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Molecular Sexing of Southeast Asian Barn Owl, *Tyto alba javanica*, using Blood and Feather

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Abstrak. Penentuan jantina burung adalah penting untuk tujuan kajian ekologi dan biologi evolusi, serta program pembiakan dan konservasi terutamanya bagi burung yang mempunyai ciri monomorfik. Bagi burung pungguk jelapang *Tyto alba* pengesahan jantina adalah penting untuk konservasi serta program pengenalan bagi kawalan tikus perosak. Penentuan jantina secara molekular untuk subspecies Asia Tenggara, *Tyto alba javanica,* telah dijalankan menggunakan PCR diikuti 3% gel agaros elektroforesis. Primer P2/P8 and 2550F/2718R untuk amplifikasi gen CHD (Chromo Helicase DNA-binding gene) diuji dan kedua-dua set primer memberi keputusan yang berjaya. Set primer 2550F/2718R memberi hasil yang lebih baik kerana jurang di antara jalur berganda lebih terang. DNA yang diekstrak dari darah, darah yang dicairkan, serta DNA yang diekstrak dari bulu burung diguna untuk menentukan jantina burung. DNA yang diekstrak dari bulu memberi keputusan yang kurang memuaskan akibat pencemaran serta kuantiti DNA yang rendah. Penentuan jantina menggunakan darah yang dicairkan merupakan kaedah yang menjimatkan kos serta masa. Penjujukan gen CHD dari *Tyto alba javanica* menunjukkan 98% hingga 99% kesamaan identiti bila dibandingkan gen CHD *Tyto alba alba*.

Kata kunci: Burung Pungguk Jelapang, Kaedah Molekular, Penentuan Jantina,

Abstract. Sexing of birds is important for ecology and evolutionary biology studies, as well as breeding and conservation programs especially for sexually monomorphic birds. As for barn owls, *Tyto alba*, confirmation of sex is important for conservation as well as introduction programs to control rodent pest populations. Molecular sexing of Southeast Asian subspecies, *Tyto alba javanica* was carried out using PCR amplification followed by 3% agarose gel electrophoresis. Primers P2/P8 and 2550F/2718R for the amplification of CHD gene (Chromo Helicase DNA-binding gene) were tested and both gave successful results. 2550F/2718R primer set gave better results as the gap between double bands was larger. DNA extracted from blood, whole diluted blood, and DNA extracted from feathers was used to molecularly sex owls. DNA extracted from feather gave the least effective results owing to contamination and low DNA concentration, while sexing owls using direct whole diluted blood provided a cost and time effective method. Sequencing of CHD gene from *Tyto alba javanica* showed 98% to 99% similarity in identity when compared to CHD gene of *Tyto alba alba*.

Keywords: Barn Owl, Molecular Sexing, Sex Determination.

INTRODUCTION

Sex determination is important in many studies of ecology, evolutionary biology, breeding and conservation. The ability to identify sex is vital for captive-propagation programs, whether such programs are designed for commercial or restoration purposes of endangered and protected species (Ghorpade et al. 2012; Morinha et al. 2012). In birds, the absence of juvenile sexual dimorphism often makes it difficult to determine a chick's sex on the basis of external morphology. A similar problem exists for fully grown individuals of many bird species as most species show minimal pronounced adult sexual dimorphism. Over the years, molecular sexing using polymerase chain reaction (PCR) has been gaining popularity due to its accuracy and reproducibility compared to morphological data (Kesler et al. 2006; Pitzer et al. 2008). The DNA can be sampled from blood, feather, faeces and even buccal swabs (Idaghdour et al. 2003; Jensen et al. 2003; Harvey et al. 2006; Brubaker et al. 2011). However, the most commonly used DNA samples are from blood and feathers because is the samples are relatively easy to obtain and are less prone to cross-contamination.

Bird sex chromosomes are thought to have evolved from a pair of autosomes (Fridolfsson et al. 1998). Females are heterogametic with ZW sex chromosomes, while males are homogamous with ZZ chromosomes (Ellegren 1996). Most birds have around 80 chromosomes, but due to their difference in size, the sex chromosomes are usually easy to distinguish from one another (Dubiec and Zagalska-Neubauer 2006). The chromo-helicase-DNA-binding (CHD) gene was discovered to be present in both Z (CHD-Z) and W (CHD-W) chromosomes in most non-ratite bird species with different length of introns making it a suitable marker for sex determination (Griffiths et al. 1996; Ellegren 1996). Based on this, different primer sets were developed to molecularly determine the sex of birds and the most common being P2/P8 (Griffiths et al. 1998) and 2550F/2718R (Fridolfsson and Ellegren 1999). In most cases, the female CHD-W gene yields a larger PCR product due to the bigger intron region. The PCR product size also differs between species. These two primers have been used multiple times and yield consistent results throughout all bird species (Harvey et al. 2006; Brubaker et al. 2011; He et al. 2013; Khaerunnisa et al. 2013).

Barn Owls *(Tyto alba*) are the most widely distributed species of owl, and one of the most widespread of all birds. About 36 subspecies of Barn Owls have been identified varying in body size and plumage coloration, aspects of behaviour and ecology (Taylor 1994). Barn Owls are sexually monomorphic. Sex identification is especially important for conservation of Barn Owls and more successful breeding programs. Sex identification can lead to a confirmed male-female pair release, ensuring successful breeding and propagation. In this study, we focused on molecularly sexing the Southeast Asian Barn Owl subspecies, *Tyto alba javanica*. We conducted molecular sexing using P2/P8 and 2550F/2718R on DNA extracted from blood, direct blood PCR and DNA extracted from feathers to identify the more efficient and accurate primer set and DNA source. We then sequenced the CHD gene from *T. alba javanica* and compared them against the *Tyto alba alba* CHD gene sequence in GenBank to compare for similarities between the CHD genes of both subspecies.

MATERIALS AND METHODS

Blood and Feather Collection

We harvested six fledgling Barn Owls, *Tyto alba javanica*, in their nestbox within oil palm plantations in Jengka, Pahang, Malaysia (3.7685° N, 102.5454° E). The owls had their heads covered with a breathable cloth material to keep them calm during field blood and feather sample collection. For each individual, we

collected approximately 1ml of blood from the basilic vein of the owl using a sterile 25 gauge needle. Bloodletting procedure of owls followed that of Wong (2005). Blood was then placed into a Vacutainer® tube containing EDTA to prevent coagulation of blood and were temporarily stored in an icebox, transported to the lab and stored at -20°C. Several down feathers were plucked from two different parts of the body; the ventral (underside) of their wings and the chest and subsequently placed in container bottles containing absolute ethanol. Collected samples were analysed in a molecular lab in Universiti Sains Malaysia.

Blood DNA extraction

DNA was extracted from blood using a Qiagen DNeasy® Blood and Tissue Kit. The concentrate was measured using the SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, California).

Feather DNA extraction

Feather DNA was extracted using the rapid alkaline extraction method (Malagó et al. 2002). The feathers of each sample were carefully cut directly into 1.5mL Eppendorf tubes using sterilized cuticle scissors. 20 µL of 0.2M NaOH was added to each tube and placed in a water bath of 75°C for 20 minutes. Once removed, 180 µL of 0.04M Tris-HCl pH 7.6 solution was added to each tube and centrifuged at 12, 000 rpm for 3 minutes and stored in a -20°C freezer.

PCR and Gel Electrophoresis

P2 - 5'TCTGCATCGCTAAATCCTTT3' and P8 - 5'-CTCCCAAGGATGAGRAAYTG-3' (Griffiths et al. 1998) and 2550F – 5'-GTTACTGATTCGTCTACGAGA-3' and 2718R – 5'-ATTGAAATGATCCAGTGCTTG-3' (Fridolfsson and Ellegren, 1999) were used to conduct PCR . A 20 µL reaction of 1x Buffer (75 mM Tris, 20 mM Ammonium sulphate, 0.1% Tween-20 and 1.5 mM $MgCl_2$), 1 µL Pfu-SSO7d polymerase (Wang et al. 2004) and 1 µM of each primers and 250 µM of dNTP were used. For DNA template, 100 ng of extracted DNA, 1 µL of extracted feather sample or 1 µL of blood diluted 1:50 in PBS, was used. PCR reactions were carried out using; pre-denaturation for 5 minutes at 98°C, followed by 35 cycles (or 40 cycles for feather) of 95°C denaturation for 30 seconds, 60°C annealing for 30 seconds and 72°C extension for 30 seconds, and a final extension at 72°C for 1 minute. PCR products were visualized on a 3% agarose gel stained with RedSafe (Intron).

DNA Sequencing

The bands from the PCR products with the P2/P8 primer sets were cut and gel purified using MYgen Gel & PCR purification system (Gene Xpress). Purified bands were sent to First Base Sdn Bhd for sequencing using the same P2 and P8 primers. Sequencing results were aligned and assembled using Snapgene (GSL Biotech LLC) and BLAST search was performed to identify the CHD locus using the GenBank database.

RESULTS

PCR with DNA Extracted From Blood

The two different sets of primers (P2/P8 and 2550F/2718R) were first used with DNA extracted from blood. As kit-extracted DNA produces a relatively pure DNA sample, the initial run of both primer sets with extracted DNA enabled a comparison of band results between both primer sets.A single band indicated the sample as male while a double band indicated that the sample as female in both primer sets. PCR run using P2/P8 primers gave bands of about 300 bp in size. The double bands present when using P2/P8

primers were about 50bp apart (Figure 1A). PCR run with 2550F/2718R primer gave bands around 600bp and 1000 bp in size (Figure 1B). Results from both P2/P8 and 2550F/2718R primers showed that samples 1 and 4 are males, while samples 2, 3, 5 and 6 were females (Figure 1). However, as 2550F/2718R primer set showed a more obvious and definite band separation, this primer set was chosen as a comparison with diluted blood and DNA extracted from feathers (Figure 1C).

PCR with Diluted Whole Blood and DNA Extracted from Feather

PCR analysis using undiluted blood produced no results. Various dilutions were then attempted and conduct the PCR. A 1: 50 dilution ratio of blood with PBS gave the most consistent results (Figure 2).

PCR protocol that was used for DNA extracted from feather differed slightly from previously used PCR protocol. The difference was that the PCR analysis with feather-extracted DNA had an extended denaturation cycle of 40 cycles, due to low DNA concentration from feather extraction. PCR using DNA extracted from feathers only gave similar results to DNA extracted from blood for sample 1, 4 and 6 (Figure 3). PCR of sample 2 gave only a faint second band and multiple bands for sample 3 while there was no result for sample 5 (Figure 3).

DNA Sequencing

The DNA sequencing of the CHD gene for *Tyto alba javanica*, compared with the CHD gene sequencing of *Tyto alba alba*, obtained from GenBank is shown in Table 1. There was a 98% to 99 % similarity between the CHD genes of the two subspecies.

DISCUSSION

Both sets of primers, P2/P8 and 2550F/2718R, can amplify the CHD gene and produce two bands to differentiate between males and females samples. Based on our results, the 2550F/2718R primer set showed clearer distinction between bands as they were further apart than bands produced by the P2/P8 primer set. Furthermore, optimization of the PCR annealing temperature improved the specificity of 2550F/2718R primer set results. PCR annealing temperature of 55°C gave more than 2 bands in female samples while an annealing temperature of 58°C produced two sharp bands without any unspecific amplifications.

Fridolfsson and Ellegren (1999) tested the 2550F/2718R primers on 50 avian species. Among their tested species, the closest species related to Barn Owls were owls from the family Strigiformes: *Aegolius funereus* and *Strix nebulosi*. They reported band fragments of 600-650 bp and 1200 kb in size, while our results were band fragments sizes of 600 bp and 1000 kb in size.

Griffiths *et al.* (1998) tested P2/P8 primers on 28 avian species and the species most closely related to Barn Owls was the Tawny Owl (*Strix aluco*). Griffiths et al. (1998) reported that bands of the Tawny Owl could not be distinguished on a 3% agarose gel and a 8% denaturing acrylamide gel had to be used to see results. They reported bands about 340bp in size with double bands separated by about 20-50bp. In contrast, our PCR run with Barn Owl samples gave bands roughly 300 bp in size and were successfully distinguished on 3% agarose gel, with double bands approximately 50bp apart. Another method to improve the results from P2/P8 primers is to use restriction enzymes (Gábor et al. 2014). However this method is costly as well as time consuming.

Molecular Sexing using Whole, Diluted Blood

We found that direct PCR amplification can be done using diluted whole blood, a method successful for analysis of human blood (Mercier et al. 1990; Bu et al. 2008). Direct amplification of blood without dilution was unsuccessful, probably due to the inhibitors in blood (Mercier et al. 1990). However, diluting the blood with PBS in a 1:50 ratio provided results similar to PCR amplification using DNA extracted from blood. This method is more time and cost effective as it eliminates the need for DNA extraction and expensive extraction kits. Additionally, 1µL of blood can roughly be used for 50 PCR reactions, hence making this method significantly effective in studies with high number of trials. It is important to note that blood diluted in a 1:100 ratio also produced results.. However, the bands produced were not as bright as bands produced with blood with a 1:50 dilution.

Molecular Sexing using Feather Extracted DNA

Molecular sexing using feather DNA extraction is a non-invasive method, but DNA is harder to extract. In our samples, PCR amplification with DNA extracted from feathers gave similar results with molecular sexing with diluted whole blood and DNA extracted from blood. However, its major disadvantages are a very low DNA concentration as well as feathers being highly susceptible to contamination. In our results, sample from owl 5 produced no bands, indicating a very low DNA concentration, while results from owl 3 gave multiple bands, probably due to DNA contamination. Potential problems of a low DNA concentration are the absence of results or inconsistent sex assignment due to W allele dropout (Taberlet et al. 1999; Rudnick et al. 2007). W allele dropout causes a female to be wrongly assigned as a male and is usually caused when DNA is present in low concentrations in the sample, possibly caused by poor storage procedures resulting in DNA degradation or contamination (Grant, 2001). There are also ethical issues of feather collection for samples as it has been argued that plucking of feathers could affect fitness and survival of owls if collected incorrectly (McDonald and Griffith 2011).

In conclusion, our studies show primer set 2550F/2718R gave the clearest results for sexing of Barn Owls, *Tyto alba* and the most accurate DNA source was DNA extracted from blood. This would enable faster and simpler sexing for Barn Owls in introduction programs as well as breeding and conservation programs. Additionally, our study shows that molecular sexing can be done using whole blood; a cost and time efficient method for sexing of bird species.

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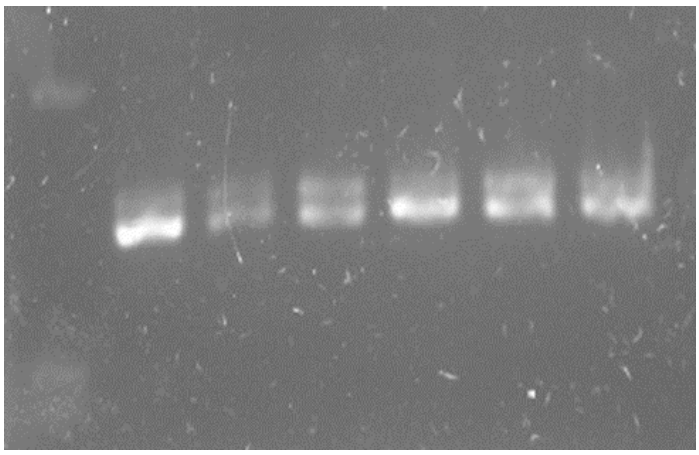

Plate A

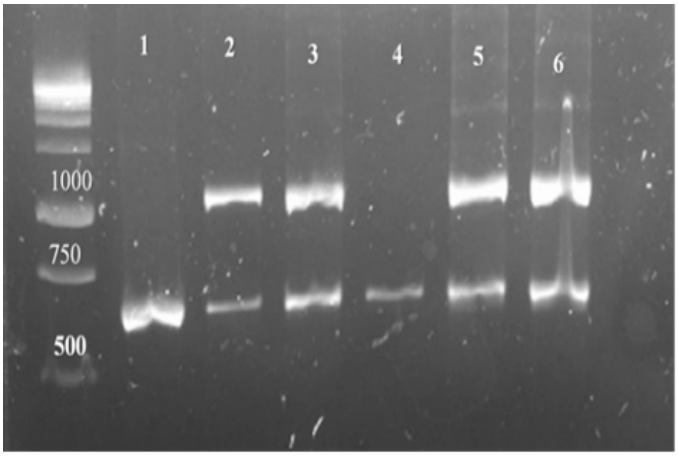

Plate B

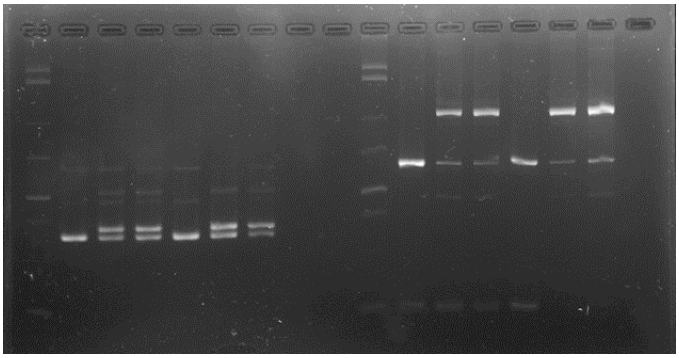

Plate C

Figure 1 (A-C): Molecular sexing of *Tyto alba javanica* DNA extracted from blood using PCR amplification followed by 3% gel electrophoresis. First lane is the DNA base ladder and samples 1 till 6 were loaded in consecutive lanes. Plate A = PCR amplification using P2/P8 primers, single band (sample 1 and 4) indicate male while double bands (sample 2, 3, 5, 6) indicate female. Plate B = PCR amplification using 2550F/2718R primers, single band (sample 1 and 4) indicate male while double bands (sample 2, 3, 5, 6)

indicate females. Plate C = Side-by-side comparison of bands produced with P2/P8 primers (left lanes) and 2550F/2718R primers (right lanes).

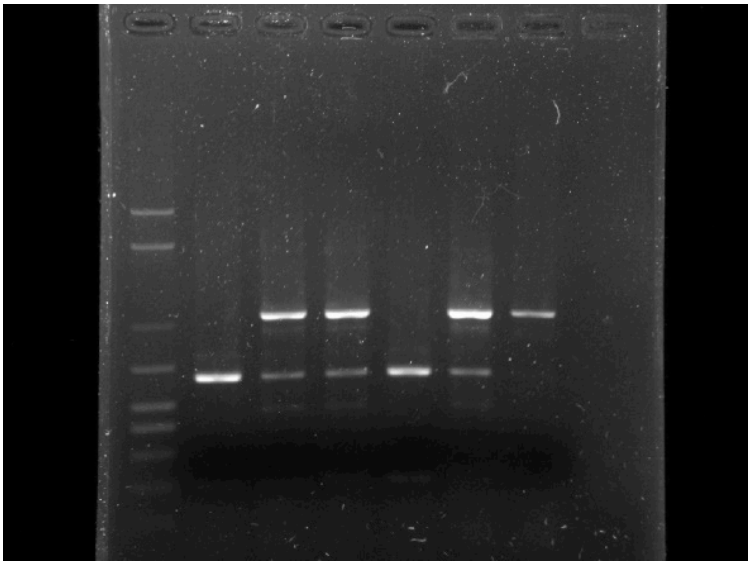

Figure 2: Molecular sexing of *Tyto alba javanica* using PCR amplification of direct diluted blood with 2550F/2718R primers followed by 3% gel electrophoresis. Single band indicate males (Sample 1 and 4) while double bands indicate females (Sample 2, 3, 5, 6).

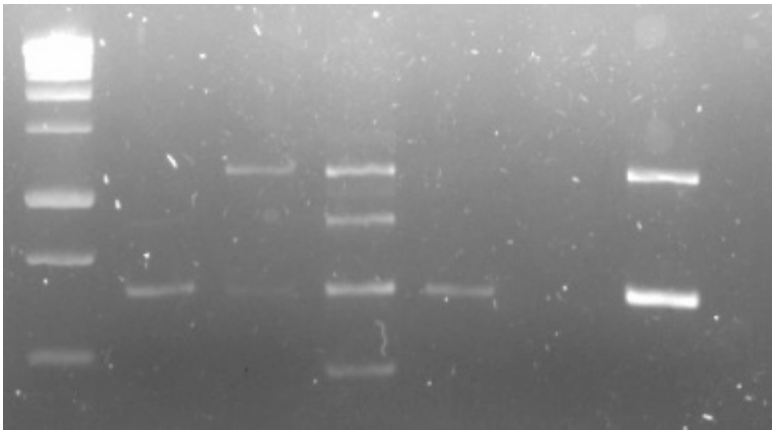

Figure 3: Molecular sexing of *Tyto alba javanica* by PCR amplification using DNA extracted from feather with primers 2550F/2718R followed by 3% gel electrophoresis. Single band indicate male (Sample 1 and 4) while double bands indicate female (Sample 6).

Table 1: CHD gene sequencing results of *Tyto alba javanica* versus *Tyto alba alba*

Sample	Sequencing Primer	Score	Expect	Identities	Gaps
B1	P2	544 bits (294)	8.00E-151	303/307 (99%)	1/307 (0%)
	P8	538 bits (291)	4.00E-149	304/310 (98%)	1/310 (0%)
B4	P2	542 bits (293)	3.00E-150	302/306 (99%)	1/306 (0%)
	P8	536 bits (290)	1.00E-148	301/306 (98%)	1/306 ()%)