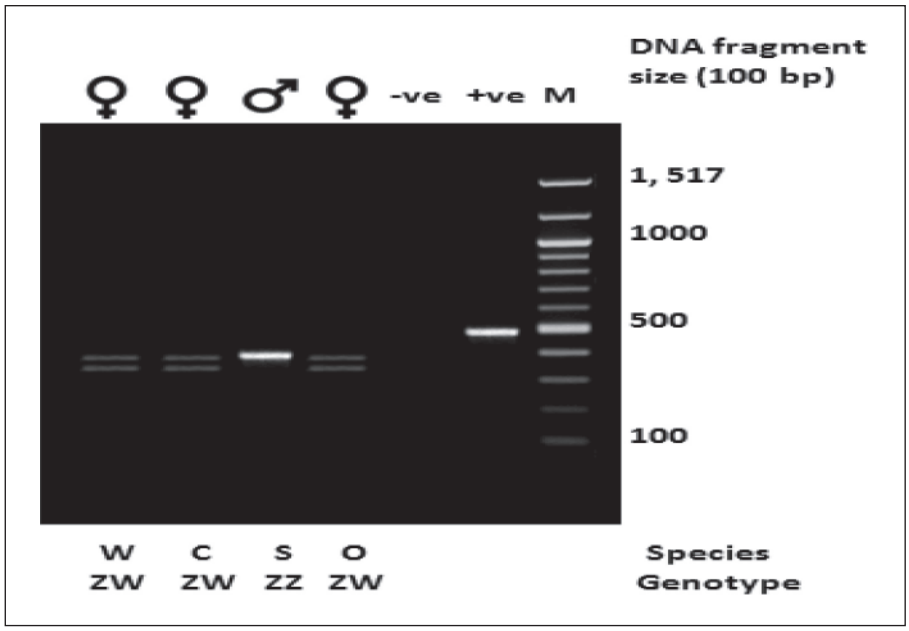

Fig. 1. PCR product of amplification using P2/P8 primer pair in four species of monomorphic bird: White-throated Kingfisher (W), Collared-stripe Owl (C) Stripe-headed Bulbul (S) and Olive-winged Bulbul (O).

very common intronic sizes in CHD-W and CHD-Z genes, but the use of restriction enzyme has solved this problem (Han *et al.,* 2009). In this case, other method such the RFLP (Restriction fragment length polymorphism) would be preferable in order to gain the accuracy of sexing depends on how it deals with the small differences between CHD1 amplicon (Constantini *et al.,* 2008).

In conclusion, it was found that PCR test alone works well in sexing monomorphic birds. From the data analysis, all four birds were successfully identified thus it was confirmed to be fast, accurate and applicable in others monomorphic avian species. This technique has potential for studies and conservation programmes for threatened and rare birds. Further works with potential method with high specificity in intron variation among species needs to be done as to improve the existing database on avian species in for the Malaysian Biodiversity.

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