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Sex Determination by PCR-RFLP in the Oriental White Stork *Ciconia boyciana*

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Jae-Ik Han, Hye-Jin Jang, Seokhwan Cheong, Sukyung Kim, Shi-Ryong Park, and Ki-Jeong Na (2009) Sex determination by PCR-RFLP in the Oriental White Stork *Ciconia boyciana*. *Zoological Studies* **48**(5): 619-624. The Oriental White Stork (*Ciconia boyciana*) is difficult to sex based on phenotype alone, and thus alternative methods capable of producing an unambiguous result are necessary. Griffiths' polymerase chain reaction (PCR) method based on intronic length polymorphism in the chromo-helicase DNA-binding (CHD) protein gene is widely used in birds; however, we were able to show that it is not appropriate for sexing this species of stork, as it resulted in a single band of approximately 380 bp in both males and females on a 2% agarose gel. However, Griffiths' sexing method can be applied to many birds, and we made applicable that method to this stork using a restriction enzyme. Sequencing of the male's CHD-Z PCR product revealed a 377 bp fragment with several specific restriction sites, including *Hae*III. A restriction fragment length polymorphism (RFLP) analysis of the female PCR product with *Hae*III and subsequent sequencing showed that the 380 bp band was, in fact, composed of 2 fragments, one of 377 bp (CHD-Z) and a larger fragment of 384 bp (CHD-W), specific to females. A number of restriction enzymes specific for CHD-W were also identified. Consequently, we demonstrate that a combination of PCR together with RFLP analysis (PCR-RFLP) is a simple and rapid method for sexing this stork species. http://zoolstud.sinica.edu.tw/Journals/48.5/619.pdf

Key words: Oriental White Stork, Chromo-helicase-DNA-binding (CHD) protein, PCR, Restriction fragment length polymorphism (RFLP), *Hae*III.

The Oriental White Stork *Ciconia boyciana* belongs to the family Ciconiidea and the order Ciconiformes. This stork is listed as an endangered species in the *Red List of Threatened Species* of the International Union for Conservation of Nature (IUCN) (Birdlife International 2001) and has also become extinct as a permanent resident in South Korea. At present, this stork mainly inhabits the Heilong River and Wusuli River basins along the border between Russia and China (Smirenski 1991). The Korea Institute of Oriental White Stork Rehabilitation Research (KIOWSRR) at Korea National University of Education, Cheongwon, Korea and the Laboratory of Veterinary Laboratory Medicine at Chungbuk National University, Cheongju, Korea are undertaking a combined effort to reintroduce them as breeding birds in Korea. As a first step, routine health screening and artificial breeding, following sex determination based on phenotypic and behavioral features, have already been introduced. However, sexing based on phenotypic and behavioral characteristics is difficult in young storks, since these birds show little sexual dimorphism; hence, more accurate and definitive

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sexing techniques are required.

There are 3 major approaches to sexing avian species. The 1st approach is at the cellular level using techniques such as karyotyping and flow cytometry to identify sex chromosomes. The 2nd approach is at the molecular level and involves techniques such as polymerase chain reaction/ random amplified polymorphic DNA (PCR-RAPD), amplified fragment length polymorphism (AFLP), amplification of microsatellite loci, restriction fragment length polymorphism (RFLP), and simple PCR using primer pairs based on intronic size variations, which can be used to differentiate between the chromo-helicase DNA-binding (CHD) proteins, CHD-Z and CHD-W (Griffiths' et al. 1996, Cavallo et al. 1997, Lessells and Mateman 1998, Griffiths' and Orr 1999, Nesje and Roed 2000, Cheng et al. 2006). The 3rd approach is based on behavioral features, for example the acoustic structure of the clatter produced by male and female birds (Eda-Fujiwara et al. 2004).

Based on the fact that male birds have 2 identical sex chromosomes (ZZ), whereas females are heterogametic (ZW), Itoh et al. (1997) used a W chromosome-specific primer pair to differentiate between males and females using a PCR. However, as this method does not amplify a product for the male stork, all reactions must include a positive control in order to preclude a false-negative result. Griffiths' et al. (1998) suggested a method using a single set of PCR primers to amplify homologous sections of 2 conserved CHD genes, located on the sex chromosomes of all birds. The CHD-Z gene is found in both sexes, while the CHD-W gene is unique to females. The primers amplify homologous sections of the 2 genes, incorporating introns, which usually vary in size. This method is very simple, is definitive for almost all birds, and is therefore widely used.

Some bird species, however, have no intronic size variations or only very small variations under normal conditions, and thus several modifications of Griffiths' method were reported (Ito et al. 2003, Sacchi et al. 2004, An et al. 2007, Cerit et al. 2007, Reddy et al. 2007). In this report we demonstrate that Griffiths' method is not suitable for sexing this stork species due to the difficulty in separating 2 very similarly sized PCR fragments from each of the CHD-W and CHD-Z genes on a 2% agarose gel. In order to resolve this problem, we sequenced the CHD-Z and CHD-W gene fragments that were amplified by the primer pair reported by Griffiths' et al. (1998), and, in addition, we investigated the use of restriction enzymes in conjunction with the PCR method for sexing the Oriental White Stork.

MATERIALS AND METHODS

DNA extraction

Genomic DNA was extracted from heparinized blood taken from 4 male and 4 female Oriental White Storks at the KIOWSRR facility, using a genomic DNA purification kit (Promega, Madison, WI, USA). All samples were pre-sexed based on phenotypic and behavioral characteristics, and females were only included if they had previously produced chicks.

PCR-RFLP and sequencing

A segment of the CHD gene was amplified using the primer pair P2 and P8 as described by Griffiths' et al. (1998). PCR amplification was carried out in a total volume of 50 μl. Reaction conditions were as described by Griffiths' et al. (1998), and the PCR products were visualized on a 2% agarose gel. The amplicons from a male and female stork were then bi-directionally sequenced with the P2/P8 pair using an ABI Prism™ BigDye™ terminator cycle sequencing ready reaction kit vers. 3.1 (Applied Biosystems, Foster City, CA, USA).

The sequenced product from a male stork's CHD-Z gene was analyzed for restriction sites using the CLC sequence viewer vers. 4.6.2 (CLC bio, Aarhus, Denmark). Amplicons from both male and female storks were subsequently digested using the enzyme, *Hae*III (Takara Bio, Shiga, Japan), at 37°C for 1 h. The restriction fragments were separated by electrophoresis on a 2% agarose gel for 50 min at 100 V and stained with ethidium bromide for visualization under UV light. Each restriction fragment was then extracted from the gel using a MEGA spin™ Agarose Gel Extraction Kit (iNtRON Biotechnology, Sungnam, Korea) and was bi-directionally sequenced with the P2/P8 primer pair by the same method as for the PCR products.

RESULTS

The results of the PCR based on Griffiths'

method are shown in figure 1A. A single band of approximately 380 bp was visible in each of the male and female samples with no obvious size variation between the 2 fragments.

Sequencing of the male's PCR product, amplified by the primers P2 and P8, showed that the amplified fragment of the CHD-Z gene consisted of a 377 bp product containing an intron of 194 nucleotides in length (Fig. 2). However, sequencing of the female's product showed a double peak indicating the presence of PCR fragments of similar size but of different sequences.

After sequencing, an analysis by the CLC sequence viewer identified several restriction enzymes specific for CHD-Z (Table 1). Among them, *Hae*III, which cuts between nucleotides 312 and 313, and should result in 2 restriction fragments of 312 and 65 bp in the male, was used for restriction of the purified PCR products using a MEGA spin™ Agarose Gel Extraction Kit (iNtRON Biotechnology) from both sexes.

Electrophoresis of the male's *Hae*III restriction digested product revealed only the 312 bp PCR product on a 2% gel. The 65 bp fragment was too faint to see. The female stork had 2 bands of approximately 320 and 380 bp (Fig. 1B). The larger band in the female (presumed to be from the CHD-W gene) appeared to be the same size as the unrestricted fragment. The sequencing of the digested gel fragments indicated that the similarly sized bands in the male and female were the 312 bp restriction products of the CHD-Z gene, while the female's larger 384 bp band was the

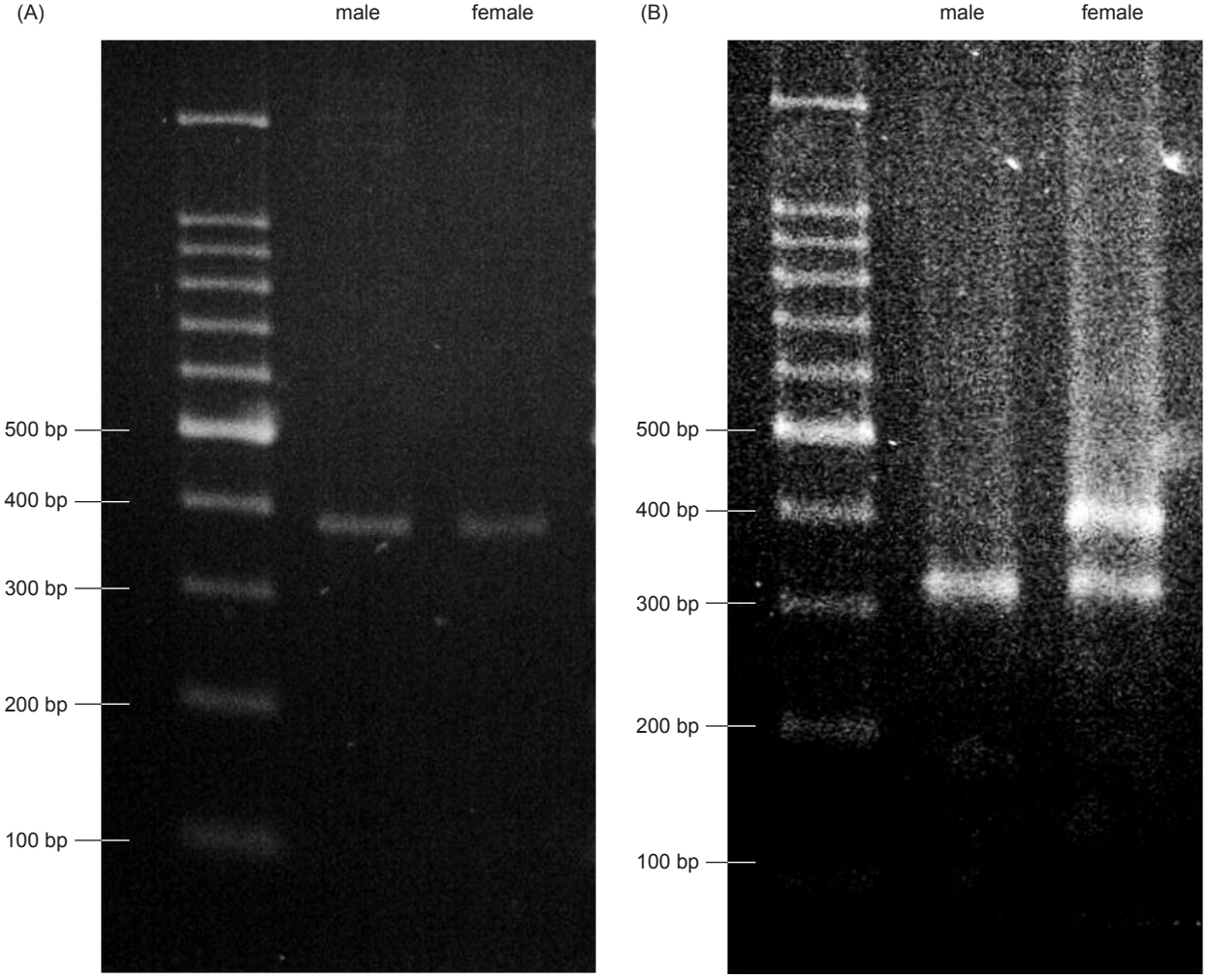

Fig. 1. (A) PCR products of CHD genes in male and female Oriental White Storks amplified by the P2 and P8 primers on a 2% agarose gel with a 100 bp ladder. (B) RFLP analysis with *Hae*III of the CHD genes in male and female storks, with a 100 bp ladder. Male storks showed only 1 band (of approximately 320 bp), whereas female storks showed 2 bands (of approximately 380 and 320 bp).

product of the CHD-W gene. In both sexes, the 65 bp fragment from the CHD-Z gene was too faint to visualize on the 2% gel. Sequencing of the female's larger fragment showed that the intron of the CHD-W gene was 201 bp (Fig. 2), which was only 7 nucleotides longer than the 194 bp of the CHD-Z intron. The introns showed only 64% sequence homology while the exons showed 94% sequence homology.

In the analysis using the CLC sequence viewer, several restriction enzymes specific for CHD-W were also identified (Table 1).

The partial CHD-Z and CHD-W nucleotide sequences of *C. boyciana* were deposited in GenBank under the accession numbers EU871825 and EU871826, respectively.

DISCUSSION

Griffiths' et al. (1998) reported that the P2/P8 PCR method based on intronic length polymorphism is capable for differentiating the sex of a number of bird species. This sexing method is simple and rapid, so we decided to use it to sex *C. boyciana*. Unfortunately, Griffiths' P2/P8 PCR primers produced similarly sized bands of approximately 380 bp in both males

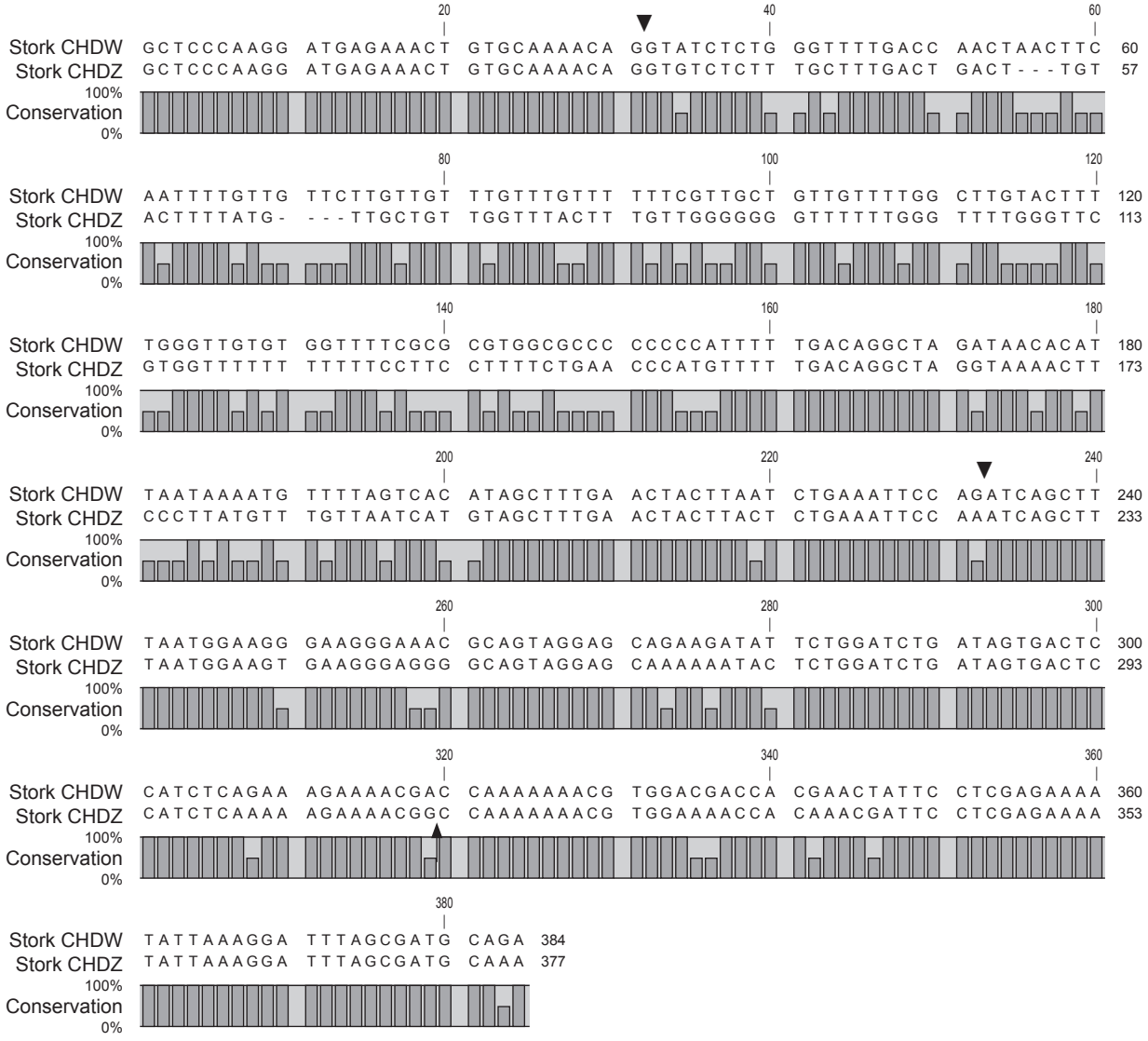

Fig. 2. Sequence alignment of the partial exon, intron, and partial exon of the CHD-Z and CHD-W genes. The 2 arrowheads indicate the boundaries of the intronic sequence. The arrow indicates the CHD-Z-specific *Hae*III restriction site between nucleotides 312 and 313. Sequence alignment was performed using CLC sequence viewer vers. 4.6.2.

and females. The male band was confirmed by direct sequencing to be a 377 bp fragment of the CHD-Z gene. The female PCR product was finally resolved as being composed of 2 different fragments of almost identical size. Digestion with *Hae*III and direct sequencing of the restriction products confirmed the female's apparently single PCR product as a 377 bp fragment of the CHD-Z gene and a 384 bp fragment of CHD-W gene. On the basis of intronic size variation between CHD-Z and CHD-W, this result indicated that the P2/P8 primer pair is capable of amplifying both CHD genes in this stork species. However, since the intron varies by only 7 nucleotides, this method alone cannot differentiate between the 2 CHD genes. Similar observations were reported in some other birds including several vultures in the order Accipitriformes and the Cockatiel Parakeet (*Nymphicus hollandicus*) in the order Psittaciformes (Ito et al. 2003, Cerit and Avanus 2007, Reddy et al. 2007).

Direct sequencing of the P2/P8 PCR product in the male Oriental White Stork revealed a number of restriction sites in the stork's CHD-Z gene, among which *Hae*III is a restriction enzyme specific for CHD-Z in the Great Tit (*Parus major*) and Short-toed Eagle (*Circaetus gallicus*) (Griffiths' et al. 1996, Sacchi et al. 2004). Thus, *Hae*III was selected and tested, and found to be specific for CHD-Z. It is therefore applicable for use in sexing this stork speices. We also identified a number of restriction enzymes specific for CHD-W following sequencing of the female Oriental White Stork (Table 1). Consequently, we identified that PCR-RFLP using a restriction enzyme specific for CHD-Z is a simple and rapid method for sexing this stork species.

Murata et al. (1998) reported a multiplex PCR method for sexing the Oriental White Stork. This method is simple and rapid; however, this is only

Table 1. List of restriction enzymes specific to the partial CHD-Z and CHD-W gene sequences of *Ciconia boyciana*

	Recognition site	Recognition site pattern (5' to 3')	Restriction enzymes
Partial CHD-Z (377 bp)	33rd	GTCTC	*Alw*26I, *Bsm*AI, *Bso*MAI, *Bst*MAI
	145th, 190th	CATG	*Fat*I, *Cvi*AII, *Fae*I, *Hin*1II, *Hpy*CH4I, *Hsp*92II, *Nla*III
	247th	ACNNNNNCTCC	*Bsa*XI
	308th	ACGGC	*Bce*AI, *Bce*fI
	310th	GGCC	*Ban*AI, *Bec*AII, *Bim*19II, *Bme*361I, *Bse*QI, *Bsh*FI, *Bsh*I, *Bsp*ANI, *Bsp*BRI, *Bsp*KI, *Bsp*RI, *Bsu*RI, *Bfe*I, *Clt*I, *Dsa*II, *Esa*BC4I, *Fru*DI, *Hae*III[a], *Mch*AII, *Mfo*AI, *Ngo*PII, *Nsp*LKI, *Pa*II, *Pch*133I, *Pfl*KI, *Pho*I, *Pla*I, *Pru*2I, *Sbv*I, *Sua*I
	310th	YGGCCR	*Aco*I, *Bfi*89I, *Cfr*I, *Eae*I, *Eco*HK31I
	310th	CGGCCR	*Gdi*II
	338th	GAWTC	*Pfe*I, *Tfi*I
Partial CHD-W (384 bp)	48th	CCANNNNNNNTC	*Cje*PI
	143rd	GGYRCC	*Acc*B1I, *Ban*I, *Bbv*BI, *Bsh*NI, *Eco*64I, *Hgi*CI, *Hgi*HI, *Msp*B4I, *Pfa*AI
	143rd	RGCGCY	*Acc*B2I, *Bme*142I, *Bsp*143II, *Hae*II, *Lpn*I
	143rd	GRCGYC	*Acy*I, *Aha*II, *Aso*II, *Ast*WI, *Asu*III, *Bbi*II, *Bsa*HI, *Hgi*DI, *Hgi*GI, *Hgi*HII, *Hgi*I, *Hin*1I, *Hsp*92I, *Msp*17I, *Pam*II
	143rd	GGNNCC	*Asp*NI, *Bmi*I, *Bsp*LI, *Nla*IV
	143rd	GGCGCC	*Bbe*I, *Bins*II, *Din*I, *Eco*78I, *Ege*I, *Ehe*I, *Kas*I, *Mch*I, *Mly*113I, *Mar*I, *Nda*I, *Nun*II, *Sse*AI
	178th	ATTAAT	*Ase*I, *Asn*I, *Bpo*AI, *Psh*BI, *Sru*4DI, *Vsp*I
	272th	GAANNNNTTC	*Asp*700I, *Bbv*AI, *Mro*XI, *Pdm*I, *Xmn*I
	272th	GAAGA	*Mbo*II, *Ncu*I
	303rd	CTNAG	*Hpy*F3I
	303rd	CTCAG	*Bse*MII, *Bsp*CNI

[a]Restriction enzyme used in this study.

useful for this stork species. Griffiths' method and the *Hae*III, restriction enzyme have a broad coverage (Sacchi et al. 2004, Reddy et al. 2007), and thus our PCR-RFLP based on the CHD gene sequence is more useful for institutes where several species of birds need to be examined.

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