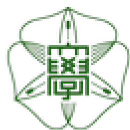

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Genomic DNA sex identification in pet red whiskered bulbul (*Pycnonotus jocosus*) in Thailand

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Abstract

The red whiskered bulbul (*Pycnonotus jocosus*) is a common pet bird in Thailand. Accurate and sensitive sex identification is necessary for breeding. This study developed a sex identification method using duplex PCR with a combination of a female-specific gene and 18S ribosomal RNA gene primers. EE0.6 sequences were used as primers for the female-specific gene. The results of this method were the same as mating observations for 117 known-sex red whiskered bulbuls. Amplification limitations of genomic DNA were 0.1 ng/µl and 0.08 ng/µl for males and females, respectively. These findings are more sensitive than the results from PCR of *CHD1* gene reported before. Thus, this assay is an accurate, sensitive and suitable method from molecular sex identification in red whiskered bulbuls.

Key Words: *Pycnonotus jocosus*, red whiskered bulbul, sex identification

Sex identification in avian species is an important tool for aviculture, conservation and forensic studies[11)]. The differentiation between males and females in avian species is complex and difficult to determine by visual observation in sexually monomorphic birds and nestlings[7)]. Common sex identification methods for sexually monomorphic and juvenile birds are cloacal inspection, steroid sexing, cytological sex identification and surgical sex identification[3)]. These methodologies have low accuracy, are expensive and time consuming, and entail a high level of injury to birds[7)]. Polymerase chain reaction (PCR) methods for sex identification have been developed based on sex chromosome-related genes such as the chromo-helicase-DNA-binding 1 gene (*CHD1*) and the 0.6 kb *Eco*RI fragment (EE0.6) and consider the differences of nucleotide sequences between males (two Z chromosomes) and females (Z and W chromosomes)[6,8,9,12)]. The *CHD1* gene is located on the avian sex chromosomes of non-ratite birds[8)]. The differences

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in intron length of the *CHD1* gene between the Z and W chromosomes are used to determine sex in many non-ratite birds[8]. The EE0.6 sequence is likely a part of a pseudogene and conserved on the W chromosomes of Carinatae birds[12]. These techniques are reliable, cheap, rapid, and simple and results in low levels of damage to birds[3,5,7].

It is difficult to differentiate between males and females for many Passeriformes species[5]. One passerine bird, the red whiskered bulbul (*Pycnonotus jocosus*), is a highly vocal, common pet in Thailand[16]. Breeding of the red whiskered bulbul in Thailand has expanded and has a high economic value[10]. The red whiskered bulbul is protected under the 1992 Wildlife Conservation and Protection Act and thus the species can be hunted, owned, bred and traded only with permission[10,16]. Breeding of the red whiskered bulbul can help decrease hunting of this species in nature[13]. The traditional sex identification of red whiskered bulbul is performed by observe differences of general appearance between males and females such as body size, feather and voice[4]. Head, breast and tail of male is bigger than females[4]. Males have taller erect black crest than females[4]. Males have darker red feathers at face patch and under tail coverts while their cheeks and underparts are whiter[4]. Moreover, the melodic vocal is always found in male[4]. These observations have limitations that is investigators must be had a great expertise and experience[16]. Besides, juvenile birds are difficult to distinguish between sexes by using the traditional method[16]. These troubles are resolved by PCR for avian sex identification. A previous study in Thailand suggested P2/P8 primers targeted to the *CHD1* gene as *CHD1* primers for sex identification in red whiskered bulbuls[16]. However, the amplified products of P2/P8 primers generally difficult to distinguish in a standard agarose gel electrophoresis in females that may be misinterpreted as males[5]. Accordingly, this study focused to develop a duplex PCR for red whiskered bulbul sexing that amplified a female-specific gene and an avian species-specific gene (18S ribosomal RNA (rRNA)). The primer set for female sex identification was derived from EE0.6, which has been suggested as a successful primer set for female sexing in Passeriformes except red whiskered bulbuls[9,12]. The 18S rRNA gene is a housekeeping gene recommended as an internal control[17]. However, sex identification in red whiskered bulbuls with a combination of EE0.6 and 18S rRNA primers has not been reported in the previous literature. Thus, the present study compared the accuracy rates and the sensitivities of sex identifications in red whiskered bulbuls between the duplex PCR with using EE0.6 and 18S rRNA primers and the PCR with using *CHD1* primers (P2/P8).

Between July 2016 and October 2016, feather specimens were collected from the chests or wings of red whiskered bulbuls. Feather sampling is a noninvasive and simple technique, and feather specimens are a valuable resource for genetic studies[14,15]. The specimens consisted of 117 known-sex birds (n = 60 males and n = 57 females) from 8 breeding farms in Central (Bangkok, Nakhon Pathom and Nonthaburi province) and Western (Kanchanaburi and Phetchaburi province) of Thailand. Sex information for all bird samples was provided by the owners. The sex of the birds was determined by observation during mating to determine if the samples were parent breeding stock and produced fertilized eggs. Genomic DNA of the specimens was extracted from approximately 2 to 10 mm of the basal tip of 1 to 3 feathers[1,14,15]. To extract DNA, the QIAamp DNA Mini kit (QIAGEN, Germany) was used according to the manufacturer's protocols. All DNA samples were stored at −20°C until testing.

USP1/USP3 primers that amplify the sex-related EE0.6 gene on the W chromosome were used for sexing the red whiskered bulbul. In addition, 18S-F/R primers that amplify the avian 18S rRNA gene were used as an internal control. The primer sequences for identification and product sizes are presented in Table 1. The 18S rRNA fragment can be a positive control for amplified detection of template quality and technical errors.

Table 1. PCR primer sequences and product sizes for avian sex identification and internal control

Primer name	Sequence (5' to 3')	Target gene	PCR product size (bp)
USP1[a]	CTATGCCTACCACMTTCCTATTTGC	female-related gene	380
USP3[a]	AGCTGGAYTTCAGWSCATCTTCT		
18S-F[b]	AGCTCTTTCTCGATTCCGTG	18S rRNA gene	256
18S-R[b]	GGGTAGACACAAGCTGAGCC		
P2[c]	TCTGCATCGCTAAATCCTTT	*CHD1* gene	332 (*CHD-Z*)
P8[c]	CTCCCAAGGATGAGRAAYTG		384 (*CHD-W*)

[a]reference number 12
[b]reference number 18
[c]reference number 8

The total volume of the amplification reaction was 25 μl and consisted of 3 μl of DNA sample, 1X DreamTaq™ Buffer (Thermo Scientific, Lithuania), 0.5 μM of the USP1/USP3 primers, 0.04 μM of the 18S rRNA primers, 0.2 mM of each deoxynucleotide triphosphate (dNTP) and 0.625 U of DreamTaq™ DNA polymerase (Thermo Scientific, Lithuania), with the final volume adjusted by the addition of nuclease-free water. The amplification conditions included initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec and an extension step at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were separated in 2% agarose gel and visualized with GelRed™ (Biotium, United States) under UV illumination. Males and females showed a single band (256 bp) and a double band (256 bp and 380 bp), respectively (Fig. 1).

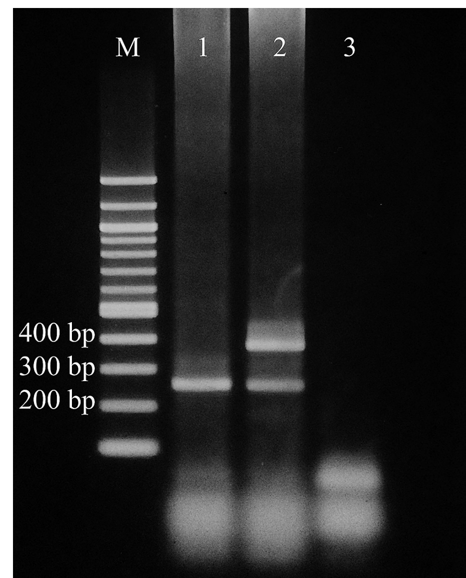

Fig. 1. Specific bands for sex identification in red whiskered bulbuls using duplex PCR with USP1/USP3 and 18S rRNA primers on 2% agarose gel. Lane M: 100 bp DNA standard ladder (New England Biolabs, United Sates), Lane 1: Male sample (256 bp of 18S rRNA), Lane 2: Female sample (380 bp of USP1/USP3 and 256 bp of 18S rRNA) and Lane 3: Blank.

To compare the sensitivity of identification, genomic DNA concentration and yield were measured by NanoDrop One (Thermo Scientific, USA). One μl of a 10-fold dilution of genomic DNA from each sex was used as templates for PCR for each primer set. Genomic DNA concentration of males and females ranged from 0.01 to 100 ng/μl and 0.008 to 80Qng/μl, respectively (Fig. 2). The primer set of P2 and P8 was designed to target *CHD1*[8] and was used for comparison. The primer sequences of the P2/P8 primers set and product sizes are showed in Table 1. The PCR reaction mixture for the P2/P8 primers contained 1X DreamTaq™ Buffer (Thermo Scientific, Lithuania), 0.5 μM of each primer, 0.2 mM of each dNTP, 0.625 U of DreamTaq™ DNA polymerase (Thermo Scientific, Lithuania) and nuclease-free water up to 25 μl. The conditions for the P2/P8 primers set was similar to the conditions for the USP1/USP3 primers set, but the primer annealing step was optimized at 52°C for 45 sec[16]. The PCR products (10 μl) of the two primer sets were compared by electrophoresis. The results of the P2/P8 primers showed that males and females display a single band (332 bp) and a double band (332 bp and

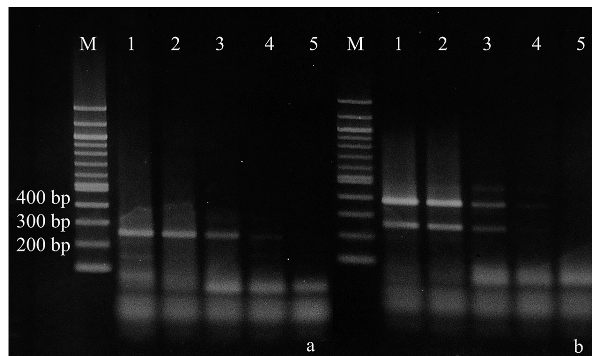

Fig. 2. Sensitivity results of sex identification by PCR with a primer combination of USP1/USP3 and 18S rRNA primers. (a) A series of male DNA concentration; Lane M: 100 bp DNA ladder, Lanes 1–5: 100, 10, 1, 0.1, and 0.01 ng/μl, respectively. (b) A series of female DNA concentration; Lane M: 100 bp DNA standard ladder, Lanes 1–5: 80, 8, 0.8, 0.08, and 0.008 ng/μl, respectively.

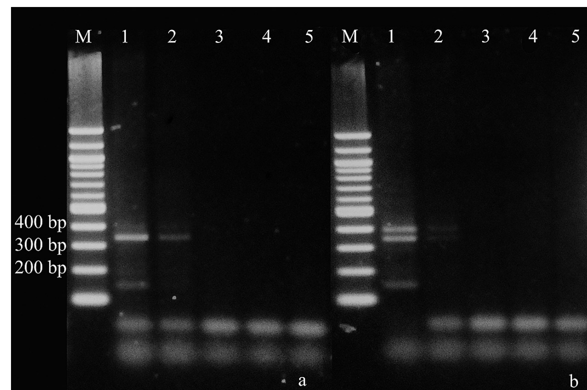

Fig. 3. Sensitivity results of avian sex identification by PCR with P2/P8 primers P2/P8. (a) A series of male DNA concentration; Lane M: 100 bp DNA standard ladder, Lanes 1–5: 100, 10, 1, 0.1, and 0.01 ng/μl, respectively. (b) A series of female DNA concentration; Lane M: 100 bp DNA ladder, Lanes 1–5: 80, 8, 0.8, 0.08, and 0.008 ng/μl, respectively.

384 bp), respectively (Fig. 3). Furthermore, the accuracy rate of this study was determined by comparing the USP1/USP3 and P2/P8 primer sets. A total of 117 DNA samples were tested for sex identification using these primer sets in parallel. DNA was amplified in a 25-μl reaction mixture containing a 3-μl DNA sample.

The sex of mature red whiskered bulbuls was determined by duplex PCR assay using genomic DNA from feather specimens. Both male and female birds showed a 256 bp fragment of the 18S rRNA gene (Fig. 1), and all female birds showed a 380 bp band of the W-specific gene (Fig. 1). In this study, no samples had a mismatch between results of the USP1/USP3 primer set and mating observations (100% accuracy). Conversely, the accuracy rate of the P2/P8 primers set was 96.6%, which is similar to the accuracy rate for Passeriformes in a previous report (96.4%)[2]. Thus, these results demonstrate highly accurate sex identification by duplex PCR of the female-specific gene and an internal control.

To test the sensitivity of the sex identification method, the USP1/USP3 primers and internal control primers were compared with the P2/P8 primers. PCR for the USP1/USP3 primers amplified positive results up to genomic DNA dilutions of 0.1 ng/μl and 0.08 ng/μl for males and females, respectively (Fig. 2); the P2/P8 primers set was detected at dilutions of 10 ng/μl and 8 ng/μl for males and females, respectively (Fig. 3). The sensitivity results indicate that sex identification by USP1/USP3 and 18S rRNA primers is more sensitive than sex identification by P2/P8 primers. Moreover, the size difference between Z- and W-specific fragments from P2/P8 amplification is approximately 50 bp. For this reason, it may be difficult to determine the difference between Z- and W-bands on agarose gel for the PCR products amplified with the P2/P8 primers. Previous recommendations suggest that products of P2/P8 amplification should be separated in polyacrylamide gel[5,11]. However, the fragments of USP1/USP3 and 18S rRNA primers in this study differed in length by 124 bp, making it easy to distinguish males and females in agarose gel.

In conclusion, the present study develops an accurate and sensitive duplex PCR assay sex identification method, which is an important tool for breeding, management and conservation of the domesticated red whiskered bulbuls.

Acknowledgements

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