UNIVERSITI PUTRA MALAYSIA

FINGERPRINTING THE PAINTED STORKS (Mycteria leucocephala PENNANT), MILKY STORKS (Mycteria cinerea RAFFLES), AND THEIR SUSPICIOUS HYBRIDS IN A ZOO IN MALAYSIA

YEE YOKE SIM

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By

YEE YOKE SIM

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FINGERPRINTING THE PAINTED STORKS (*Mycteria leucocephala* PENNANT), MILKY STORKS (*Mycteria cinerea* RAFFLES), AND THEIR SUSPICIOUS HYBRIDS IN A ZOO IN MALAYSIA

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March 2017

Chair: Associate Professor Janna Ong Abdullah, PhD.
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Painted storks (*Mycteria leucocephala*) and milky storks (*Mycteria cinerea*) are large wading birds of southern Asia, and listed as endangered species by IUCN (International Union for Conservation of Nature). Most painted storks inhabit India while milky storks mainly colonise east Sumatra and the west coast of Peninsular Malaysia. In the past decade, no wild milky stork has settled in Peninsular Malaysia. Both species were captive bred at the Zoo Negara for conservation purposes. Yet, storks with intermediate plumage traits between two stork species were observed, and suspected to be their hybrids. Based on the intermediate plumage characteristics, hybrids of two storks were suspected at natural breeding sites of Cambodia, Singapore, and Thailand. However, mere plumage appearances are often insufficient to confirm the bird hybrids, and hence, molecular markers are used. Unbalanced sex ratio in a population could be one of the reasons that two species hybridised. Females and males of both stork species have no differentiation in plumage traits, and therefore, molecular-sexing markers are used to identify genders. The objective of this study was to fingerprint individual storks using different classes of DNA markers including sex-linked marker to identify hybrids, and to estimate sex ratio. Based on the plumage characteristics of each stork, blood was sampled systematically onto FTA cards (Whatman, classic) (FTA – fast analysis of nucleic acids). DNA was extracted from a disc of FTA cards (3 mm), and PCR was run with RAPDs (random amplified polymorphism DNA), ISSRs (inter-specific sequence repeat), and cross species microsatellites, electrophoresed on agarose gels and stained with ethidium bromide. Six out of 44 screened primers were selected to fingerprint each stork, and two distinctive sets of banding patterns were generated, which differentiated pure storks from hybrids. These six markers included two wood stork microsatellites (WSU09U/WSU09L and WSU13U/WSU13L), two short RAPDs (Operon D-03 and Operon D-05), one long RAPD (LR7), and one 5’-anchored ISSR (RAM2). The data revealed that most loci of hybrids were combinations of two pure storks, few loci were exclusively found in hybrids alone. The fingerprinting data were compared to plumage characteristics, and it was found out these hybrids possessed six different types of intermediate plumage characteristics between two parental species, and they were not a typical kind of hybrid only. Sex-linked microsatellites P2/P8 was run with PCR, electrophoresed PCR products on 7.5% PAGE gel (non-denaturing) and stained with silver staining. The result showed that sex ratio of female to male in painted stork population was 1 to 5, while all hybrids and milky storks were males, indicating that males were the majority. It can therefore be concluded that stork hybrids were identified through DNA fingerprinting using six selected DNA markers. The hybrids of intermediate plumage traits were confirmed by DNA fingerprinting. Sex ratio in the breed storks was not balanced.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Sarjana Sains

FINGERPRIN BURUNG JONG (*Mycteria leucocephala* PENNANT), BURUNG UPEH (*Mycteria cinerea* RAFFLES), DAN HIBRID SANGSI DALAM ZOO DI MALAYSIA

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Burung Jong (*Mycteria leucocephala*) dan Burung Upeh (*Mycteria cinerea*) adalah burung-burung yang besar di Asia selatan dan disenaraikan sebagai spesies yang terancam oleh IUCN (*International Union for Conservation of Nature*). Kebanyakan burung Jong mendiami India dan majority burung Upeh mengeduduki Sumatera timur dan pantai barat Semenanjung Malaysia. Dalam dekad yang lalu, tidak ada burung Upeh liar menetap di Semenanjung Malaysia. Kedua-dua spesies ini diternakkan di Zoo Negara bertujuan untuk pemeliharaan. Namun, bulu-bulu bercorak pertengahan diantara dua spesies burung diperhatikan dan disyaki adalah hibrid mereka. Berdasarkan ciri-ciri bulu pertengahan, hibrid kedua spesies burung berkenaan disyaki di tempat pembiakan semulajadi Kemboja, Singapura dan Thailand. Walau bagaimanapun, observasi ciri-ciri bulu sahaja, selalunya tidak mencukupi untuk mengesahkan hibrid burung dan oleh itu, penanda molekul digunakan. Rasio seks yang tidak seimbang di dalam sesuatu populasi boleh menjadikan salah satu sebab, bahawa dua spesies hibridisasi. Jantan dan betina dari kedua-dua spesies burung Jong dan burung Upeh, tiada pembezaan dalam ciri-ciri bulu dan sebab itu, penanda seks molekul digunakan untuk mengenal pastikan jantina. Objektif kajian ini adalah untuk fingerprintin burung-burung individu menggunakan kelas penanda-penanda DNA yang berlainan, termasuk penanda-seks DNA, untuk mengenal pastikan hybrid-hibrid dan untuk menganggarkan rasio seks. Berdasarkan ciri-ciri bulu setiap ketikan, darah disampel secara sistematik ke kad FTA (klasik). DNA diekstrak daripada disk kad FTA (3 mm) dan PCR dijalankan dengan RAPDs (*random amplified polymorphism DNAs*), ISSRs (*Inter-specific sequence repeat*), dan microsatellit spesies berkarabat, elektroforesis pada gel agarose dan diwarnakan dengan etidium bromida. Enam dari 44 penanda-penanda yang dipilih untuk fingerprin setiap burung dan dua jenis corakan loreng yang berlainan dihasilkan, telah membezakan burung-burung tulen dari hibrid-hibird. Enam penanda-penanda termasuk dua mikrosatellit (WSU09U / WSU09L dan WSU13U / WSU13L), dua RAPDs pendek (Operon D-03 dan Operon D-05), satu RAPD panjang (LR7) dan satu 5'-penambat ISSR (RAM2). Data mendedahkan bahawa kebanyakan *loci* hibrid adalah gabungan dua spesies burung tulen, beberapa *loci* eksklusif didapati dalam hibrid-hibrid sahaja. Data fingerprintin dibandingkan dengan ciri-ciri bulu dan ia mendapati hibrid ini mempunyai enam jenis ciri-ciri bulu pertengahan di antara dua spesies burung, dan mereka bukan hibrid tipikal sahaja. Mikrosatellit seks P2 / P8 dijalankan dengan PCR, produk PCR elektroforesis pada 7.5% gel PAGE (*non-denaturing*) dan berwarna dengan pewarna perak (*silver staining*). Hasilnya menunjukkan bahawa rasio seks betina kepada jantan dalam populasi burung Jong adalah 1 hingga 5, manakala semua hibrid dan burung Upeh adalah jantan, menunjukkan bahawa jantan adalah majoriti. Oleh itu, konklusifnya adalah bahawa hibrid stork dikenal pasti melalui fingerprintin DNA menggunakan enam penanda-penanda DNA terpilih. Data berbanding dengan ciri-ciri bulu dan disahkan hibrid-hibrid berbulu variasi. Rasio seks yang tidak seimbang didapati dalam burung-burung berikut.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABRREVIATIONS

APS	Ammonium persulfate $(NH_4)_2S_2O_8$
bp	base pairs
DNA	Deoxyribonucleic Acid
FTA	Fast Technology Analysis of nucleic acid
dNTP MIX	deoxy-N-triphosphate (N = A / T / C / G) (A - adenosine, T - thymine, C - cytosine, G-guanine)
F1	Felial 1 (offspring of parental species)
F2	Felial 1 cross with felial 1 to give felial 2 offspring
F3	Felial 2 cross with felial 2 to give felial 3 offspring
HCOH	Formaldehyde
$MgCl_2$	Magnesium chloride
NaOH	Sodium hydroxide
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
10XTBE	Tris-base(1mM), Boric Acid(1mM), Na_2EDTA(20mM)
1X TBE	Tris-base(0.1mM), Boric Acid(0.1mM) Na_2EDTA (2mM)
1XTE	Tris-Cl(10mM), Na_2EDTA(1mM)
TEMED	N,N,N',N'-tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

The painted stork (*Mycteria leucocephala*) and the milky stork (*M. cinerea*) are large migratory birds of southern Asia and listed as endangered species by IUCN (International Union for Conservation of Nature). The largest population of painted storks is found in India but their numbers are declining significantly primary due to deforestation (Kalam and Urfi, 2008, Urfi, 2011). Milky storks, on the other hand, are found mainly in east Sumatra, Indonesia and at one time, on the west coast of Peninsular Malaysia (Verhrught, 1987; Iqbal, 2008). However, in the past decade, no wild milky stork population has been detected in Peninsular Malaysia (Li et al., 2006). Both stork species are captive-bred at the Zoo Negara in Malaysia for conservation purposes. According to the breeders of the Zoo Negara, the breeding programme has been successful, and these storks now inhabit the breeding site. Both species have similar body sizes and shapes but are distinguished by plumage colourations and patterns. Yet, storks with intermediate plumage traits between two stork species have been observed at the Zoo Negara, and these are suspected hybrids. The breeders separated hybrids from pure storks according to feather traits, as they had discovered that the fertility of hybrid storks was uncertain and some hybrids could even be barren (Schilthuizen et al., 2011), hence, identification of these hybrids is important. Although bird hybrids are frequently detected by their feather characteristics, yet in many cases, mere physical appearances are insufficient to ascertain the hybrids. Therefore, molecular markers have become main approach to distinguish hybrids from pure breeds (Stenzler et al., 2004; Huang et al., 2004; Gonzalez et al., 2005). Combination of genetic data and morphological data was recommended for hybrid identification (Jorstad et al., 2007, Irwin et al., 2009). Hybridization of birds is commonly in the wild and bird hybrids are often spotted through feather traits or songs (Randler, 2002). Based on intermediate plumage traits, hybridisation between painted storks and milky storks was suspected at the Thailand National Park, the Cambodian National Park, and the Singapore National Zoo *(www.thainationalparks.com/species.paintedstork)*.The sex ratio of a population plays an important role in hybridisation (Backström & Välli, 2011). Two different species with compatible mating behaviour tend to hybridise, especially when opposite sex number is low (Mallet, 2007). Thus, a balanced sex ratio is crucial to prevent hybridisation in breed populations. There is no feather differentiation between females and males of painted storks, and of milky storks, hence, molecular-sexing method is used to identify genders of these storks. The results of DNA fingerprinting, plumage characteristics and sex ratio were provided the breeders of Zoo Negara, for separating hybrids from pure storks, and improving the breeding programme. In this study, there were two sections of DNA fingerprinting, the first one was identifying the hybrids by using selected DNA markers and the second one was molecular-sexing of the storks through sex-linked DNA marker.

1.1 Objectives of the Study

The main objective of this study was to carry out fingerprinting of individual storks for identifying hybrids, and molecular-sexing. Identification of hybrids was the main part while the molecular-sexing was the minor part of the project.
The sub-objectives were:

1. To screen a range of different classes of DNA markers, searching for suitable DNA markers that generating different banding patterns between hybrids, and pure storks. Selected DNA markers were used to fingerprint individual storks of each population, and to differentiate the hybrids from pure storks.

2. To collect morphological data based on plumage colourations and patterns of pure storks and hybrids. The fingerprinting data were compared to plumage characteristics, and separated pure storks and hybrids accordingly.

3. To estimate the sex ratio of the stork populations by using P2/P8 sex-linked microsatellite markers.

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