ONLINE RESOURCES

Molecular sex identification of painted storks (*Mycteria leucocephala*): using FTA cards, horizontal PAGE and quick silver staining

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Introduction

Painted storks (*Mycteria leucocephala*) are large wading birds of southern Asia, widely distributed across the subcontinent of India, Indochina and parts of Southeast Asia (Ali and Ripley 1987; Hancock *et al.* 1992; Urfi and Kalam 2006). Due to loss of natural habitats, the numbers of these storks are declining and they are listed as a near threatened species (IUCN Red List of Threatened Species 2012. milky stork: www.iucnredlist.org/details/106003825/0; painted stork: www.iucnredlist.org/details/106003827/0). Painted storks and milky storks (*M. cinerea*), which is another species indigenous to Southeast Asia (Khoo 2006) were captive bred at the same breeding site in the Malaysian National Zoo (Zoo Negara), Kuala Lumpur, for conservation. Painted storks have sexually monomorphic plumage characters for males and females, their sexes are identified based on sexual size dimorphism: the male storks are heavier and larger than female storks at adult stage (Urfi and Kalam 2006; Khoo 2006). However, we have been informed that there were ambiguous cases where sizes of male and female overlapped and their genders were not identified. Assignation of genders in birds based on their mass and size is used commonly in the fields and it requires to know the ages of birds. The shortcoming of using this method is when male and female weights overlap, their sexes remain unknown (Weatherhead *et al.* 2007). Yet, to know the sex ratio in a breeding population is important for effective breeding programme. Previously, a study of morphometric and molecular sexing using feathers as DNA source was conducted in an attempt to sex both painted storks and milky storks of Zoo Negara by Ong *et al.* (2011), but the results were not very conclusive. It was reported that the males of both species were perceived to have about 1 to 3 cm longer bills and about 1 to 3 cm longer tarsus (lower legs) than the females. The sex ratio of one female for five males in both stork species was confirmed by using the 2550F/2718R primers (Ong *et al.* 2011).

Most of the molecular methods for bird sexing use feathers or blood collected in anticoagulant tubes as source of DNA. Here, we used dried blood on FTA (fast technology for analysis of nucleic acids) cards for molecular sexing of painted storks. The blood of these storks in Zoo Negara was sampled on FTA cards (Whatman) by the Malaysian Wildlife veterinarian team and the bird breeders of the zoo. FTA cards are small size cards (128 × 74 mm) with two or four rings in the centre, coated with antimicrobial agents, protein lysis agents, cell lysis agents, designed for the collection of blood at ambient temperatures and made easy to carry during long field trips (Gutiérrez-Corchero *et al.* 2002; Dutton 2005). DNA on FTA cards is proven for usages such as genotyping with microsatellite markers (Carr and Appleyard 2008), sexing passerine birds with sex-linked microsatellite markers (Gutiérrez-Corchero *et al.* 2002) and to isolate microsatellites of Kirtland's warblers (King *et al.* 2005). FTA-PCR products were electropheresed on agarose gels using ethidium bromide staining (Gutiérrez-Corchero *et al.* 2002; King *et al.* 2005; Carr and Appleyard 2008). Thus far, there is no record of FTA-PCR products electrophoresed on PAGE system (polyacrylamide gel electrophoresis) and stained with silver staining. Since most of the blood samples of Malaysian

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wildlife species including wild bird species were sampled on FTA cards, it was crucial for us to establish protocols to analyse DNAs from these cards such as DNA extraction, PCR formulas, electrophoresis system and staining protocols. Here, we described a molecular sexing method for the painted storks using dried blood on FTA cards (Whatman), P2/P8 sex-linked microsatellite primers designed by Griffiths *et al.* (1998), horizontal PAGE system (Yee *et al.* 2010) and quick silver staining protocol (Byun *et al.* 2009).

Materials and methods

The sexes of these free flying storks were engraved on a ring tag around its leg based on their sizes and weights according to zoo procedure (Khoo, Record Book of Zoo Negara). Sampling was done randomly without choosing the bird genders for genetic studies. One mL of blood was drawn invasively using a 5 mL syringe from the lower leg of the storks by veterinarians and dropped on a ring of a FTA card. The blood stains on these cards were dried under ambient temperature for 1–2 h, away from sunlight. Each blood drop was dried completely to prevent fungus growth in humid tropical climate. These cards were kept in tightly closed metal containers to prevent insects and rats from eating the dried blood. The Whatman protocol recommended users to punch out a 1–3 mm disc with a scientific puncher (Harrison). The disc was washed with Whatman Purification agent and rinsed with 1× TE buffer (10 mM Tris-Cl/0.1 mM Na_2 EDTA). Fifty microlitres of PCR mixture was loaded directly onto the disc to perform the PCR (http://www.whatman.com). This method allowed each disc to run 1× PCR each time. As our samples were scarce, this recommended method was not used. Instead, a 3 mm disc was punched and DNA's was extracted with Wizard DNA Purification kit (Promega, Madison, USA) with a few modifications as described in Yee *et al.* (2008). Extracted DNA pellet was re-suspended in 50 μL of 1× TE buffer (10 mM Tris-Cl/L mM Na_2 EDTA). This volume could be used to run 50 to 100× PCRs, thus maximizing the usage of blood samples and a reduced cost of FTA Purification agents.

For most bird species, female birds are heterozygotes (WZ), two bands would be observed whereas male birds are homozygotes (ZZ) hence a single band would be observed. Chromo-helicase-DNA-binding protein gene (*CHD1*) is found as two coding alleles on Z (*CHD1Z*) and W (*CHD1W*) chromosomes in birds (Griffiths *et al.* 1996). The universal method for molecular sexing for many wild bird species and domestic birds was to amplify *CHD1Z* and *CHD1W* alleles (Fridolfsson and Ellegren 2000). Sex-linked microsatellite primers P2/P8 designed by Griffiths *et al.* (1998), flanked the highly conserved *CHD1W* and *CHD1Z* genes on the W chromosome and Z chromosome, respectively and this pair of primers had been widely used to sex most sexually monomorphic avian species (Griffiths *et al.* 1998; Nesje and Røed 2000; Morinha *et al.* 2011).

The optimized PCR mixture for painted stork molecular sexing contained 1× PCR buffer (Promega), 5.5 mM of $MgCl_2$ (Promega), 0.5 mM of dNTPs (Promega), 2.5 U of *Taq* polymerase (Promega), 0.5 μM of each P2 and P8, 0.5–1 μL (10–20 ηg) DNAs, total up to a volume of 15 μL with double distilled H_2O, overlaid with 5–10 μL of mineral oil. The sequences of P2 is 5′ - TCT GCA TCG CTA AAT CCT TT -3′ and P8 is 5′ - CTC CCA AGG ATG AGR AAY - 3′ in which R = G or A, Y = T or C (Griffiths *et al.* 1998; Gutiérrez-Corchero *et al.* 2002; Morinha *et al.* 2011). The TC-412 thermal cycler (Techne, Burlington, USA) programming was: pre-denaturing at 94°C for 2 min, 30 cycles of denaturing 94°C for 20 s, annealing 42°C for 30 s, extension 72°C for 30 s, and a final extension at 72°C for 7 min, with 4°C on hold. The PCR products were electrophoresed on a horizontal PAGE system (Yee *et al.* 2010), using 7.5% non-denaturing polyacrylamide gel (29:1, 20 × 20 cm) and run at 170 V for 4.5 h. The polyacrylamide gel components for DNA migration were same for both vertical PAGE and horizontal PAGE, taken from (Ausubel *et al.* 1999). The polyacrylamide solution was allowed to polymerize on a horizontal gel tray, with a sliding cover on top without air bubble. Ten μL PCR product mixed with dye was dropped on a piece of cut-out filter paper (5 × 5 × 1 mm) (Whatman) and inserted onto the crevice of slide gel pieces. The whole gel tray with inserted PCR samples was placed on a 20 × 20 cm gel tank for electrophoresis. The photographs of setting up of horizontal PAGE and details of modification in chemical compositions to make different gel percentages for electrophoresis of DNA fragment sizes are described in Yee *et al.* (2010). Gels were stained with quick silver staining method by Byun *et al.* (2009). There were three solutions to be prepared: fixing solution, developer solution and de-staining solution. The fixing solution was 0.5% acetic acid, 0.2% $AgNO_3$, 10% ethanol; the developing solution was of 5% NaOH, 0.1% HCOH (pre-heat to 55°C); and the de-staining solution was of 0.5% acetic acid and 10% ethanol. The whole staining process took only three steps such as fixing, developing and de-staining (Byun *et al.* 2009). The stained gel was placed under a white light illuminator and photographs were taken with a zoom-in digital camera. The two separated fragments of *CHD1W* allele (400 bp) and *CHD1Z* allele (385 bp) were seen clearly. A specific DNA-ladder (Cambrex, Weisbaden, Germany, 20 bp–800 bp, markers fragment 20 bp apart) was used for comparing and scoring. These two alleles were only 15 bp different in size, which required a long electrophoresis run to separate two fragments unambiguously. Of the 31 samples tested, five were females (ZW) and 26 were males (ZZ).

Results and discussion

To compare the methods between using a FTA disc and isolating DNA from a disc to run PCR, DNA extracted from a disc required less chemicals. To run a 3 mm FTA disc,

PCR mixture of 50 μL is needed as each disc has to be fully immersed in solution when performing PCR in the thermocycler (Yee *et al.* 2008). As stated above, 0.5–1 μL DNA was loaded to each 15 μL PCR mixture and DNA isolated from each 3 mm disc allowed us to run 50–100× PCRs. Therefore, DNA extracted from a disc not only saved limited blood samples but it also saved PCR chemicals to run more samples (Yee *et al.* 2008). In our lab vertical PAGE is used for electrophoresis of DNA fragments whereas horizontal PAGE is used for electrophoresis of allozymes and proteins. We experimented and adapted the horizontal PAGE to run DNA fragments successfully, based on basic theory of electrophoresis in that negatively charged DNA molecules moved from negative electrode to positive electrode. This protocol was tested on PCR products amplified from a wide range of primers including single-locus markers and multi-loci markers as described in Yee *et al.* (2010). We replaced vertical PAGE system with horizontal PAGE system because it was faster to assemble horizontal gel-tray. It has no leakage and technically less demanding which allowed us to run large population samples in shorter time. At initial stage of our studies, we electrophoresed FTA-PCR products of P2/P8 on 6% Metaphor agarose gels (Cambrex), but only one band was observed in all the painted stork samples. To remedy the situation, we then electrophoresed the PCR products on 7.5% nondenaturing polyacrylamide gels in horizontal PAGE to obtain a better resolution. The staining efficacy of ethidium bromide was reduced by polyacrylamide gels for this species therefore we switched to the simplified silver staining protocol of Byun *et al.* (2009) which gave us the staining quality we wanted for Z and W bands. Although a few silver staining protocols were compared by Byun *et al.* (2009), yet this protocol was faster, used less hazardous chemicals and required less staining solutions. This quick silver staining protocol took three steps to complete whereas the conventional silver staining protocol took six to nine steps.

In conclusion, the method of utilizing the dried blood on FTA cards as DNA source, P2/P8 primer pairs, horizontal PAGE system and quick silver staining protocol could be used as an alternative molecular protocol to sex painted storks. This has also shown that two closely sized DNA fragments, amplified from FTA cards could be separated on horizontal PAGE system. The quality of DNA on FTA cards was confirmed as not being degraded by the humidity of the tropics during transportation and storage. It has made it possible for us to collect wildlife animal blood samples with ease during long trips with assurance that small DNA fragments were tagged from these cards. Both our data and those of Ong *et al.* (2011) showed that the sex ratio of the painted storks in Zoo Negara population was a ratio of 5 : 1 (male : female). Based on these stork plumage characteristics, we suspected that hybrids between the painted storks and the milky storks were present in this breeding population and most hybrids were males and sterile (Haldane 1922). Therefore, further genetic and morphological studies on the pure breeds and the suspicious hybrids of these storks will be done in order to monitor, manage and to enhance the stork breeding program of Zoo Negara.

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