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PCR-based Method for Sex Identification of Eastern Sarus Crane (*Grus antigone sharpii*): Implications for Reintroduction Programs in Thailand

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Due to human activity and a reduction in the size and quality of wetland habitats, populations of the Eastern sarus crane (*Grus antigone sharpii*) have declined dramatically across their range in Southeast Asia. Conservation efforts in Thailand have focused on reintroduction of the founders harboring the highest genetic diversity. One of the most important requirements to ensure the persistence of the reintroduced populations is a balanced sex ratio. In this study we tested three simple PCR-based methods which may be used for reliable sex identification in *G. a. sharpii.* The first method employs two combined primer sets based on a 0.6 kb *EcoRI* fragment (*EE0.6*). The second method is based on the intronic length polymorphism of the chromo-helicase DNA binding protein (*CHD*). The last technique relies on PCR-RFLP technique. The sex of six known and 24 unknown cranes were successfully identified by all three methods. These PCR-based sex identification methods are also useful for captive breeding management of *G. a. sharpii*.

Key words: *EcoRI* fragment, *Grus antigone sharpii*, PCR-based, PCR-RFLP, sex identification

INTRODUCTION

Sarus Cranes (*Grus antigone*) are nonmigratory and currently found in isolated areas of northwestern India, Southeast Asia, and Australia (Wood et al., 1996; Meine and Archibald, 1996). There are four recognized subspecies: the Indian (*G. a. antigone*), Eastern (*G. a. sharpii*), Australian (*G. a. gillae*) and the extinct Philippine sarus (*G. a. luzonica*). In the past, populations of the Eastern sarus crane have severely declined throughout its historic range in Southeast Asia. The reduction in the size and quality of remaining wetland habitats mean that remnant populations face a high risk of extinction. The Eastern sarus crane is listed as vulnerable according to the IUCN Red List of Threatened Species and is protected under the Thai Wildlife Preservation and Protection Act, B.E. 2535 (1992).

The Eastern sarus crane has been extinct in the wild in Thailand. Thailand has greatly increased its conservation efforts to recover *G. a. sharpii* in the northeastern region. The recovery plan and reintroduction program includes establishment of founder populations with high genetic diversity to enhance the evolutionary potential of the species to adapt to environmental changes. At Nakhonratchasima Zoo, captive populations have been successfully expanded from five founders to more than 100 individuals (Tanee et al., 2009). Twenty-four offspring of the third generation individuals were reintroduced to wetlands in northeastern Thailand during 2011–2012. In order to complete the effective reintroduction program, it is critical to determine sex ratio of the population prior to reintroduction process. The balance of the sex ratio in small populations is one of the stochastic events that can radically affect the size of the next generation.

To achieve a balanced sex ratio, the development of reliable sex identification techniques play a critical role in captive breeding program. Based on morphology alone, adult male cranes appear identical to females. The field researchers use a unison call to differentiate between the sexes during the breeding season (Archibald, 1976) a technique unusable when birds are not in breeding season. Therefore, reliable molecular sexing in cranes has become a vital tool for determining the sex of these sexually monomorphic birds.

Chromosomal sex determination in birds differs from that in mammals. In contrast to mammals, male birds have

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two identical sex chromosomes (ZZ), whereas females are heterogametic (ZW). Molecular sexing in birds is therefore mostly based on the detection of the W chromosome or the difference between Z and W allelic sequences (Griffiths et al., 1992; Ioh et al., 2003). The primers based on a 0.6 kb *EcoRI* fragment (*EE0.6* sequence) were designed based on the W chromosome of chickens. This combined with primers for a homolog sequence on the Z chromosome, has been used for sexing the red-crowned cranes and white-naped cranes (Itoh et al., 2001). Wen-Bin et al. (2009) successfully identified the sex of seven species of Chinese Cranes using the combined primer set based on the *EE0.6* sequences. Several other conserved genes have previously been discribed, including chromo-helicase DNA binding protein (*CHD*) gene, located on both the Z and W chromosome (Griffiths and Tiwari, 1995; Ellegren, 1996). *CHD* contains at least two introns, the length of which on the Z and W chromosome differs, enabling sex identification in several avian species (Griffith et al., 1998; Fridolfsson and Ellegren, 1999; Fridolson, 1999; Jensen et al., 2003; Ito et al., 2003; Dubiec and Zagalska-Neubauer, 2006 and Cerit and Avanus, 2007). The three *CHD*-related primer pairs used in sex identification were designed to flank each of these different introns i.e. I P2/P8 (Griffith et al., 1998), 1237L/1272H (Kahn et al., 1998), 2550F/2718R (Fridolfsson and Ellegren, 1999).

In our study, we have tested three reliable PCR-based methods using the primer sets according to the *EE0.6-Z, EE0.6-W*, *CHD-Z* and *CHD-W* sequences for sex identification in the Eastern sarus crane. These simple, cost-effective, and robust PCR techniques facilitate management of human-assisted breeding program with the purpose of reintroduction of the Eastern sarus crane.

MATERIALS AND METHODS

Sample collection and DNA extraction

We sampled three couple of captive founders (three males and three females) and 24 unknown-sex chicks due to be released into the wild in Thailand. Genomic DNA was extracted from blood samples collected from the parents and chicks using a guanidine/phenol/chloroform DNA extraction method (Nelson and Krawetz, 1992; Tavitchasri et al., 2011). In brief, blood was treated by lysis buffer, consisted of NaCl, Tris-HCl (pH 8.0), EDTA, SDS, followed by agitation for 10 minutes. The samples were treated with the combination of phenol: chloroform: (1:1), mixed, and separated. The samples were phenol-extracted, and then was DNA isopopanol precipitated and washed twice in 75% ethanol. The DNA samples were resuspended in TE, consisted of 10 mM Tris and 1 mM EDTA, pH 8.0, and kept at –20. The quality and quantity of extracted DNA was checked by 1.0% agarose gel electrophoresis with ethidium bromide staining and UV visualization.

EE0.6 *fragment amplification*

Genomic DNA was amplified using the two sets of primers: 1) AWS05/NRD4 (W-specific) and SINT-F/SINT-R (control); and 2) USP1/USP3 (W-specific) and CPE15F/CPE15R (control) (Table 1). PCR reaction was carried out in 10 μL volumes containing 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 200 μM dNTPs, 1 μM of each primer, 0.5 U Tag polymerase™ Fermentas (5 U μL^{-1}), 100 ng genomic DNA, and dH_2O to make the reaction volume up to 10 μL. The amplification reaction was performed as follows: an initial denaturation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 50 sec, annealing at 56°C for 50 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min.

CHD-Z *and* CHD-W *gene amplification*

Genomic DNA was amplified using the primer pair 2550F/2718R described by Fridolfsson & Ellegren (1999). PCRs were carried out in 10 μL volumes containing 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 200 μM dNTPs, 1 μM of each primer, 0.5 U Tag polymerase™ Fermentas® (5 U μL^{-1}), 100 ng genomic DNA, and dH_2O to make the reaction volume up to 10 μL. The amplification reaction was performed as follows: an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 5 min.

PCR-RFLP

First, genomic DNA was amplified using the P2 and P8 primer pair which target the CHD gene as described by Griffith et al. (1998). PCRs were carried out in 10 μL volumes containing 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 200 μM dNTPs, 1 μM of each primer, 0.5 U Tag polymerase™ Fermentas® (5 U μL^{-1}), 100 ng genomic

Table 1. Nucleotide sequences of primers used for sex identification in this study.

No.	Primer	Nucleotide sequence (5'-3')	Location	Source
1	AWS05	ACAGTTTGTCTGTCTCCGGGGAA	EE0.6W	Itoh et al. (1997)
	NRD4	TCAGAGCACTCTTTCCAGGAA		
2	SINT-F	TAGGCTGCAGAATACAGCAT	SPIN-Z/W	Itoh et al. (2001)
	SINT-R	TTGTGCAGTTCTAGTCCATA		
3	USP1	CTATGCCTACCACMTTCCTATTTGC	EE0.6W	Ogawa et al. (1997)
	USP3	TCGACCTRAAGTCWAGTAGAAGA		
4	CPE15F	AAGCATAGAAACAATGTGGGAC	SPIN-Z/W	Itoh et al. (2001)
	CPE15R	AACTCTGTCTGGAAGGACTT		
5	2550F	GTTACTGATTCGTCTACGAGA	CHD-Z/W	Fridolfsson and Ellegren (1999)
	2718R	ATTGAAATGATCCAGTGCTTG		
6	P2	TCTGCATCGCTAAATCCTTT	CHD-Z/W	Griffiths et al. (1998)
	P8	CTCCCAAGGATGAGRAAYTG		

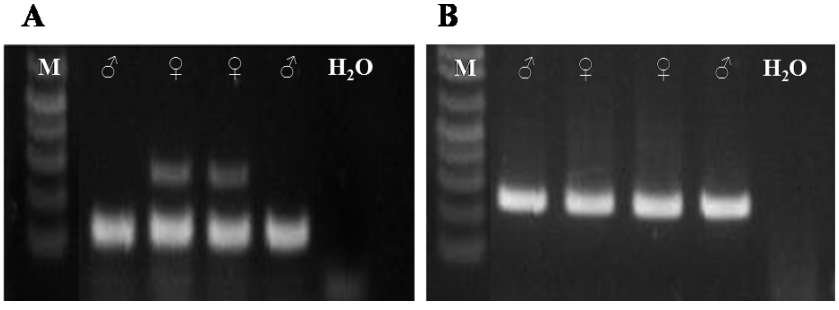

Fig. 1. PCR amplifications of EE0.6 in the Eastern sarus crane in Thailand with primer combination **(A)** SINT-F/SINT-R (*EE0.6Z* 150 bp) and AWS05/NRD4 (*EE0.6W* 300 bp); **(B)** USP1/USP3 (*EE0.6W* 370 bp) and CPE15F/CPE15R (*EE0.6Z* 250 bp). The "M" lane contains a 100 bp molecular weight size standard. Lane 1 = male, 2 = female and H_2O = negative control.

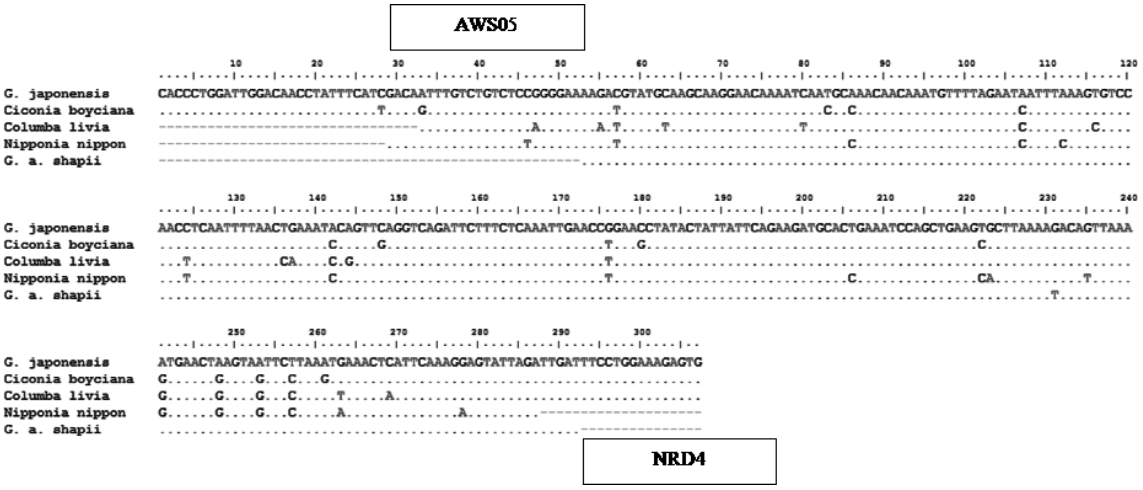

Fig. 2. Position and sequences of the primers and sequences for the *EE0.6* fragment. AWS05 and NRD4 primers are as described by Itoh et al. (1997). Dots indicate identity in the nucleotide sequences with the reference sequence. *Grus a. shapii* sequences in this study (Accession no. KC737840) were compared to *Grus japonensis* (Accession no. AY114731), *Ciconia boyciana* (Accession no. D85617), *Nipponia nooppon* (Accession no. D85616) and *Columba livia* (Accession no. AB045309).

DNA, and dH_2O to make the reaction volume up to 10 μL. The amplification reaction was performed as follows: an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 5 min. PCR products were digested with 1 U *Hae*III (Fermentas®) for 2 h at 37°C.

DNA sequence analysis

All PCR and restriction digest products were visualised by gel electrophoresis in 1.5% agarose gel and purified with a GeneJET™ Gel Extraction Kit before sequencing. *EE0.6* and *CHD* PCR products were purified and further sequenced directly. The PCR-RFLP products were first cloned with the TA-cloning kit (Invitrogen) and transformed into *E. coli* strain DH10β. Selected clones were reamplified with the same primers and then purified as for *EE0.6* and *CHD*. Purified products obtained from both males and females were sequenced by First BASE Laboratory (Malaysia). Sequences were aligned using Bioedit version 7.0.5.2 (Hall, 1999).

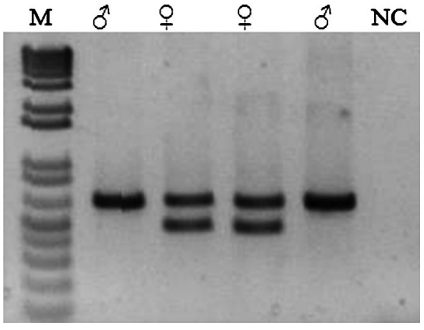

Fig. 3. Sex identification based on 2550F/2718R primers used to amplify CHD sequences. Males were identified by a single band (635 bp) and females by two bands (635 and 457 bp) on a gel. The "M" lane contains a 100 bp molecular weight size standard. Lane NC = negative control.

RESULTS

Sex identification based on the *EE0.6* sequence

Genomic DNA isolated from six known-sex (three males and three females) and 24 unknown-sex individuals were amplified for the *EE0.6* sequences using primer combinations of 1) AWS05/NRD4 (W-specific) and SINT-F/SINT-R (control); and 2) USP1/USP3 (W-specific) and CPE15F/CPE15R (control). Using AWS05/NRD4 and SINT-F/SINT-R primers, all female cranes produced two products with different length (150 and 300 bp), whereas all male cranes produced one product of the same length (150 bp). In contrast, using the USP1/USP3 and CPE15F/CPE15R primers, all males and females generated products of the same length (250 bp) because USP1/USP3 primer could not be amplified in female *G. a. sharpii* (Fig. 1).

We performed sequence analyses to confirm these results. The sequences amplified from SINT-F/SINT-R, AWS05/NRD4 and CPE15F/CPE15R primers were blast with GenBank nucleotide sequence data bases. The result showed that, the sequences amplified from SINT-F/SINT-R and CPE15F/CPE15R primers are the part of *spindlin* gene that amplifies fragments of identical size from both the Z and W chromosomes. While, the sequences generated from AWS05/NRD4 were 93–97% identical to chromosome-W repetitive genome sequences of *Grus japonensis* (AY114731), *Ciconia boyciana* (D85617), *Nipponia nooppon* (AB045309) and *Columba livia* (EJ598049) (Fig. 2). *EE0.6* sequences of one founder female crane have been deposited in GenBank (accession number KC737840).

The result from this method showed that combination of AWS05/NRD4 and SINT-F/SINT-R correctly identified the sex of all six known-sex samples (100% success rate of sex identification), while the second combination, USP1/USP3 and CPE15F/CPE15R, failed to identify sex of all sample (0% success rate).

Sex identification based on *CHD* intronic length

The results of the sex identification method based on intronic length polymorphism are shown in Fig. 3. The 2550F/2718R primers successfully identified sex of all known-sex samples (100% success rate). A single band (620 bp) was observed in males and two bands (620 bp and 450 bp) in females. These results were confirmed using

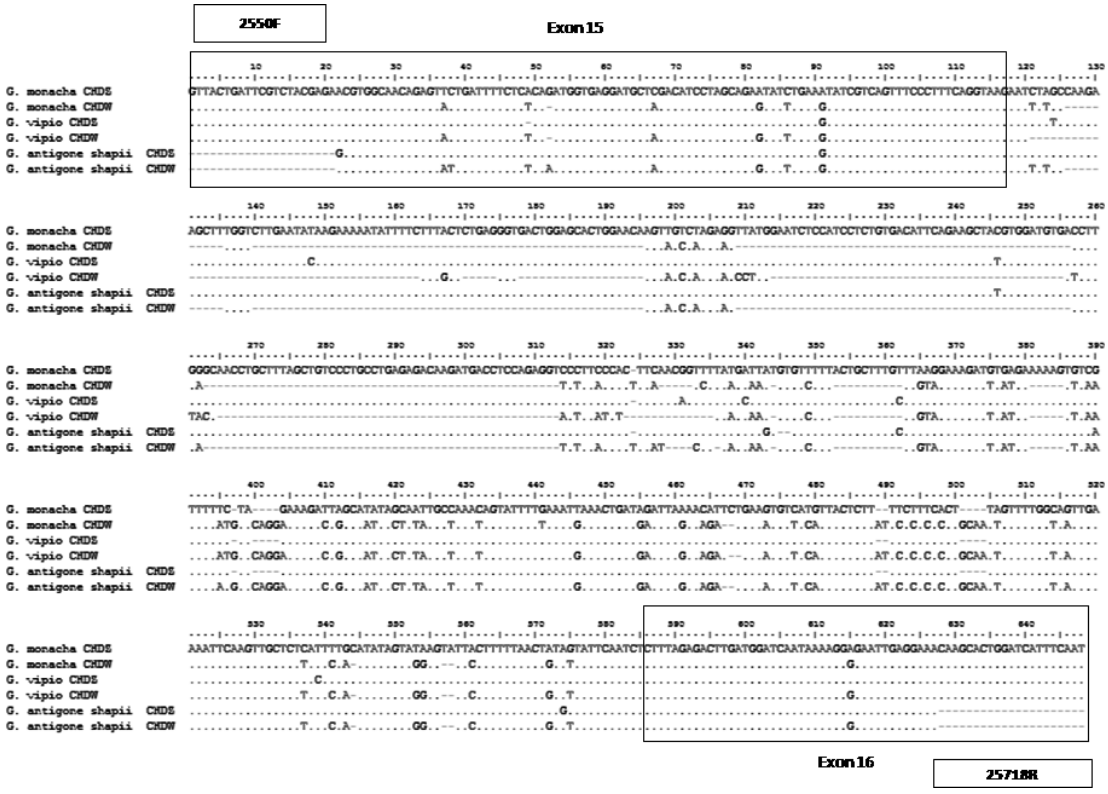

Fig. 4. Position and sequences of primers and sequences of *CHD* partial exon 15, intron 15, and partial exon 16. The illustrated sequences included exon 15, the poorly conserved intron 15 and exon 16. The 2550F and 2718R primers were described by Fridolfsson and Ellegren (1999). Dots indicate identity in the nucleotide sequences with the reference sequence. The *CHDZ* and *CHDW* sequences of *G. a. shapii* in this study (Accession nos. KC676707 and KC676708 respectively) were compared with *Grus monacha* (Accession nos. EU014905 and EU014912) and *Grus vipio* (Accession nos. EU014906 and EU014913).

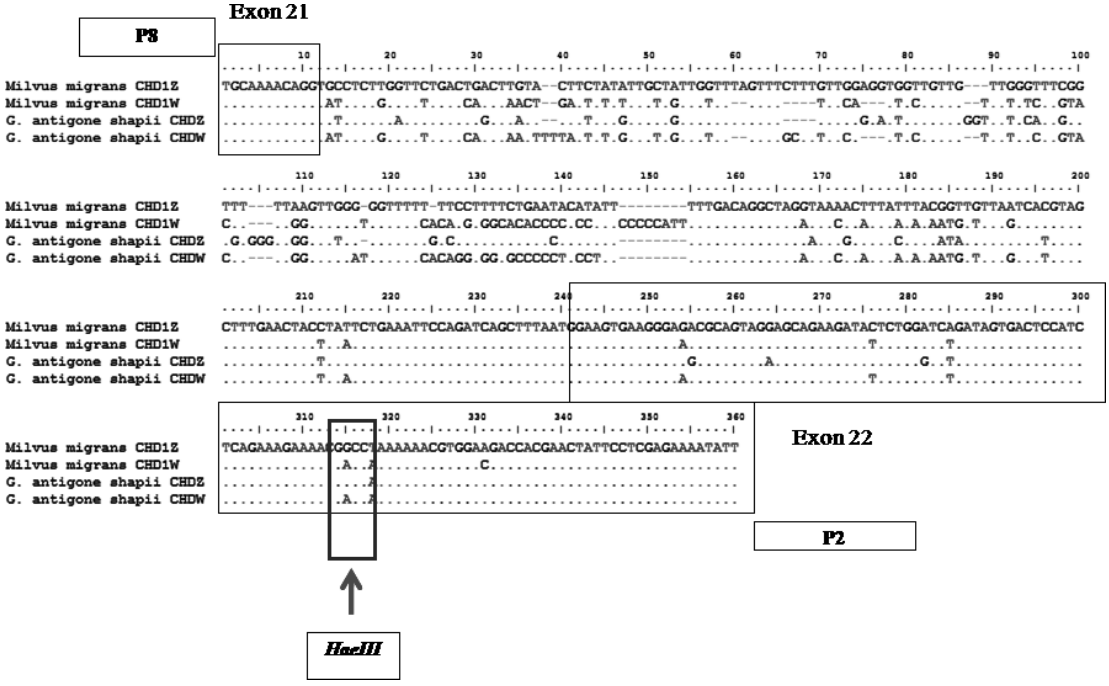

Fig. 5. Position and sequences of primers and sequences of *CHD* partial exon 21, intron 21, and partial exon 22. The illustrated sequences include exon 21, the poorly conserved intron 21 and exon 22. P2 and P8 primers were described by Griffith et al. (1998). Dots indicate identity in the nucleotide sequences with the reference sequence. The *CHDZ* and *CHDW* sequences of *G. a. shapii* in this study (Accession nos. KC676707 and KC676708 respectively) compare with *Milvus migrans* (Accession nos. AB096142 and AB096141). *CHD-Z* sequence contains a *HaeIII* restriction enzyme cleavage site (red arrow).

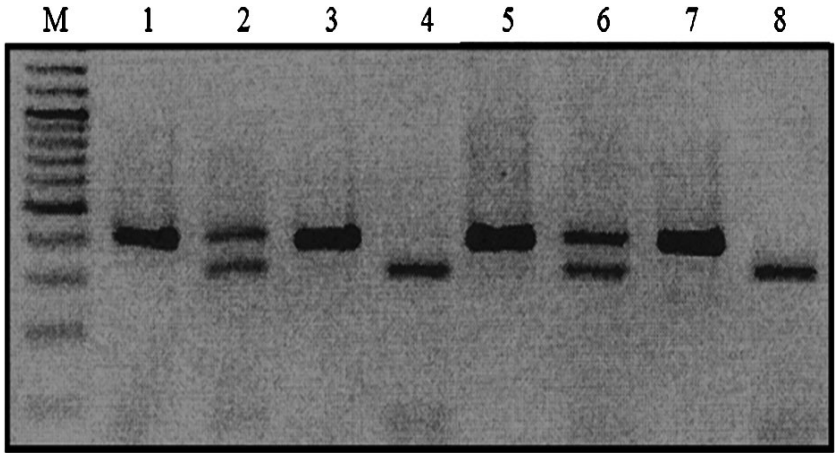

Fig. 6. PCR products and corresponding *HaeIII* enzyme digested from males and females of the Eastern sarus crane (*G. a. sharpii*). Lanes nos. 1, 2, 5, and 6 represent the female samples; the lane nos. 3, 4, 7 and 8 represent male samples. Lane nos. 1, 3, 5, and 7 show the 385 bp PCR products generated using primer P8/P2. Lane nos. 2, 4, 6, and 8 show the results from the *HaeIII* enzyme digestion. The "M" lane contains a 100 bp molecular weight size standard.

sequence analyses (Fig. 4). The *CHDZ* and *CHDW* sequences of *G. a. sharpii* were compared with *Grus monacha* and *Grus vipio*. Sequence comparison showed that these amplified fragments include exon 15, intron 15 and exon 16. Likewise *G. monacha* and *G. vipio*, *G. a. sharpii* contained the different size and sequence between intron 15 of CHD-Z and CHD-W. This difference in size caused two distinct bands on gel. *CHD-Z* and *CHD-W* sequences of one founder female crane have been deposited in GenBank (accession number KC676707 and KC676708).

Sex identification based on PCR-RFLP

The *CHD* product amplified by using primers P2 and P8 could not distinguish between the sexes as only a single band (385 bp) was observed on gel for both males and females. The sequence analysis by BLAST revealed that the target fragment mostly related to partial exon 20, intron 20 and partial exon 21 of *CHD* gene of the black kite (*Milvus migrans*) (accession number AB091642). The undetectable difference of two products on gel is due to a small 2-bp difference between *CHDZ* and *CHDW* fragments (Fig. 5). Moreover, we found that exon 21 of *CHD-Z* contains a *HaeIII* restriction enzyme cleavage site which produces three bands in females and two bands in males (Fig. 6). This result also indicated the applicability of sex identification based on PCR-RFLP in *G. a. sharpii*.

Sex identification of 24 unknown-sex cranes for reintroduction

The AWS05/NRD4 and SINT-F/SINT-R, 2550F/2728R and P2/P8 primers were tested in 24 unknown-sex cranes. The sexes of the 24 cranes were identified according to band patterns. Sex identification by using three methods showed the same results that the sexes of 24 cranes including 16 males and eight females (data not shown).

DISCUSSION

Sex identification is a considerable element for captive management and selection of the effective population for reintroduction program in several species, especially the sexual monomorphic birds such as cranes. Molecular techniques have been increasingly applied for sex identification in bird species. Herein, we compared three different primer sets to assess the efficiency of sexing and determine a suitable protocol for application in *G. a. sharpii*.

The first method is based on amplification of the *EE0.6* sequence which is a female-specific gene sequence on the W chromosome. In this study, we achieved a 100% success rate of sex identification of *G. a. sharpii* using combined AWS05/NRD4 (W-specific) and SINT-F/SINT-R (control) primer sets. However, PCRs based on the USP1/USP3 (W-specific) and CPE15F/CPE15R (control) primers failed to discriminate between the sexes. The failure of sex identification by USP1/USP3 in *G. a. sharpii*, might be caused by the sequence variation between species.

The second and third primer sets relate to *CHD* gene which has been used for sex identification in a wide range of bird species (Fridolson and Ellegren, 1999; Jensen et al., 2003; Ito et al., 2003; Dubiec and Zagalska-Neubauer, 2006; Cerit and Avanus, 2007). In the presented study, we also successfully identified sex in *G. a. sharpii* using PCR-based intronic length polymorphism (2550F/2718F), PCR band presence (AWS05/NRD4) and restriction enzyme (P2/P8) digestion. For the intronic length based method, we found that the difference in size between *CHD-Z* and *CHD-W* fragment is 170 bp, therefore they could be identified by agarose gel. For the other birds, the difference in size between *CHD-Z* and *CHD-W* fragment amplified with the same primer set ranges from 150 to 250 bp (Fridolfsson and Ellengren, 1999; Jensen et al., 2003; Dubiec and Zagalska-Neubauer, 2006 and Cerit and Avanus, 2007).

The fragments amplified with the P2 and P8 primer set could not be used for sex identification in *G. a. sharpii*. The failure was due to the small size difference (2 bp) between *CHD-Z* and *CHD-W* fragment. In other avian, the difference in size ranged from 10 to 80 bp (Griffiths, 1998; Jensen et al., 2003; Dubiec and Zagalska-Neubauer, 2006 and Cerit and Avanus, 2007). This size difference can be detected using polyacrylamide gel which provides better resolution (Dubiec and Zagalska-Neubauer, 2006). However, in some birds, such as auklets (Dawson et al., 2001) and Accipitridae (Ito et al., 2003), both polyacrylamide and agarose gels fail to detect any size differences between *CHD-Z* and *CHD-W* fragments.

Although the fragments did not have any detectable differences in size, males can be differentiated from females by a *HaeIII* restriction site in the *CHD-Z* exon 22 (P2/P8 amplification), which is not present in the *CHD-W* exon 22. Griffiths et al. (1996) successfully sex identified 13 species of bird by using enzyme *HaeIII* cut PCR product. Väli and Elts (2002) amplified an intron of *CHD* from Woodcock blood by using P2/P8 primer set. Then restriction enzyme *HaeIII*

were applied a cleavage site in the W chromosome. As a result, sex of the Eurasian woodcock could be identified: three bands in females and single band in males (Väli and Elts, 2002). Contrastingly, we found a cleavage site of this enzyme on the Z chromosome of *G. a. sharpii*. Therefore, this method successfully identified the sex of *G. a. sharpii* based on three bands in females and two bands in male.

Grus a. sharpii has been extinct in the wild in Thailand. The reintroduction program is a hopeful scenario for re-establishing the population in natural sources. Not only the appropriate number of reintroduced cranes but also the balanced sex ratio promotes the sustainable conservation. The presented application might be a significant component in sex determination and selection tool for balancing reintroduction. This study suggested that 24 reintroduced cranes were 16 males and eight females, which is unbalance sex ratio. Therefore, male cranes should be more released in the next reintroduction to compensate male-biased ratio.

In conclusion, sex identification of *G. a. sharpii* in Thailand was successfully performed by using a combination of primers based on *EE0.6* sequence, the 2550F and 2718R primer set based on *CHD* gene intronic length pholymorphism, and the P2 and P8 primer set with restriction enzyme based on *CHD* gene. The 2550F and 2718R primer set was the most simple and robust method.

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