Sexing the Oriental White Stork *Ciconia boyciana* by PCR Using a Single Plucked Feather as a Source of DNA

Koichi Murata¹, Yuichiro Itoh², Akira Ogawa² and Shigeki Mizuno³

¹Kobe Municipal Oji Zoo, 3-1 Oji-cho, Nada-ku, Kobe 657, Japan
²Laboratory of Molecular Biology, Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981, Japan

A small amount of DNA extracted from a single plucked contour feather from four captive young Oriental White Storks *Ciconia boyciana*, a sexually monomorphic bird, was used for gender determination by the method of polymerase chain reaction (PCR) based on its sex chromosome-specific DNA sequences, the 0.6-kb *Xba I-Hind III* fragment (*XH0.6*) on the W chromosome and the region homologous to the *XH0.6* sequence (*XH0.6-RSM*) on the Z chromosome. After the agarose gel electrophoresis of the PCR products, two bands which correspond to the W and Z chromosome-specific fragments were observed from the female DNA. By contrast only one Z chromosome-specific fragment was observed from the male DNA. These results were consistent with those obtained by Southern blot hybridization using the DNA from red blood cells and the discriminant analysis of body measurements. This PCR method using a single feather is easy, fast, accurate, and also relatively stress-less to the birds which are not used to being handled for sampling. It will become one of useful tools in the captive propagation program and reintroduction plan for the *C. boyciana*, a special natural monument of Japan.

**Key Words:** *Ciconia boyciana*, feather, Oriental White Stork, PCR, sex identification, W chromosome-specific DNA

It is important to identify the gender of a bird not only for field research but for captive breeding, and especially for captive propagation plan of the endangered species. However, many bird species appear sexually monomorphic (Ritchie et al. 1994, Hildebrandt et al. 1995, Griffiths et al. 1996). Even in dimorphic species, it is often difficult to identify the gender of their juveniles by the external features.

To date, various techniques for sex determination on the monomorphic birds have been developed such as chromosome analysis by tissue culture, hormone assay to compare estrogen conjugate/testosterone levels in fecal samples, laparoscopy or ultrasonography for direct visualization of gonads, and multi-parameter discriminant functions of body measurements (Bercovitz et al. 1978, Bush et al. 1978, Murata et al. 1987, 1989, 1997, Ritchie et al. 1994, Hildebrandt et al. 1995). These methods, however, have some demerits; they are laborious, less accurate, risky, or they need special veterinary skills. Furthermore, it should be considered to minimize the stress at the time of handling for sexing or sampling.

Recently, molecular techniques for sexing birds have been developed. Flow cytometry is applied to measure the difference of DNA contents in red blood cells between male and female birds (Nakamura et al. 1990). Some W chromosome-
specific DNA probes have enabled to identify the sex of chickens, geese, wrens, and others (Tone et al. 1984, Quinn et al. 1990, Rabenold et al. 1991). The disadvantages of these methods are that they need time, cost or special facility for using radioisotopes.

Comparing with these methods, polymerase chain reaction (PCR) to amplify W chromosome-specific DNA is more useful because of its speed, ease and reliability (Sabo et al. 1994, Griffiths et al. 1996, Itoh et al. 1997). Furthermore, PCR method doesn’t require a large quantity of DNA and it is possible to amplify the target sequence with a very small amount of tissue samples, which means noninvasive to the birds, especially to chicks, at the time of collecting samples. Therefore, a single plucked feather was recommended as the source of DNA for genetic studies in birds (Taberlet & Bouvet 1991). It may be possible to collect feathers from nestling without exerting bad influence on nursing.

We describe here the method of sex determination of the Oriental White Stork *Ciconia boyciana*, one of the special natural monuments in Japan, using a single plucked contour feather by PCR. The primer sets used in this paper were the one to amplify a W chromosome-specific DNA sequence of *Ciconia boyciana* and the other to amplify a sequence on the Z chromosome, which serves as an internal positive control to ascertain amplification of sex specific DNA sequences from such a small tissue sample.

**MATERIALS AND METHODS**

Four captive young Oriental White Storks of six months old, which were captive born and kept in Toyooka Oriental White Stork Breeding Center (Nojo, Toyooka, Hyogo 668, Japan) were examined.

A single contour feather, around 9 cm long (Fig. 1), is plucked from the breast.
Sexing Oriental White Stork by PCR

of each bird by hand wearing clean plastic gloves. Five ml of heparinized blood sample was also collected from the brachial vein at the same time. The sex of the storks were determined by Southern blot hybridization with the W chromosome-specific probe on genomic DNA prepared from the blood sample (Itoh et al. 1997, Ogawa et al. 1997) and discriminant analysis of body measurements (Murata et al. 1989, 1997). The sampled feathers were sealed in plastic bags separately and stored in a refrigerator at 5°C.

DNA was extracted from a single feather following the method of Walsh et al. (1991) and Murata & Masuda (1996) with a slight modification. Briefly, about 5mm long calamus from the proximal tip (inferior umbilicus) was immersed and cut into small pieces in 200 µl of 5% (w/v) Chelex® 100 (Bio-Rad), incubated at 56°C for at least 10 hours, and then boiled in a water-bath for 8 min. After centrifuging at 12,000rpm for 5 min, the supernatant was used as a DNA extract. Primers used for amplifying the W chromosome-specific DNA sequence, 0.6-kb Xba I-HindIII fragment (XH0.6), of Oriental White Stork were 5'-CACCCTGGATTGGACAACCTATTTTC-3' (forward) and 5'-CACTCTTTCCAGGAAATCAA-3' (reverse) as reported by Itoh et al. (1997). For an internal positive control, the XH0.6-related sequence in male (XH0.6RSM), supposed to be present on Z chromosome, was PCR-amplified using a primer set of 5'-TACAGATAAAAAGTGCAGTCATTGGC-3' (forward) and 5'-TCTTTGAGGACACACTCAGAGGCAC-3' (reverse). The detailed information on these primers is given elsewhere (Itoh et al. 1997, Ogawa et al. 1997).

PCR was performed in a 50 µl mixture containing 2 µl of the DNA extract, 200 µM each of dNTP, 50 pmoles each of primers, 0.25 units of Taq DNA polymerase (Perkin Elmer), and 5 µl of 10x buffer (Perkin Elmer). The PCR conditions in a DNA thermal cycler (TSR-300, IWAKI Glass, Inc.) were as follows: initial DNA denaturation at 95°C for 180 sec followed by 40 cycles of 95°C 80 sec for denaturation; 59°C, 90 sec for annealing; and 72°C, 60 sec for extension, and lastly 72°C, 540 sec for final elongation. The 10 µl of PCR products were electrophoresed on a 2% agarose gel (NuSieve 3 : 1, FMC® Bio Products) in 1x TBE buffer at 100V for 30 min, and stained with ethidium bromide.

RESULTS AND DISCUSSION

The sex of four young Oriental White Storks, 2 males and 2 females, determined with the present PCR method, coincided with the one identified by the method of Southern blot hybridization (Itoh et al. 1997, Ogawa et al. 1997) and the discriminant analysis of body measurements (Murata et al. 1989, 1997). The expected sized band of about 580 base pairs (bp) which represents the Z chromosome-specific DNA sequence was amplified from a male feather extract (Fig. 2, lane 3). In contrast, the bands of about 580bp and about 300bp, which represents the Z and W chromosome-specific DNA sequences, respectively, were amplified from a female (Fig. 2, lane 4).

Identification of the sex by PCR using universal primers which amplify the sex determining region Y (SRY) has been possible in some mammals (Murata & Masuda 1996). In birds, there have been no report about universal primers like SRY for amplifying the sex determining region conserved in all the bird species.

Griffiths et al. (1996) recently discovered a gene for chromodomain-helicase-
Fig. 2. Z chromosome-specific DNA fragment (about 580 base pairs, bp) and W chromosome-specific DNA fragment (about 300 bp) amplified by PCR from the DNA extracted from a single plucked contour feather of a male or a female Oriental White Stork *Ciconia boyciana* using sex chromosome-specific primer sets. lane 1, DNA marker (φ x174/Hae III); lane 2, blank; lane 3, male; lane 4, female and lane 5, no template DNA.

DNA-binding protein W-linked (CHD-W) and a non-W-linked CHD gene (CHD-NW) in the Great Tit *Parus major*, and they suggested both genes could be used for the gender determination in a wide variety of bird species. Their method, however, needs digestion with a restriction enzyme to distinguish fragments derived from the male and female. Furthermore, the primers amplify a human DNA sequence, and thus, precaution against the contamination is needed. For amplification of the CHD-W gene in Collared Flycatchers *Ficedula albicollis*, Ellegren (1996) designed conditions of PCR amplification with three primers. As our primer sets are specific to *C. boyciana*, there needs little caution on the human DNA contamination during examination, and they can amplify both Z and W chromosome-specific DNA fragments of different sizes by a single PCR reaction with two sets of primers and without using any restriction enzyme.

It has not been checked whether XH0.6 and XH0.6-RSM sequences can be used for sexing other species of Ciconiiformes, except for the European White Stork *C. ciconia* whose gender could be identified with this method (Murata, unpublished data). The XH0.6 and XH0.6-RSM were cloned as EE0.6-related sequences from Oriental White Stork (Itoh *et al.* 1997). EE0.6 is a non-repetitive sequence cloned from the W chromosome of chicken *Gallus gallus domesticus*, and it was demonstrated that EE0.6 could be used as a universal probe for identification of genetic
Sexes of Carinatae birds by Southern blotting (Ogawa et al. 1997). Previous (Itoh et al. 1997) and present results suggest that the sex of other sexually monomorphic birds may also be identified by PCR using primer sets designed from the EE0.6-related sequences on the W and Z chromosomes of the species.

The wild population of C. boyciana in Japan became extinct in 1971, but the long-term effort of captive breeding is progressing, and moreover the reintroduction is planned at Toyooka, where the last wild Oriental White Stork in Japan was caught and placed in captivity (Murata 1997). One of the key points for the success of captive breeding of C. boyciana is to start pairing at young ages of birds, because this species is aggressive and it is quite difficult to make a good pair bond in adult ages in captivity (King 1992, Murata et al. 1993). We believe that this rapid, accurate, and relatively stress-less method for sexing young Oriental White Storks will be a powerful tool for both of the captive propagation and the reintroduction program for the endangered species.

We thank the staff of the board of education in Hyogo Prefectural and Toyooka City Government, and Mr. K. Matsushima and Mr. M. Sato of the Toyooka Oriental White Stork Breeding Center. We also thank Dr. R. Masuda of Hokkaido University for supplying Chelex®-100 and useful comments on the DNA extraction with it.

LITERATURE CITED


Murata, K., Miyashita, M., Nagase, K., Komiya, T. & Matsushima, K., 1989. Sex


(Received 26 August 1997; Accepted 27 October 1997)
和文抄録  Japanese Summary of Papers in English

コウノトリ Ciconia boyciana の羽根１本からの抽出 DNA を用いた PCR 法による性鑑別

（Sexing the Oriental White Stork Ciconia boyciana by PCR Using a Single Plucked Feather as a Source of DNA. 46: 157-162）

村田浩一・伊藤裕一郎・小川 見・水野重樹

1 神戸市立王子動物園, 〒657 神戸市灘区王子町 3-1
2 東北大学農学部応用生物化学科分子生物学講座, 〒981 仙台市青葉区堤通西宮町 1-1

外形からは性判別が困難なコウノトリ（ニホンコウノトリ）Ciconia boyciana より採取した胸部の正羽１本から DNA を抽出し、ポリメラーゼチェーンリアクション（PCR）法での性判別を試みた。対象個体は豊岡コウノトリ保護増殖センターで繁殖した若鳥４羽で、性別はあらかじめ血液から抽出した DNA の Southern blot hybridization および体側値の判別分析で鑑定した。DNA は長さ約5 mm の羽柄１本（Fig. 1）から Chelex®-100 を用いて抽出した。ニワトリ W 染色体上の DNA 配列（EE0.6）と相同性を有するコウノトリ W 染色体上の配列（XH0.6）、およびそれと相同性を持ちコウノトリの Z 染色体上にあると考えられる配列（XH0.6-RSM）をプライマーとして PCR を行った結果、目的とする DNA 領域を增幅することができた。增幅産物のアガロースゲル電気泳動では、雌に Z および W 染色体上の特異的配列を示す 2 本のバンドが認められたが、雄には Z 染色体上の特異的配列を示す 1 本のバンドしか出現しなかった（Fig. 2）。本法は、簡易、迅速、正確であり、試料採取時の保定期間でいない鳥に対して与える影響は比較的小さいものである。このため、国の特別天然記念物であるコウノトリの飼育下繁殖および野生復帰計画にとっては、有用な技術のひとつとなる。

タンチョウ（Grus japonensis）の越冬給餌場の利用

（Feeding-Station Use of Wintering Japanese Cranes Grus japonensis in Eastern Hokkaido in Relation to Age and Social Status. 46: 163-174）

胡 東宇・正宣宏之・福田弘巳