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Application of *CHD1* Gene and EE0.6 Sequences to Identify Sexes of Several Protected Bird Species in Taiwan

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ABSTRACT: Many bird species, for example Crested Serpent Eagle (*Spilornis cheela hoya*), Collared Scops Owl (*Otus bakkamoena*), Tawny Fish Owl (*Ketupa flavipes*), Crested Goshawk (*Accipiter trivirgatus*), Grass Owl (*Tyto longimembris*), *etc.*, are monomorphic, which is difficult to identify their sex simply by their outward appearance. Especially for those monomorphic endangered species, finding an effective tool to identify their sex beside outward appearance is needed for further captive breeding programs or other conservation plans. In this study, we collected samples of Crested Serpent Eagle, Collared Scops Owl, Tawny Fish Owl, Crested Goshawk, and Grass Owl, five protected monomorphic species in Taiwan, as well as Black Swan (*Cygmus atratus*) and Nicobar Pigeon (*Caloenas nicobarica*), two aviary introduced monomorphic species served as a control group. We used sex-specific primers of avian *CHD1* (chromo-helicase-DNA-binding) gene and EE0.6 (*Eco*RI 0.6-kb fragment) sequences to identify the sex of these birds. The results showed that *CHD1* gene primers could be used to correctly identify the sex of Black Swans, Nicobar Pigeons and Crested Serpent Eagles, but it could not be used to correctly identify sex in Collared Scops Owls, Tawny Fish Owls, and Crested Goshawks. In the sex identification using EE0.6-sequence fragments, A, C, D and E primer sets could be used for sexing Black Swans; A, B, C, and D primer sets could be used for sexing Crested Serpent Eagles; and E primer set could be used for sexing Nicobar Pigeons and the two owl species. Correct determination of sex is the first step if a captive breeding measure is required. We have demonstrated that several of the existing primer sets can be used for sex determination of several captive breeding and indigenous bird species.

KEY WORDS: Collared Scops Owl, Crested Serpent Eagle, Crested Goshawk, sex identification of bird, Tawny Fish Owl.

INTRODUCTION

The Crested Serpent Eagle (*Spilornis cheela hoya*), Crested Goshawk (*Accipiter trivirgatus*), Tawny Fish Owl (*Ketupa falvipes*), and Collared Scops Owl (*Otus bakkamoena*) are native bird species in Taiwan and all are enlisted as "Rare and Valuable" wildlife species and legally protected by Taiwan government. Breeding programs in captivity are important for their conservation. However, all the species are sexually monomorphic which makes it difficult to discriminate between sexes via outward appearance. Therefore, it is important to develop accurate methods to enhance accuracy of sex determination (Duan and Fuerst, 2001; Dubiec and Zagalska-Neubauer, 2006; Cerit and Avanus, 2007) and therefore, improve captive breeding success.

Sex chromosomes in birds are Z and W; ZZ and ZW chromosome combination are male and female, respectively. Chromo-helicase-DNA-binding gene (*CHD1* gene) is located on sex chromosomes and *CHD-W* on W (Griffiths and Tiwari, 1995), and *CHD-Z* on *Z* (Griffiths and Korn, 1997). Because there are possible differences in intron length of the *CHD1* gene between Z and W, primer sets of P2/P8 (Griffiths et al., 1998), 1237L/1272H (Kahn et al., 1998) and 2550F/2718R (Fridolfsson and Ellegren, 1999) were designed for PCR to determine the sex of birds. Due to the genetic differences, some birds have similar intron length in *CHD-W* genes in both sex chromosomes that make these primers inapplicable to detect sex differences (Kahn et al., 1998; Griffiths et al., 1998; Fridolfsson and Ellegren, 1999; Ito et al., 2003; Wang et al., 2007; Chang et al., 2008). Therefore, the applicability of each set of primer needs to be tested for the accuracy of detection in sex determination. The different EE0.6 (*Eco*RI 0.6-kb fragment) sequences located on the avian Z and W sex chromosomes are also available for sex determination (Ogawa et al., 1997, 1998; Itoh et al., 2001).

We previously reported several reliable sex-specific genetic markers for sex determination in the Crested Serpent Eagle and Crested Goshawk (Hsu et al., 2009). In this study, we tested the primer sets and report their applicability in determining the sex of four native Taiwanese bird species.

Table 1. The primer sequences and PCR conditions of EE0.6 sequence for sex identification.

Primer name	Primer sequences[a] (5'-3')
USP1	CTATGCCTACCACMTTCCTATTTGC
USP3	AGCTGGAYTTCAGWSCATCTTCT
AWS03	ACAGTTTGTCTGTCTCCGGGGAA
NRD4	TCAGAGCACTCTTTCCAGGAA
AWS05	CACCCTGGATTGGACAACCTATTTC
KM81F	TACAGATAAAAAGTGCAGTCATTGTGGC
KM81R	TCTTTGAGGACACACTCAGAGCAC
CPE15F	AAGCATAGAAACAATGTGGGAC
CPE15R	AACTCTGTCTGGAAGGACTT
INT-F	ATAGAAACAATGTGGGAC
INT-R	CTCTGTCTGGAAGGACTT
SINT-F	TAGGCTGCAGAATACAGCAT
SINT-R	TTGTGCAGTTCTAGTCCATA

Name	Sexing primer	Control primer	Denaturation	Annealing	Elongation
A	USP1/USP3	CPE15F/CPE15R	95ºC, 80 sec	60ºC, 90 sec	72ºC, 60 sec
B	USP1/USP3	INT-F/INT-R	95ºC, 80 sec	56ºC, 90 sec	72ºC, 60 sec
C	AWS03/USP3	CPE15F/CPE15R	95ºC, 80 sec	59ºC, 90 sec	72ºC, 60 sec
D	AWS03/USP3	INT-F/INT-R	95ºC, 80 sec	56ºC, 90 sec	72ºC, 60 sec
E	USP1/NRD4	CPE15F/CPE15R	95ºC, 80 sec	59ºC, 90 sec	72ºC, 60 sec
F	AWS05/NRD4	SINT-F/SINT-R	95ºC, 80 sec	59ºC, 90 sec	72ºC, 60 sec

[a] Source of the primer sequences: Itoh et al. (2001).

MATERIALS AND METHODS

Animals

Blood samples of 41 Crested Serpent Eagles, 16 Collared Scops Owls, eight Crested Goshawks (these birds were also used in Hsu et al. (2009)), 13 Grass Owls and three Tawny Fish Owls were taken from the brachial vein (V. Ulnaris) with a heparinized syringe. To confirm the accuracy of sex identification, the sex of 20 Crested Serpent Eagles and eight Grass Owls were also determined by using laparoscopy. The autopsy muscle samples of Black Swans (*Cygmus atratus*) and Nicobar Pigeons (*Caloenas nicobarica*) were also taken to serve as positive controls for the sex determination study.

DNA isolation

The DNA isolation procedure was previously described in Hsu et al. (2009). In brief, the blood cells were isolated and incubated with 3 mL of lysis buffer (10 mM Tris-Cl; 150 mM NaCl; 10 mM EDTA; pH=8.0), 60 μL proteinase K (10 mg/mL; Amersco, Ohio, USA) and 200 μL 10% SDS at 55ºC overnight. The DNA was then extracted using the phenol: chloroform: isoamyl alcohol solution (25:24:1: v/v/v) and chloroform (Wu et al., 2007). For autopsy muscle tissue DNA extraction, 0.1 g of tissue was minced in 500 μL lysis buffer and treated with 100 mL 10% SDS and 60 μL proteinase K (10 mg/mL) for 12 h at 55ºC. Phenol/chloroform extraction procedure was used to extract the DNA from the tissue. The DNA was washed twice with 70% ethanol to get rid of contaminants. The quality and concentration of genomic DNA was determined by measuring the absorbance at 260 and 280 nm (Sambrook and Russell, 2001).

The PCR procedure for *CHD1* gene to determine sex

The primer set of 1237L/ 1272H (Kahn et al., 1998) was used to determine the sex of birds. PCR amplification was performed with 30 ng of genomic DNA, 0.4 μM of each primer and Taq DNA Polymerase Master Mix RED (Ampliqon, Herlev, Denmark). The PCR procedure was described by Kahn et al. (1998). A 4% agarose gel electrophoresis was applied to separate the target DNA products stained with ethidium bromide for DNA visualization.

The PCR procedure for EE0.6 sequence to determine sex

The primer sets from EE0.6 sequence, A, B, C, D, E and F (Itoh et al., 2001; Table 1), were used to amplify sex-specific fragments of birds. PCR amplification was performed with 30 ng of genomic DNA, 0.4 μM of each primer and Taq DNA Polymerase Master Mix RED (Ampliqon). The PCR procedure was described by Itoh et al. (2001). The annealing temperature for each set of primer was optimized empirically (Table 1). A 1.5% agarose gel electrophoresis was applied to separate the target DNA products stained with ethidium bromide for DNA visualization.

RESULTS AND DISCUSSION

Utilization of *CHD1* gene to determine sex

Utilizing CHD1 primer set (1237L/1272H) to determine the sex of birds was successful for Grass Owls, Nicobar Pigeons and Black Swans (Fig. 1). The female birds produced at least one female-specific PCR band whereas the males either produced no band or only one non-sex-specific band. This primer set cannot be used to distinguish sex in Crested Goshawk, Collared

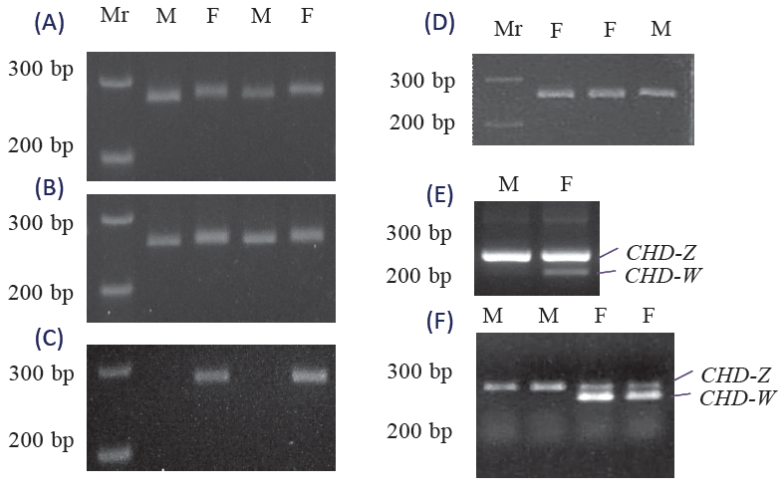

Fig. 1. Sex identification of Crested Goshawk (A), Collared Scops Owl (B), Grass Owl (C), Tawny Fish Owl (D), Nicobar Pigeon (E), and Black Swan (F), using primers 1237L/1272H of *CHD1* gene. PCR products were separated on a 4% gel. (M: Male. F: Female. Mr: 100 bp ladder markers).

Table 2. Comparison of *CHD1* gene, EE0.6 sequence, laparoscopy and other methods in sex identification of Crested Serpent Eagle, Crested Goshawk, Collared Scops Owl, Tawny Fish Owl and Grass Owl[a].

Species	Crested Serpent Eagle[c]		Crested Goshawk[c]		Collard Scops Owl		Tawny Fish Owl		Grass Owl	
Sex / Methods	M	F	M	F	M	F	M	F	M	F
CHD1 gene	13/13	8/8	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
EE0.6 sequence	13/13	8/8	4/4	4/4	10/10	6/6	2/2	1/1	5/5	8/8
Laparoscopy	13/12	7/8	0/0	0/0	0/0	0/0	0/0	0/0	5/5	8/8
Other methods[b]	1/1	0/0	2/2	1/1	0/0	1/1	2/2	1/1	0/0	0/0

[a] The data represent test result /actual number of male or female birds. (M: male, F: female)
[b] Other methods : anatomy or observation of laying eggs.
[c] Part of the data were summarized from Hsu et al. (2009) for comparative purpose.

Scops Owl, and Tawny Fish Owl. The results suggest that other molecular markers are needed to identify the sex of these bird species. When we compared the results of sex determination for 21 Crested Serpent Eagles, the endoscopy technique mistakenly identified a premature bird (Table 2), suggesting that proper molecular technique is reliable method for sex determination. Although the CHD1 primer can be used to correctly identify (100%) the sex of Crested Serpent Eagles, it requires separation of PCR products on a 4% gel and an extended electrophoresis time (Hsu et al., 2009). Therefore, others have developed Crested Serpent Eagle specific primers for sex identification (Hsu et al., 2009; Chou et al., 2010). For the Grass Owls, only females generated a PCR product, presumably from *CHD-W* gene. The results were identical to that from endoscopy examination; therefore, this primer set is useful to identify sex in the Grass Owl. However, addition of an internal control primer pair to generate a common product for both sexes will be helpful to reduce misidentification.

Utilization of EE0.6 sequence to determine sex

The A, C, D, and E sets of primers from EE0.6 sequence could be used to correctly determine the sex of Black Swans (Fig. 2). The female generated one sex-specific band for these primer sets. Primer sets B and F were not suitable to determine the sex of Black Swans because it did not generate any sex differential PCR products. The A, C, and D sets of primers from EE0.6 sequence could be used to correctly determine the sex of Crested Goshawks (Fig. 2). The male generated at least one common PCR band and the female generated at least one sex-specific band for these primer sets. Primer sets B and E were not suitable to determine the sex of Crested Goshawks because it did not generate sex differential PCR products. Primer set E could be used to distinguish the sex of Collared Scops Owls and Tawny Fish Owls (Fig. 2). For Grass Owls, the primer sets C, D, and F could be used to identify the sex. As reported by Hsu et al. (2009), the A, B, C, and D sets of primers from EE0.6 sequence can be used to correctly determine the sex of Crested Serpent Eagles. Primer set E was not suitable to determine the sex of Crested Serpent Eagles because it did not generate sex differential PCR products. We summarized the sex

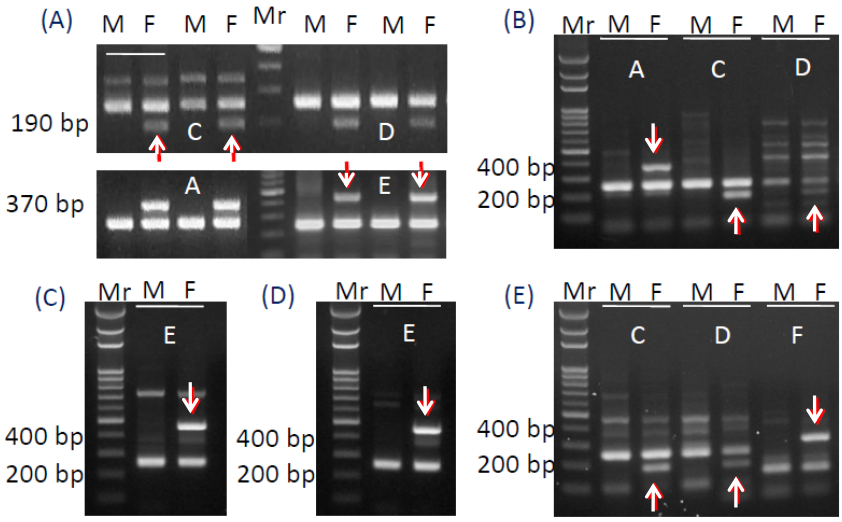

Fig. 2. Sex identification of Black Swan (A), Crested Goshawk (B), Collared Scops Owl (C), Tawny Fish Owl (D), and Grass owl (E) using EE0.6 sequence with different primer sets (A, B, C, D, E and F). Arrows indicate the sex specific PCR product. (M: Male. F: Female. Mr: 100 bp ladder markers).

Table 3. Comparison of *CHD1* gene and EE0.6 sequence in sex identification for Crested Serpent Eagle, Crested Goshawk, Collared Scops Owl, Tawny Fish Owl and Grass Owl.

Loci	*CHD1* gene[a]	EE0.6 sequence[b]					
Primers / Species	1237L/ 1272H	A	B	C	D	E	F
Crested Serpent Eagle	+[c]	+++	+++	++	+	-	-
Crested Goshawk	-	+++	-	++	+	-	-
Collared Scops Owl	-	-	-	-	-	++	-
Tawny Fish Owl	-	-	-	-	-	+++	-
Grass Owl	+	-	-	+	+	-	+++

[a] *CHD1* gene primers (1237L/ 1272H) (Kahn et al., 1998).
[b] EE0.6 sequence A-F primer sets (Itoh et al., 2001).
[c] + + + Clearly identified.
+ + Clearly identified but with one of the following situations: weak bands, little differential bands, or too many non-specific bands.
+ Possible to identify but with two of the following situations: weak bands, little differential bands, or too many non-specific bands.
- Impossible to identify.

determination performance of the seven primer sets used in the current study for the five species tested on Table 3. The comparison was based on the clarity and specificity of the sex differential PCR products and its applicability for sex determination.

The Z- and W-linked sequence allows sex discrimination using a suitable combination of primers for *CHD1* gene or EE0.6 sequence (Griffiths and Tiwari**,** 1995; Kahn et al., 1998; Itoh et al., 2001). However, there are also genetic polymorphisms that make the sex detection using these sequences impossible. Each bird species needs to be tested individually to get a proper combination of primers for sex determination. In the current study, we have tested seven pairs of primers from two different genetic regions and found that there were suitable individual primer sets for sex determination in Crested Serpent Eagle, Crested Goshawk, Collared Scops Owl, Grass Owl and Tawny Fish Owl (Table 3). In the previous study (Hsu et al., 2009), we successfully utilized random amplified polymorphic DNA (RAPD) technique (Hadrys et al., 1992) to find several new sex specific gene sequences for Crested Serpent Eagles. We have also cloned sequences for determining the sex of the Crested Goshawks. These are novel sequences that are powerful for determining the sex of these birds because a large sex difference makes it much easier to be detected in a gel electrophoresis. Real-time PCR technique which does not require an agarose gel electrophoresis procedure can simplify the sex determination technique (Chang et al., 2008; Chou et al., 2010). These improvements will enhance the power of correct identification of sex in monomorphic birds. In conclusion, we have found that several suitable primer pairs for sex determination for several indigenous conserved bird species. These primer sets can be

utilized to correctly identify the sex of several important native birds of species in Taiwan. The correct sex identification is a critical step for successful captive breeding programs.

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利用 *CHD1* 及 EE0.6 分子標幟進行臺灣保育鳥類性別鑑定

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摘要：在臺灣許多保育鳥類，例如：大冠鷲(Crested Serpent Eagle, *Spilornis cheela hoya*)、領角鴞（Collared Scops Owl, *Otus bakkamoena*）、黃魚鴞（Tawny Fish Owl, *Ketupa flavipes*）、鳳頭蒼鷹（Crested Goshawk, *Accipiter trivirgatus*）和草鴞（Grass Owl, *Tyto longimembris*）等，由於屬於單外表型（monomorphic），公母外表相似，因此常無法由其外貌型態的不同，而判定其公母性別，故除了以傳統外貌分辨公母的方法，發展有效率的方法鑑別這些保育鳥類的公母，對於其保育及繁殖計畫極為重要。本研究以黑天鵝（Black Swan, *Cygmus atratus*）及綠簑鴿（Nicobar Pigeon, *Caloenas nicobarica*）兩種鳥類作為對照組，設計針對鳥類 *CHD1* 基因及 EE0.6 DNA 序列，利用聚合酵素連鎖反應法可產生不同片段大小之特異性引子，進行大冠鷲、領角鴞、黃魚鴞、鳳頭蒼鷹和草鴞公母性別鑑定。試驗結果顯示，利用所設計 *CHD1* 基因特異性引子，針對黑天鵝、綠簑鴿、大冠鷲及草鴞可準確進行公母鑑別，但是領角鴞、黃魚鴞及鳳頭蒼鷹等則無法利用此基因檢測進行性別鑑定。另在 EE0.6 DNA 序列特異性引子檢測結果顯示，利用 A、C、D 及 E 引子組可進行黑天鵝公母鑑別；A、B、C 及 D 引子組可進行大冠鷲公母鑑別；E 引子組可進行綠簑鴿、領角鴞和黃魚鴞公母鑑別；C、D 及 F 引子組可進行草鴞公母鑑別。正確的性別鑑定，為進行保育類鳥類保育繁殖計畫最重要的第一步，本研究結果確立可利用 *CHD1* 基因及 EE0.6 DNA 序列特異性引子，進行上述保育鳥類之公母鑑別，而準確性高的保育鳥類公母鑑別分子檢測技術的建立，可提高人工配對繁殖效率，進而提昇對於上述臺灣稀有的保育鳥類的保育繁殖效率。

關鍵詞：領角鴞、大冠鷲、鳳頭蒼鷹、鳥類性別鑑定、黃魚鴞。