MEDWELL PUBLISHING
Journal of Animal and Veterinary Advances 8 (6): 1137-1140, 2009
ISSN: 1680-5593

Sex Identification of Seven Species of Cranes in China by PCR

[1]Bao Wen-Bin, [1]Wu Sheng-Long, [1]Zhang Hong-Xia,
[1]Luan De-Qin, [2]H.H. Musa and [1]Chen Guo-Hong
[1]College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China
[2]Faculty of Veterinary Science, University of Nyala, P.O. Box 155, Nyala, Sudan

Abstract: Sexes of cranes cannot be distinguished visually due to the morphological similarities between male and female. To facilitate the protection program for the endangered birds, red-crowned cranes in particular, genomic DNA was extracted from feather samples and a PCR-based sexing method was established using three primer combinations of avian sex-specific EE0.6 sequence. The specificity of the PCR was confirmed by amplifying genomic DNA from 22 known-sex cranes of seven species. After 30 cycles of amplification, easily distinguishable single bands for males and two bands for females were displayed by agarose gel electrophoresis. The sexes of 50 unknown-sex cranes of seven species were successfully identified using the PCR. The noninvasive PCR-based sexing method can not only facilitate the human-assisted breeding of cranes, but also provide useful reference for accurate sexing of other endangered birds.

Key words: Crane, EE0.6 sequence, sex identification, PCR, DNA, China

INTRODUCTION

Sex identification in avian species is one of the key points of avian breeding and evolutionary studies. About 50% of the world's bird species do not show sex-linked morphology (Griffithsm *et al*., 1998). The sexes of cranes also cannot be distinguished visually due to the morphological similarities between male and female birds. Of the 15 species of cranes, seven are vulnerable to extinction, making cranes one of the most threatened families of birds in the world (Duan and Fuerst, 2001). The traditional sex identification methods used in monomorphic birds such as vent sexing, laparoscopy, steroid sexing and karyotyping, are unreliable and time-consuming (Cerit and Avanus, 2007). Recently, Random Amplified Polymorphic DNA (RAPD) approach for noninvasive identification of crane gender has been established by using the PCR primers based on crane sex-linked DNA on W chromosome (Duan and Fuerst, 2001). A 0.6 kb EcoRI fragment (designated EE0.6) has been cloned from the W chromosome of chickens, which is conserved in all bird species examined and the homolog sequence is present on the Z chromosome of cranes (Itoh *et al*., 2001). If one PCR system for this sex-related gene on W chromosome can be constructed, it will be easy to identify sex in cranes.

There are nine crane species in China, some of them such as red-crowned cranes (*Crus japonensis*), hooded cranes (*Grus monacha*) and Siberian cranes (*Grus leucogeranus*) are the 1st class protected animal by nation. The national protection program of crane has been launched in China including human-assisted breeding efforts to increase the bird populations. During the execution of the program, sex identification is fundamentally important for management and conservation, captive management and basic research of the cranes. To establish a quick noninvasive method for crane sex determination, in this study we established a PCR-based method using three sets of primers according to the EE0.6 sequence (Itoh *et al*., 1997, 2001; Ogawa and Irina, 1997) and genomic DNA from feather samples of 7 species cranes as the template. The results may help to solve the key problem during the human-assisted breeding of crane and give a guide and reference for the accurate sex identification of other endangered birds.

MATERIALS AND METHODS

Sampling: The cranes used in this study were from Wuxi Zoo (Wuxi city, Jiangsu province, China), Yangzhou zoo (Yangzhou city, Jiangsu province, China) and Hefei Wild Animals park (Hefei city, Anhui province, China). The secondary feathers were collected from the wings of 50 immature unknown-sex cranes of seven species. As the known-sex controls, feather samples were also collected from 2 couple white-naped cranes (*Grus vipio*), one

Corresponding Author: G.H. Chen, College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China

Table 1: Primer sequences used in this study

	Primer	Sequence
A	AWS05/NRD4	5'-CACCCTGGATTGGACAACCTATTTC-3'
		5'-TCAGAGCACTCTTTCCAGGAA-3'
B	SINT-F/SINT-R	5'-TAGGCTGCAGAATACAGCAT-3'
		5'-TTGTGCAGTTCTAGTCCATA-3'
C	USP1/USP3	5'-CTATGCCTACCACMTTCCTATTTGC-3'
		5'-AGCTGGAYTTCAGWSCATCTTCT-3'
D	CPE15F/CPE15R	5'-AAGCATAGAAACAATGTGGGAC-3'
		5'-AACTCTGTCTGGAAGGACTT-3'

couple demoiselle cranes (*Anthropoides virgo*), one couple red-crowned cranes (*Grus japonensis*), 2 couple common cranes (*Grus grus*), two couple black-necked cranes (*Grus nigricollis*), one couple hooded cranes (*Grus monacha*) and two couple Siberian cranes (*Grus leucogeranus*). The gender of the couple has been confirmed by reproduction.

Genomic DNA preparation: Each feather tip of 0.5 cm long was mixed first with 10 μL 1 mol L DTT^{-1} and then with 450 μL 1× TNE (10 mmol L^{-1} Tris-Hcl, 1 mmol L^{-1} EDTA, 100 mmol L^{-1} NaCl, pH 8.0). After addition of 45 μL SDS (10%) and 10 μL proteinase K (10 mg mL^{-1}), the mixture was incubated for 24 h at 56°C with gentle agitation. The genomic DNA was extracted with phenol/chloroform mixture, washed with 70% ethanol and resuspended in TE (Tris-EDTA) buffer at the final concentration of 100 ng μL^{-1} and preserved at -20°C.

PCR Amplification: Three combinations of primers (A and B, B and C, C and D) shown in Table 1 were used. PCR were carried out in 10 μL volume containing 200 μM dNTPs, 2 pM of each primer, 0.5 U Taq polymerase (5 U μL^{-1}), 100 ng genomic DNA and ddH_2O up to 10 μL. The kit was from Takara Biotechnology Dalian Co. LTD, China. The amplification reaction was performed as follows: an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 50 sec, annealing at 56-58°C for 50 sec and extension at 72°C for 50 sec, with a final extension at 72°C for 10 min. Exactly 6 μL of the PCR products were separated by 1.5% agarose gel at 120 V, visualized with gold view under UV light by Gene Genius gel imaging apparatus.

RESULTS

Specificity of the PCR: To validate the specificity of PCR amplification, genomic DNA from 22 known-sex cranes of 7 species were amplified for the EE0.6 sequences using primer combinations of A and B, B and C and C and D. Agarose gel electrophoresis showed that single or 2 distinct bands of different sizes could be amplified from male or female birds using all the three primer combinations, including all seven crane species (Fig. 1).

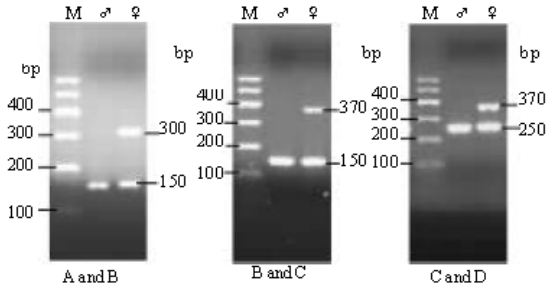

Fig. 1: PCR amplifications of known sex of cranes with primer combinations A and B, B and C and C and D A and B (EE0.6W 300 bp, EE0.6Z150 bp). B and C (EE0.6W 370 bp, EE0.6Z 150 bp), C and D (EE0.6W 370 bp, EE0.6Z 250 bp) M: 100 bp of DNA Ladder Marker (Takara)

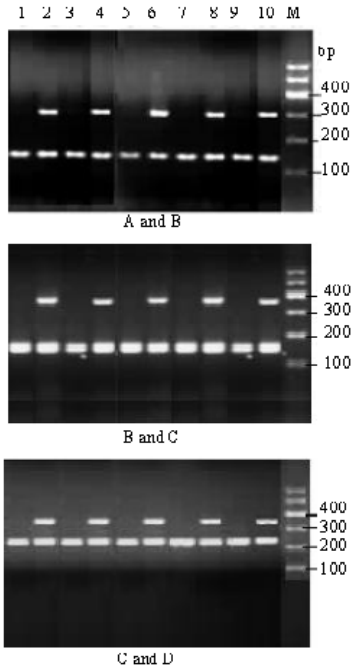

Fig. 2: Sex identification of unknown-sex cranes by PCR. The odd and even numbers represent male and female, respectively. M: 100 bp DNA Ladders

Sex identification of 50 unknown-sex cranes: To sex the unknown-sex individuals, genomic DNA from white-naped cranes, demoiselle cranes, red-crowned cranes, common cranes, black-necked cranes, hooded cranes and Siberian cranes were submitted to PCR amplification using the three different primer combinations. After 30 cycles of amplification, agarose gel electrophoresis showed the same single or two bands for the unknown-sex cranes (Fig. 2). The sexes of the 50 cranes were identified according to the band patterns.

DISCUSSION

Sex identification is necessary for management, conservation and basic research of endangered avian species. Traditional sexing methods have the disadvantages of time-consuming, low accuracy and/or physical destruction (Cerit and Avanus, 2007). Therefore, alternative convenient and potentially noninvasive methods are needed, especially using tissue sources such as feather samples (Taberlet *et al*., 1999). In the recent study on birds, genomic DNA was mainly extracted from feathers, especially from the tips of feathers (Natalia *et al*., 2001). Because this area may contain some blood or tissues, it will extract more DNA from this part, without protein and RNA. The degradation in the process of extraction is not serious. Besides, Tian *et al*. (2005) did some research on the DNA extraction from different parts of feathers of endangered birds in order to resolve the problems of sampling in the study of avian molecular biology. They reported that genomic DNA and mtDNA could be extracted from the tips portion. In this study, secondary feathers of these cranes were collected and genomic DNA was extracted from different parts of feathers such as calamus, feather marrow, quill and plumula of upper umbilicus. The results showed that genomic DNA with a certain quantity and quality was obtained from calamus, while other parts obtained too little DNA or nothing. The reason may due to the fact that the calamus contains a little blood or tissues.

RAPD analysis has been shown to be able to sex some species of birds (Griffthsm *et al*., 1998). The RAPD-PCR method was first used to identify avian sex-linked chromodomain-helicase-DNA binding protein (CHD) gene (Ellegren, 1996; Griffiths *et al*., 1996). Although, the two copies of the gene exist in most bird species, one located on the W chromosome of females and the other on the Z chromosomes of both sexes, these paralogous DNA fragments have the same length and thus additional methods (e.g., DNA sequencing and restriction enzyme analysis) are needed to further analyze the PCR products. Recently, another RAPD-based approach for sexing cranes has been developed (Duan and Fuerst, 2001). By using a set of PCR primers from crane sex-linked DNA on W chromosome of female whooping cranes, a 227-230 bp fragment can be amplified from females and the duplicated versions with larger size (231-235 bp) can be amplified from both sexes.

The EE0.6 fragment was first cloned from the W chromosome of chickens, which is conserved in all bird species examined. Although, the DNA fragment is present on W and Z chromosomes, its length is significantly different (Itoh *et al*., 2001). By using the EE0.6 specific single set of primers, a single or 2 bands were amplified from male or female chickens (Ogawa and Irina, 1997), ratitae birds (Huynen and Craig, 2002) and cranes (Chen *et al*., 2006). However, all these PCR-based methods use a single set of primers, which may compromise the accuracy of sex identification due to potential nonspecific amplification and/or sample contamination. To increase the accuracy of sex identification, two sets of sex-specific primers have been used to amplify the EE0.6 fragment in white-naped and red-crowned cranes (Itoh *et al*., 2001). In this study, we established a quick noninvasive PCR-based approach for crane sexing using three combinations of EE0.6 specific primers and genomic DNA from feather samples. To confirm its specificity, we first performed the PCR using genomic DNA from known-sex cranes. Agarose gel electrophoresis of the PCR products showed expected single or two bands for male or female cranes, sizes of which are easy to distinguish on agarose gels. By using the PCR, we identified successfully the sexes of 50 cranes belonging to seven different species. Additional advantages of the PCR method include increased specificity and decreased risk of potential sample contamination.

CONCLUSION

The sex of seven species cranes in China could be identified successfully by PCR with specific primers combinations for EE0.6 sequence, which is highly conserved. This PCR-based noninvasive method was established with improved specificity and lower risk of sample contamination, which can be used to sex not only cranes, but also other endangered birds.

ACKNOWLEDGEMENTS

This research was supported by the National 863 Program of China (grant No. 2006AA10Z1E7). We also thank the Yangzhou zoo, Wuxi zoo and Hefei Wild Animals Park for providing experimental birds and Zheng Wei-Ping, Li Wen-Bin, Wang Jin-Mei, Hu Fei and Zhang Zhi-Zhong for help on sampling.

REFERENCES

Cerit, H. and K. Avanus, 2007. Sex identification in avian species using DNA typing methods. World Poult. Sci. J., 63: 91-100.

Chen, W., S.M. Xie, G.J. Xie, M. Huang, X.J. Chen and M. Liao, 2006. Application of PCR Technique in sexing birds. Vet. Sci. China, 36 (6): 485-488.

Duan, W. and P.A. Fuerst, 2001. Isolation of a sex-linked DNA sequence in cranes. J. Hered., 92: 392-397.

Ellegren, H., 1996. First gene on the avian W Chromosome (CHD) provides a tag for universal sexing of non-ratite birds. Proc. Royal Society of London Biol. Sci., 263 (1377): 1635-1641.

Griffiths, R., S. Daan and C. Dijkstra, 1996. Sex identification in birds using 2 CHD genes. Proc. Royal Society of London Biol. Sci., 263: 1251-1256.

Griffithsm, R., M.C. Double, K. Orr and R.J.G. Dawson, 1998. A DNA test to sex most birds. Mol. Ecol., 7 (8): 1071-1075.

Huynen, L. and D. Craig, 2002. A DNA test to sex ratite birds. Mol. Ecol., 11: 851-856.

Itoh, Y., A. Ogawa, K. Murata, T. Hosoda and S. Mizuno, 1997. Identification of the sex of oriental white stork, Ciconia boyciana, by the polymerase chain reaction based on its sex chromosome-specific DNA sequences. Genes Gen. Syst., 72: 51-56.

Itoh, Y., M. Suzuki and A. Ogawa, 2001. Identification of the sex of a wide range of carinatae birds by PCR using primer sets selected from chicken EE0.6 and its related sequences. Am. Gene. Assoc., 92: 315-321.

Natalia, B., F. Olga and S. Armand, 2001. Isolation of genomic DNA from feathers. J. Vet. Diagn. Invest., 13: 162-164.

Ogawa, A. and S. Irina, 1997. Molecular characterization and cytological mapping of non-repetitive DNA sequence region from the W-chromosome of chicken and its use as a universal probe for sexing Carinatae birds. Chromo. Res., 5: 93-101.

Taberlet, P., L.P. Waits and G. Luikart, 1999. Noninvasive genetic sampling: Look before you leap. Tree, 8: 323-327.

Tian, X.H., Z. Liu and S.Y. Bai, 2005. Microsampling and DNA extraction of rare and Endangered birds. J. Econ. Anim., 9 (4): 215-218.