Zoological Studies **45**(1): 104-113 (2006)

Zoological Studies

Sex Identification of the Black-faced Spoonbill (*Platalea minor*)

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(Accepted October 14, 2005)

Yeong-Hsiang Cheng, Tzong-Fu Kuo, Der-Nan Lee, and Ching-Feng Weng (2006) Sex identification of the Black-faced Spoonbill (*Platalea minor*). *Zoological Studies* **45**(1): 104-113. The Black-faced Spoonbill (BFS), *Platalea minor*, endemic to East Asia, is a well-known species listed as globally "critically endangered". It is difficult to recognize the gender of a BFS by its appearance; this can make it extremely difficult to implement human-assisted breeding programs, as well as evolutionary and ecological studies. In this work, therefore, a molecular approach was used to determine the sex of the endangered monomorphic BFS, as opposed to a morphological or histological approach. In Dec. 2002, an outbreak of *Clostridium botulinum* toxin type C killed many birds overwintering in the Tseng-Wen estuary, southwestern Taiwan, and this provided the opportunity to obtain muscle samples for DNA extraction. The polymerase chain reaction (PCR) with a single set of primers was employed to amplify a fragment in both the chromobox-helicase-DNA-binding genes (CHD)-W and CHD-Z; after electrophoresis, the products showed a single band in males, with females having a 2nd distinctive band. The PCR products for the CHD-Z and -W genes were 658 and 464 bp, respectively. The nucleotide sequences of these bands were further confirmed after cloning and sequencing. The nucleotide sequences of the CHD-Z and -W genes were 83% homologous. When using morphological and histological examination results for comparison, 26 birds (14 males and 12 females; sex ratio, 1.16) were correctly sexed using our test. This study is the first time that the gender of the Black-faced Spoonbill has been identified using the PCR technique; there is great potential for applying this tool for further investigations into the ecology and reproductive behavior of this species. http://zoolstud.sinica.edu.tw/Journals/45.1/104.pdf

Key words: Sex ratio, Chromobox-helicase-DNA-binding gene, Black-faced Spoonbill, *Platalea minor*, PCR.

The Black-faced Spoonbill (BFS; *Platalea minor*) is one of the 50 rarest birds of the world; it reached a dramatic low in population numbers in the 1990s, resulting from reductions in the species range or in the quality of its habitat or both (Groombridge 1993, IUCN 1997). In an international winter census in 2003, an increase in population was seen, but still only 1069 individuals were counted worldwide. Migration routes are poorly known with few recorded sightings in South Korea, Japan, and China. Three major wintering sites are in Taiwan, Hong Kong, and Vietnam, with minor sites in China, South Korea, and Japan (BirdLife Asia Council 1995).

Previously, most studies regarding the Black-faced Spoonbill focused on the population and its distribution (Chong et al. 1996, Kim et al. 1998, Lee et al. 1999 2001), migration routes (Chong et al. 1997, BirdLife International Asia Council 1999), and breeding biology (Chong et al. 1996, Lee et al. 2001). Information related to the sex ratio and gender identification, however, is rare. In Dec. 2002, botulinum toxicosis broke out due to ingestion of dead fish in the Tseng-Wen estuary, in Tainan, southwestern Taiwan. This tragedy caused the death of 73 Black-faced Spoonbills, or about 6.83% of the global population, and demonstrated how easily the fate of this highly threatened species could be driven by ecological stochasticity (Lee et al. 2003). However, this unfortu-

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nate incident also offered a great opportunity to explore these invaluable body samples and gain insights into potential environmental factors which might threaten this endangered species in the future.

In birds, females are heterogametic (ZW), while males are homogametic (ZZ). DNA sequencing provides us with a versatile way of discriminating male from female birds. Unfortunately, the selection of a suitable sex-linked marker is difficult. The obvious source is the W sex chromosome, as this occurs in the female (ZW) and not in the male (ZZ). However, similar to the human Y chromosome, it is small and offers a disproportionate amount of junk DNA (Stefos and Arrighi 1971). Such sequences evolve rapidly, even between closely related species, and therefore provide sex-linked markers of a limited range (Lessells and Mateman 1998). The 1st avian W chromosome gene that was discovered was the chromobox-helicase-DNA-binding (CHD-W) gene (Griffiths and Tiwari 1995), which is well conserved and linked to the W-chromosome in a range of bird species. Afterwards, more sex-determining related genes in the W- and Z-chromosomes of avian species were investigated and reviewed (Ellegren 2000). Recently, 2 major approaches have been developed to identify avian gender. One is based on the cellular level using, for example, karyotyping and flow cytometric methods (Nakamura et al. 1990, De Vita et al. 1994); the other uses molecular methods, such as PCR-RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), amplification of microsatellite loci, and RFLP (restriction fragment length polymorphism) (Griffiths et al. 1996, Lessels and Mateman 1998, Griffiths and Orr 1999, Nesje and Roed 2000). It has been shown that a single set of PCR primers can be used to sex birds throughout the Class Aves, with the exception of ratites (Griffiths and Tiwari 1996, Griffiths et al. 1996). These primers amplify homologous parts of the CHD-W gene, and the related CHD-Z gene, at the same time (Griffiths and Korn 1997). Because the CHD-Z gene occurs in both sexes, it will always be amplified, ensuring that the PCR reaction works. However, as the 2 CHD products are of the same size, Griffiths et al. (1996) used a restriction enzyme to selectively cut a fragment from the CHD-Z version before gel electrophoresis. Upon examination, females had 2 bands and males only 1. It seems that a single, simple PCR-RFLP technique based on both genes can be used to identify gender in a wide variety of birds. Molecular sexing has been shown to be a rapid and uninvasive procedure. Survival of most endangered birds may depend on breeding programs where sex identification plays an important role (Bermudez-Humaran et al. 2002). In this study, we used PCR amplification of the CHD-Z and -W genes in an application to determine the gender of monomorphic endangered Black-faced Spoonbills, in contrast to morphological and histological examinations.

MATERIALS AND METHODS

Tissue collection and metrology of the gonads

Tissue samples (n = 26, 0.2 g in weight) were taken from the breast muscle of dead BFSs, and samples were sealed in plastic bags and frozen at -20°C before DNA extraction. The gonads of all sexually mature birds were metrologically measured.

DNA extraction

The DNA extraction procedure was carried out according to Cheng et al. (2003). Briefly, 50 mg of muscle tissue was minced and digested overnight at 37°C, with 400 μl digestion buffer (0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), and 5 mM EDTA in 0.5% SDS containing 100 μg/ml proteinase K). After the addition of 1000 μl PCI (phenol: chloroform: isoamyl alcohol, 25: 24: 1), the vial was left to stand for 10 min then centrifuged at 10,000 xg for 10 min. Total DNA in the aqueous phase was precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold 100% ethanol at 4°C for 30 min. DNA pellets were obtained by centrifugation (13,000 xg for 10 min), washed twice each with 70% and 100% ethanol, air-dried, and then resuspended in 25 μl TE buffer. The nucleic acid concentration was measured by spectrophotometry at 260 nm.

PCR program

One microliter of the supernatant (template DNA) was added to 9 μl of the PCR mixture, containing 50 mM Tris-HCl (pH 8.5), 20 mM KCl, 3 mM $MgCl_2$, 0.05% bovine serum albumin, 0.2 mM of dNTP, and 0.25 μM of each primer (5'-GTTACTGATTCGTCTA CGAGA-3' and 5'-ATT-GAAATGATCCAGGCTT-3'; published by Hornfeldt et al. 2000) and applied to 0.2 U of Taq DNA poly-

merase. The PCR program had a hot start: preheating to 95°C for 7 min, then denaturing at 95°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min for a total of 30 cycles, with a final extension at 72°C for 7 min. Electrophoresis was performed on a 2.5% agarose gel containing 0.5 μg/ml ethidium bromide.

Cloning and sequencing

PCR fragments were cloned into a PCR vector (yT and A) using a TOPO cloning kit. Sequences of clones were determined by the dye terminator technique on an automatic sequence model 373A instrument, following the manufacturer's recommendations (Applied Biosystems, Foster city, CA, USA), and DNA alignments were performed using GCG Sequence Analysis Software (Genetic Computer Group, Madison, WI, USA). The nucleotide sequences of CHD-Z and -W genes were submitted to the GenBank database (accession nos.: AY464013 and AY464014).

RESULTS

The gonad metrological data are shown in Table 1. The results indicated that testis weight was in the range of 0.226-0.262 g, with the left side being heavier than the right. The testis had a long oval-like shape, with its length being greater than its width. Ovary weight (1.750 g) and length (1.764 cm) were also determined. The PCR products for the Z and the W chromosomes were 658 and 464 bp, respectively. The male had a single band (of 658 bp), while the female had 2 different bands (of 658 and 464 bp) (Fig. 1).

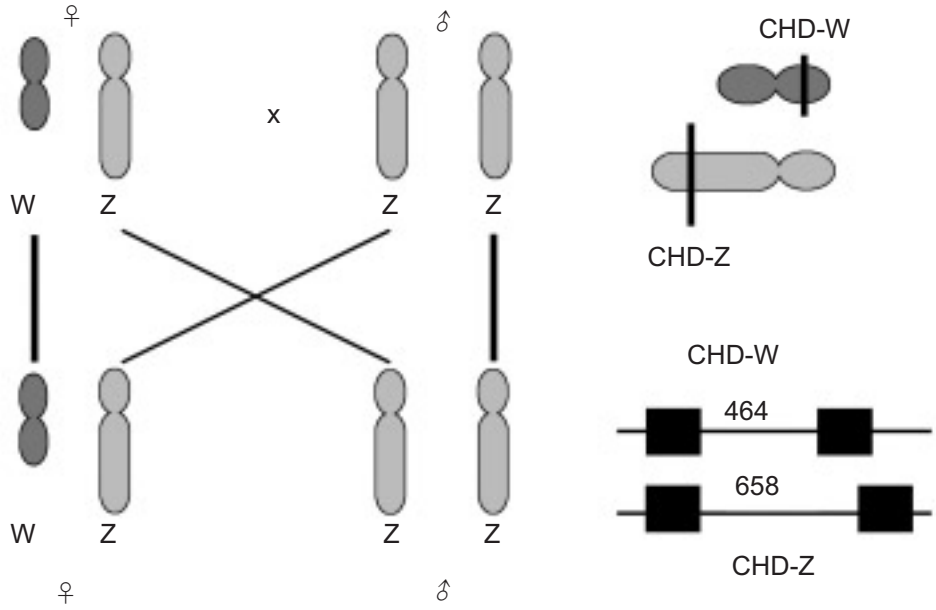

Fig. 1. Illustration of the locations of the CHD-W and -Z genes in the Black-faced Spoonbill, and lengths of the PCR products.

Table 1. Sexual gonad metrology of the Black-faced Spoonbill, *Platalea minor*

Male	Testis weight (g)	Testis length (cm)	Testis width (cm)
	L 0.262 ± 0.317 R 0.226 ± 0.275	0.857 ± 0.184	0.600 ± 0.185
Female	Ovary weight (g)[a]	Ovary length (cm)	
	1.750 ± 1.250	1.764 ± 0.495	

[a]Ovary data were only measured on the left side. Data are presented as the mean ± SD.

The partial nucleotide sequences of the BFS CHD-Z and -W genes are shown in figure 2a; after sequence alignment, they were 83% homologous. Alignment of the BFS's CHD-Z gene with the Japanese cormorant's (*Phalacrocorax capillatus*) CHD1Z gene is shown in figure 2b. Alignment of the BFS CHD-W gene with the *P. capillatus* CHD1W gene is shown in figure 2c. The nucleotide sequences of the spoonbill CHD-W and -Z genes showed high similarity with the *P. capillatus* CHD1Z and CHD1W genes, respectively. In comparing the BFS CHD-Z and -W genes with other published sequences, the homology was 98% in the CHD-Z (120 nucleotides [nt]) (Fig. 3a) and 99% in the CHD-W (103 nt) (Fig. 3b), respectively. This indicates that the nucleotide sequences of avian CHD-Z and -W are well conserved. The results obtained by the anatomic sex gland examinations of the 26 birds (with a male/female ratio of 14/12; sex ratio, 1.16) completely matched the sex-identification by CHD-Z and -W. This confirmed that gender determination, using a molecular approach, is indeed reliable in this species.

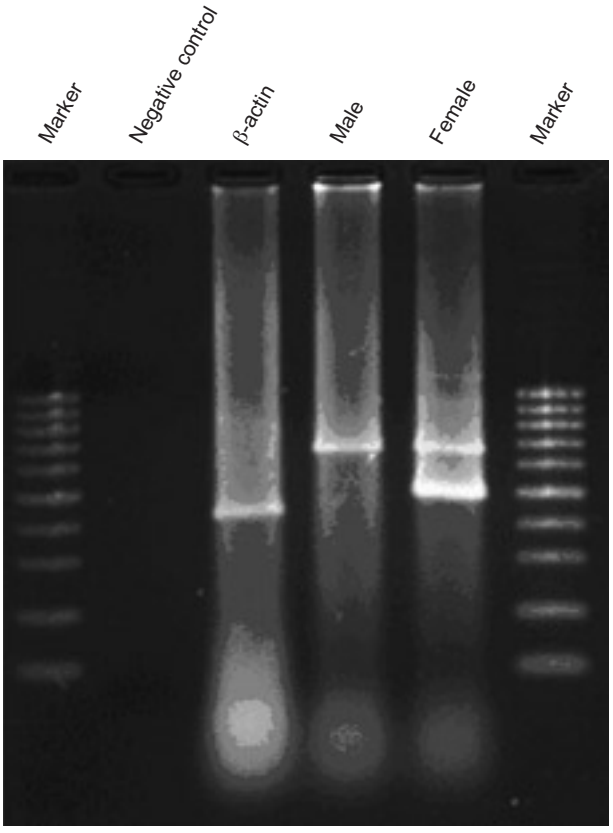

Fig. 2. Results of molecular sexing in Black-faced Spoonbills by PCR after 2.5% agarose gel separation. Marker, 100-bp ladder marker.

DISCUSSION

The present study applied a new PCR approach to determine the gender of the endangered, monomorphic Black-faced Spoonbill, as opposed to the more-commonly used morphological or histological examination methods. After the cloning and sequencing of the BFS CHD-Z and -W genes, it was obvious that these partial nucleotide sequences, CHD-Z and -W, might be good markers for identifying the sex of some other avian species.

Recently, 2 major approaches have been developed to identify avian gender. One is based at the cellular level, using, for example, karyotyping and flow cytometric methods (Nakamura et al. 1990, De Vita et al. 1994); the other uses a molecular genetic method, such as PCR-RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), amplification of microsatellite loci, and RFLP (restriction fragment length polymorphism) using restriction enzyme digestion (Griffiths et al. 1996, Lessels and Mateman 1998, Griffiths and Orr 1999, Nesje and Roed 2000). In this study, we applied a molecular approach, focusing on the 2 CHD genes, to determine the gender of BFSs. The test employed a PCR with primers that were annealed to the conserved exonic region and across an intron in both the CHD-W and -Z genes (Fig. 4). Because the intron is a non-coding region and is not well conserved, the nucleotide lengths usually differ between these 2 genes. The simplest protocol available for molecular sexing with CHD takes advantage of intronic length differences that may exist in the 2 copies (Ellegren and Sheldon 1997). The PCR, with intron-flanking primers, yielded a particular length for the CHD-W product and another length for the CHD-Z product. There are some PCR-based methods of sexing domestic poultry, using only W chromosome-specific primers, which revealed some shortcomings when no products could be observed after PCR (Saitoh et al. 1989, Clinton 1994); furthermore, it can be a laborious and tedious task when performed on large numbers of samples, using 2 different primer pairs (D'Costa and Petitte 1998). This PCR tool has the advantages over uniplex PCR of sparing reagents and requiring less sample preparation time. The primer pairs of P2/P8, designed by Griffiths et al. (1998), were tested. However, the

PCR female gender CHD-W and -Z products were too close, and therefore observing a difference between the bands, using agarose gel analysis, was too difficult (data not shown). Another possible solution is the use of 8% denaturing polyacrylamide gels which provide sufficient resolution to discriminate between these 2 products.

The metrology of the sex gonads in the BFS, including weight and other measurements, showed that during Dec. 2002, the birds were not in breeding season, as the gonads were in a resting phase. The breeding season of this bird is reported as being from late May to early Aug. at Tok-do I. (38°45'N, 124°58'E), N. Korea (Chong et al. 1996). Almost all BFSs have arrived in Taiwan by Dec. After the long migration, finding emergency energy and nutrient requirements is crucial; the majority of the birds, therefore, catch any available live or dead fish for food, becoming susceptible to a contaminated environment and botulism toxicosis exposure. Thus the conservation of the Black-faced Spoonbill, *Platalea minor*, a critically endan-

Fig. 3 (A)

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W ATTGAAATGATCCAGTGCTTGTTTCCTCAATTCCCCTTTTATTGATCCATCAAGTCTCTA 60
Z ATTGAAATGATCCAGTGCTTGTTTCCTCAATTCCCCTTTTATTGATCCATCAAGTCTCTA 60
  ************************************************************

W AAGAAATTGAATAATACAGTTAAAAAGGAA--CTCCTACTATATTCTAA-TTGGAGCAAC 117
Z AAGAGATTGAATACTACAGTTAAAAAGTAATGCTTATACTACATTCAAAATCAGAGAAGC 120
  **** ******** ************ **  **  ***** **** ** *  *** * *

W TTGAATTCTCAATTGCCAAAACAATGGGGGGGGGGGGGGGGGGAAATAAGAGTAATGTAA 177
Z CTGAATTCTCACCTGCTAAAACTGTTATGTGAAG--------GAAAAAAAAGTAATGCAT 172
   **********  *** *****  *   * *  *        **** ** ******* *

W CACTTCAT----CTTTCTAATTCATCAGTTTCATTTCAAAATACTATTCAGCATATAGTA 233
Z CACTTCATGTAATTTTTTAATTCACCAGTTTAATTTCAAAATACTACATAGGATATAGCG 232
  ********     *** ******* ****** **************   ** ******

W ATTGCCAGTCTTT-------------------TCCTGTACAT----------------- 256
Z ATTGCCACTCTTTCTAGAAAAAGAACGCTTTTTCTTATACCTTTTCTTAAGCAAAGTGGT 292
  ******* *****                  ** * *** *

W ---AAAATTAAA-----------------------------------TTTTCATAAATCT 278
Z AAAGAAATCAGAAAACAGTTGAAGTTGGAAGGGACCTCTGGAGGTCATCTTGACTAACTC 352
     **** * *                                     * ** *  **

W TTCTAC----------------AAA-----------AAGGACA-----------CTTTT- 299
Z CCCTGCTCAGGCAGGGGCGGCTAAAGCAGGTTGTCCAAGGTCATGTCCAGGTGGCTTTTG 412
  ** *               ***           **** **           *****

W ------------------------------------------------------------
Z AATGTCACAGAGAATGGAGATGCAATAACCTCTGAACAACTTGTTCCAGTGCTCTGTCAC 472


W -CTTGAGATAAAGCGG---------------TAAAGATCAAGGCTTCTTGGCTACTACC 342
Z CCTCAGAGTAAAGAAAAGATTTTTCTTATATCCAAAAATAAAAACTTCTTGGCTACTACC 532
   **     *****                  *** ** **  ****************

W AGCAAAATTCTTACCTGAAAGGGAAACTGACGATACTTCAAATACTCTGCTAGGATGTCT 402
Z ACCAAGATTCTTACCTGAAAGGGAAACTGACGATACTTCAGATATTCTGCTAGGATGTCC 592
  * *** ********************************** *** **************

W AGCATCCTCACCATCTGAGAGAAAATCAGTACTCTGTTGCCACGTTCTCGTAGACGAATC 462
Z AGCATCCTCACCATCTGTGAGAAAATCAGAACTCTGTTGCCACGTTCTCGTAGACGAATC 652
  ****************** *********** ******************************

W AG---- 464
Z AGTAAC 658
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gered species endemic to East Asia, is becoming more critical. In order to implement human-assisted breeding programs for such endangered species, it is also crucial to be able to determine the gender of these monomorphic birds. In the meantime, non-invasive and reliable methods must be developed to further study this endangered species, such as collecting and separating cells from feces. This study is the 1st evidence to identify the gender of the Black-faced Spoonbill using the PCR molecular tool; the PCR approach has great potential for ecological and reproductive-behavior research applications, particularly for future breeding programs.

Acknowledgments: We wish to acknowledge Mr. S. C. Liu (Duck Research Center, Ilan, Taiwan) for his invaluable suggestions and technical assistance, and also to express our deep compassion for the fate of the birds used in this study.

Fig. 3 (B)

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          10        20        30        40        50        60
....|....|....|....|....|....|....|....|....|....|....|....|
S ATTGAAAATGATCCAGTGCTTGTTTCCTCAATTCCCCTTTTATTGATCCATCAAGTCTCTA
P                          G.TACTG....GT..ACG.G A.CGTGG...CAGAG..

          70        80        90       100       110       120
....|....|....|....|....|....|....|....|....|....|....|....|
S AAGAAATTGAATAATACAGTTAAAAAGGAACTCCTACTA TATTCTAATTGGAGCAACTT
P CT..TT..CTC.C.G.TG..G.GG.T.CT.GA.A.C...GC.GAG..T...A.AT.T.G.

          130       140       150       160       170       180
....|....|....|....|....|....|....|....|....|....|....|....|
S GAATTCTCAATTGCCAAAACAATGGGGGGGGGGGGGGGGGGAAATAAGAGTAATGTAACA
P C.G..TC.CT..CAGGT..G...TTT.CT..TA.TA.CCAAG..GCCTT.ATC.T..C..

          190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|
S CTTCATCTTTCTAATTCATCAGTTTCATTTCAAAATACTATTCAGCATATAGTAATTGCC
P ...T.....AAG..G.      GTG.C.T...TGT.GA.AG...T.TG.A.GTT.....T.A

          250       260       270       280       290       300
....|....|....|....|....|....|....|....|....|....|....|....|
S AGTCTTTTCCTGTACATAAAATTAAATTTTCATAAATCTTTCTACAAAAAGGACACTTTT
P C..A.AGGAAGAG..TGGC.....CTAAA.GC.....AG.AT.TTG...T.A.A     C.

          310       320       330       340       350       360
....|....|....|....|....|....|....|....|....|....|....|....|
S CTTGAGATAAAGCGGTAAAGATCAAGGCTTCTTGGCTACTACCAGCAAAATTCTTACCTG
P GA...AT.GG.AA.A.G...TGTT.CAT.C..CTTA.T.C...CC.C..  ..G..TTGGC

          370       380       390       400       410       420
....|....|....|....|....|....|....|....|....|....|....|....|
S AAAGGGAAACTGACGATACTTCAAATACTCTGCTAGGATGTCTAGCATCCTCACCATCTG
P ..TT.AG..T.C.A.T.G..CTG.T..GAA.A  ...T.G.AG.TC.T.TT.A..TG.A.T

          430       440       450       460       470       480
....|....|....|....|....|....|....|....|....|....|....|....|
S AGAGAAAATCAGTACTCTGTTGCCACGTTC TCGTAGACGAATCAG
P GTTC..TC..TT..GAGAC...ATGGA.CAA.AAA..GG....TGAGGAAACAAGCACTG

          490
....|....|..
S
P GATCATTTCAAT
```

Fig. 3 (C)

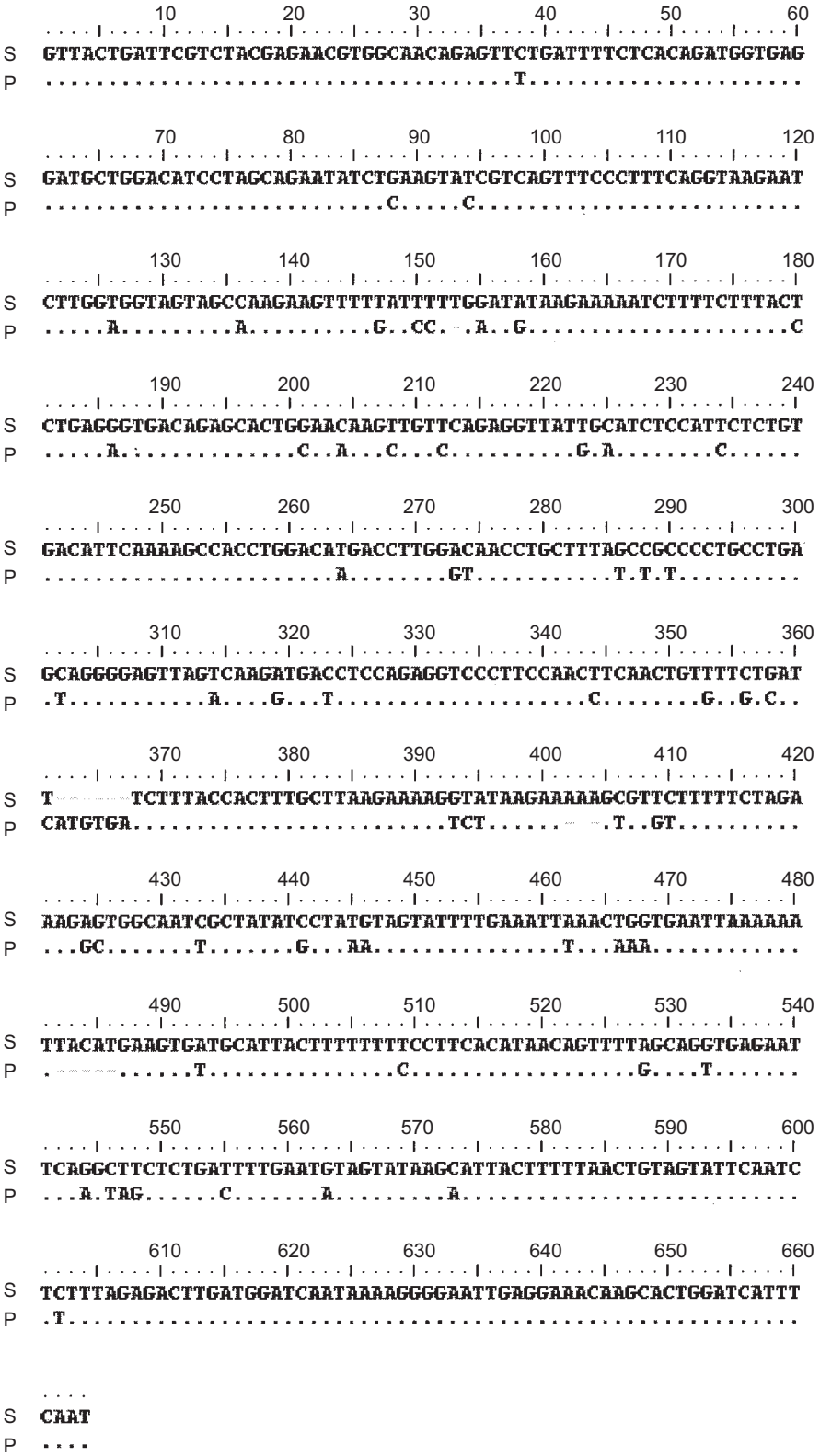

Fig. 3. (A) Alignment of CHD-Z and -W partial nucleotide sequences in the Black-faced Spoonbill. An asterisk (*) indicates an identical sequence between CHD-Z and -W. (B) Alignment of Black-faced Spoonbill CHD-Z (S) with the *Phalacrocorax capillatus* CHD1Z (P) gene. (C) Alignment of Black-faced Spoonbill CHD-W (S) with the *Phalacrocorax capillatus* CHD1W (P) gene.

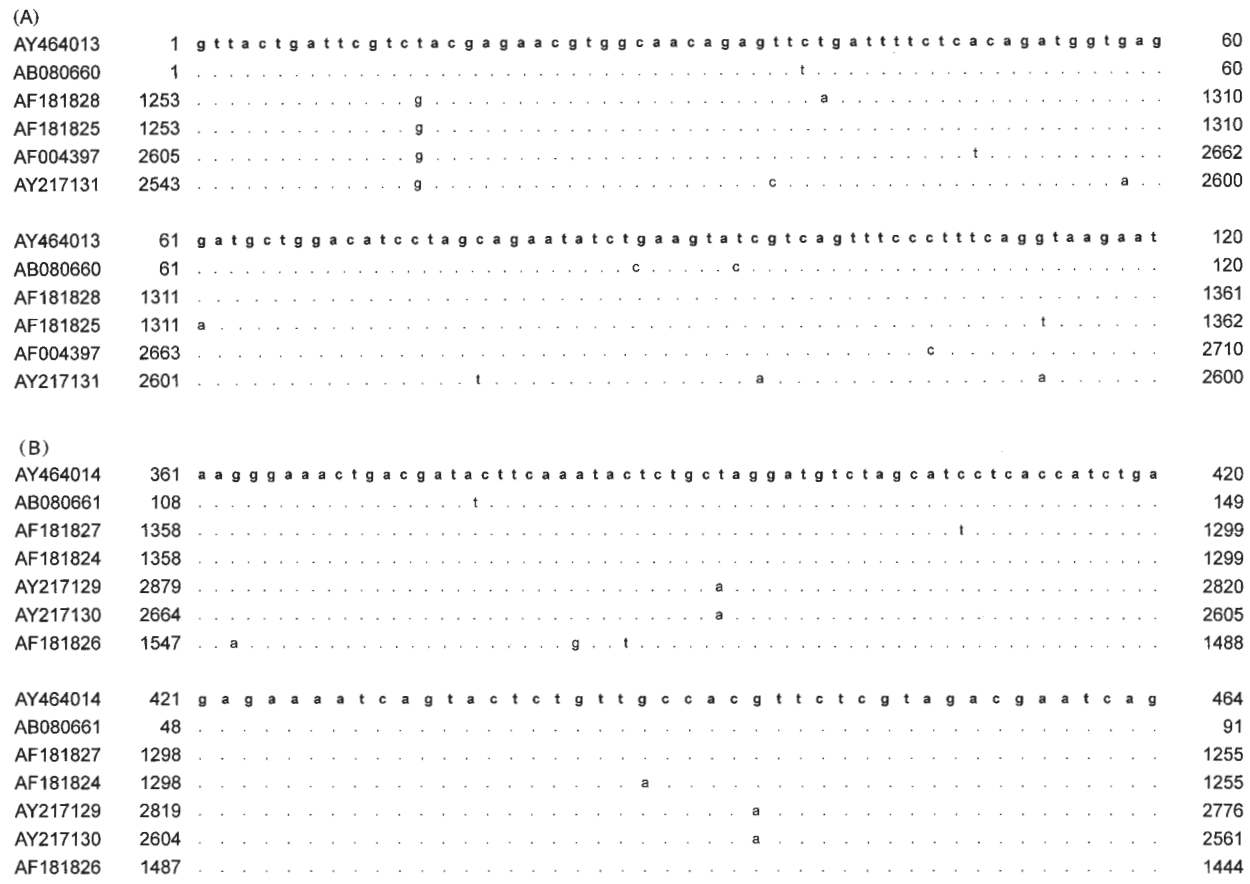

Fig. 4. (A) Comparisons of Black-faced Spoonbill CHD-Z (120 nucleotides) with the CHD-Z of some other bird species. (see footnote in appendix)

REFERENCES

Agate RJ, W Grisham, J Wade, S Mann, J Wingfield, C Schanen, A Palotie, AP Arnold. 2003. Neural, not gonadal, origin of brain sex differences in a gynandromorphic finch. Proceeding National. Academia Science U.S.A. **100:** 4873-4878.

Bermudez HLG, GA Garcia, GCH Leal, VVM Riojas, RG Jaramillo, R Montes-de-Oca-Luna. 2000. Molecular sexing of monomorphic endangered Ara birds. J. Exp. Zool. **292:** 677-680.

BirdLife Asia Council. 1995. Action plan for the Black-faced Spoonbill *Platalea minor*. Taipei, Taiwan: Wild Bird Society of Republic of China. 75 pp.

BirdLife International Asia Council. 1999. Discovery of the breeding and migration routes of Black-faced Spoonbills *Platalea minor*. Proceedings of an International Workshop on the Conservation of Black-faced Spoonbill. Taipei, Taiwan: BirdLife International Asia Council, pp. 30-42.

Cheng YH, CM Wen, ST Ding, TY Kuo. 2003. Detecting meat and bone meal in ruminant's feeds by species-specific PCR. J. Anim. Feed Sci. **12:** 851-860.

Chong JR, UI Park. 1999. The breeding sites and distribution of Black-faced Spoonbills *Platalea minor* in the Democratic Peoples Republic of Korea (DPRK). Proceeding of Conservation and Research of Black-faced Spoonbills and Their Habitats. Tokyo: Wild Bird Society of Japan, pp. 5-9.

Chong JR, UI Park, CY Rim, TS Kim. 1996. Breeding biology of Black-faced Spoonbill *Platalea minor.* Strix **14:** 1-10.

Chong JR, UI Park, CY Rim, TS Kim. 1997. Migration route and wintering ground of Black-faced Spoonbill *Platalea minor*. Bull. Korea Univ. Jpn. **15:** 68-80.

Clinton M, 1994. A rapid protocol for sexing chick embryos. Anim. Genet. **25:** 361-362.

D'Costa S, JN Petitte. 1998. Sex identification of turkey embryos using a multiplex polymerase chain reaction. Poultry Sci. **77:** 718-721.

Ellegren H, BC Sheldon. 1997. Reviews: new tools for sex identification and the study of sex allocation in birds. Trends Ecol. Evol. **12:** 255-259.

Fridolfsson AK, H Ellegren. 2000. Molecular evolution of the avian CHD1 genes on the Z and W sex chromosomes. Genetics **155:** 1903-1912.

Griffiths R, S Daan, C Dijkstra. 1996. Sex identification in birds using two CHD genes. Proc. R. Soc. Lond. B. **263:** 1249-1254.

Griffiths R, MC Double, K Orr, RJG Dawson. 1998. A DNA test to sex most birds. Mol. Ecol. **7:** 1071-1076.

Griffiths R, R Korn. 1997. A CHD1 gene is Z chromosome linked in the chicken *Gallus domesticus*. Gene **197:** 225-

229.
Griffiths R, K Orr. 1999. The use of amplified fragment length polymorphism (AFLP) in the isolation of sex-specific markers. Mol. Ecol. **8:** 671-674.
Griffiths R, B Tiwari. 1995. Sex of the last wild *Spixos* macaw. Nature **375:** 454.
Griffiths R, B Tiwari. 1996. Avian CHD genes and their use in methods for sex identification in birds. International patent publication no. WO9639505, published 12 December 1996, Oxford, UK: Isis Innovation.
Groombridge B. 1993. IUCN red list of threatened animals. Gland, Switzerland and Cambridge, UK: The International Union for Conversation of Nature and Natural Resources, IUCN.
Hornfeldt B, T Hipkiss, AK Frodolfsson, U Eklund, H Ellegren. 2000. Sex ratio and fledging success of supplementary-fed Tengmalm's owl broods. Mol. Ecol. **9:** 187-192.
Inoue-Murayama, M., Y Ueda, T Yamashita, C Nishida-Umehara, Y Matsuda, T Masegi, S. Ito. 2002. Molecular sexing of Japanese cormorants used for traditional fishing on the Nagara River in Gifu City. J. Animal Science **73:** 417-420.
IUCN. 1997. 1996 IUCN red list of threatened animals. Gland, Switzerland and Cambridge, UK: The International Union for Conversation of Nature and Natural Resources, IUCN.
Kim WB, HS Oh, HS Park. 1998. Population status and protection of the Black-faced Spoonbill on Cheju Island, Korea. Korean J. Ornigin **5:** 27-33.
Lee SH, CD Yao, KL Yang, JP Wang. 2003. Mitochondrial genetic diversity of the Black-faced Spoonbill (*Platalea minor*). Proceedings of Taiwan Biodiversity Research Symposium. Taipei, Taiwan: Council of Agriculture, pp. 8-12.
Lee WS, WH Hur, SJ Rhim. 2001. Distribution characteristics of Black-faced Spoonbill *Platalea minor* in western coast of South Korea. Korean J. Ecol. **24:** 219-222.
Lee SW, YS Kwon, JG Je, JC Yoo. 1999. Benthic animals of Kanghwa island and gut analysis of some waterbirds. Korean J. Origin **6:** 71-86.
Lessells C, A Mateman. 1998. Sexing birds using random amplified polymorphic DNA (RAPD) markers. Mol. Ecol. **7:** 187-195.
Nesje M, KH Road. 2000. Sex identification in falcons using microsatellite DNA markers. Hereditas **132:** 261-263.
Saitoh H, M Harata, S Misuno. 1989. Presence of female-specific bent-repetitive DNA sequences in the genome of turkey and pheasant and their interactions with W-protein of chicken. Chromosoma **98:** 250-258.
Stefos K, FE Arrighi. 1971. Heterochromatic nature of W chromosomes in birds. Exp. Cell Res. **68:** 228-231.

APPENDIX I:

Footnote: AY464013 *Platalea minor* chromodomain helicase DNA binding protein (CHD1Z) gene (Cheng and Kuo 2003). AB080660. *Phalacrocorax capillatus* CHD1Z gene for chromosome Z chromo-helicase-DNA binding protein (Inoue-Murayama et al. 2002). AF181828 *Nymphicus hollandicus* chromosome Z chromodomain helicase DNA binding protein 1 (CHD1Z), AF181825 *Aegolius funereus* chromosome Z chromodomain helicase DNA binding protein 1 (CHD1Z) (Fridolfsson and Ellegren 2000). AF004397 *Gallus gallus* chromo-helicase-DNA-binding on the Z chromosome protein, variant with hydrophilic domain, (CHD-Z) (Griffiths and Korn 1997). AY217131 *Taeniopygia guttata* chromo-helicase DNA-binding protein (CHD-Z) mRNA transcript B (Agate and Arnold 2003). Sex differences in structure and expression of the sex chromosome genes CHD-Z and -W in zebra finches. (B) Comparisons of Black-faced Spoonbill CHD-W (103 nt) with CHD-W sequences of some other bird species. Footnote: AY464014 *Platalea minor* chromodomain helicase DNA binding protein (CHD1W) gene (Cheng and Kuo 2003). AB080661 Phalacrocorax capillatus CHD1W gene for chromosome W chromo-helicase-DNA binding protein (Inoue-Murayama et al. 2002). AF181827 *Nymphicus hollandicus* chromosome W chromodomain helicase DNA binding protein 1 (CHD1W), AF181824, Aegolius funereus chromosome W chromodomain helicase DNA binding protein 1 (CHD1W), AF181826, *Gallus gallus* chromosome W chromodomain helicase DNA binding protein 1(CHD1W) (Fridolfsson and Ellegren 2000). AY217129 *Taeniopygia guttata* chromo-helicase DNA-binding protein (CHD-W) mRNA transcript A (Agate et al. 2003). AY217130, *Taeniopygia guttata* chromo-helicase DNA-binding protein (CHD-W) mRNA transcript B (Agate and Arnold 2003). Sex differences in structure and expression of the sex chromosome genes CHD-Z and -W in zebra finches.