Applying molecular sex identification methods to "Karma", the injured black-necked crane (Grus nigricollis) of Phobjikha

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Abstract

The black-necked crane (BNC) is endemic to the Qinghai-Tibetan Plateau, the adjacent southern Himalayas, and the Yunnan-Guizhou Plateau. They are listed as Vulnerable by the IUCN and are a Schedule I protected species in Bhutan. In early 2015, an injured crane was rescued in Phobijkha and named "Karma". Karma could be a founder for ex-situ conservation of BNCs. To determine Karma's sex, four molecular sexing methods were texted. Two of these, introduced by Fridolfsson & Ellegren (1999) and Griffiths et al. (1996), were found to be applicable to BNC and both suggested that Karma is male. It is planned that a female mate be introduced to Karma.

Keywords: Conservation genetics, endangered species, ex-situ conservation, Gruidae, molecular sexing

Introduction

The black-necked crane (BNC, *Grus nigricollis*) is 1 of 15 species and 4 genera of cranes (Family Gruidae), and is categorized as Vulnerable on the IUCN Red List (BirdLife International 2008). The BNC is endemic to an area encompassing the Qinghai-Tibetan Plateau, the adjacent southern regions of the Himalayas, and eastward to the Yunnan-Guizhou Plateau. They breed in high-altitude (4,300 m elevation) alpine wetlands and migrate to lower altitudes to overwinter. A small population of approximately 600 BNCs migrates to specific valleys in Bhutan, where they are listed as a Schedule I protected species under the Forest and Nature Conservation Rules and Regulations of Bhutan (FNCRR) (DoFPS 2007), and therefore, legally protected. BNCs are also revered by local communities, who consider them to be "heavenly" birds.

Data on the BNC wintering population, maintained by the Royal Society for the Protection of Nature since the winter of 1986, indicates an increasing trend in the BNC population (Phuntsho and Tshering, 2014). This population increase, as well as an increase in breeding populations in Tibet, is likely a result of increased habitat protection and public education efforts. However, human developments and natural threats continue to be major concerns with respect to crane habitat. In Bhutan, a lack of human resources and facilities has rendered effective local conservation difficult. Injured cranes in the wetlands of the Phobijhka valley of Bhutan often succumb to their injuries due to a lack of wildlife treatment facilities. In the winter of 2015, a juvenile crane was found in Phobjikha with multiple left-wing fractures (RSPN, 2015). The staff of the Black-necked Crane Visitor Centre (BNCVC), along with livestock extension officers from Phobjikha

and Gangtey Gewog in the Wangduephodrang district, transported the crane to the center. After disinfecting its wounds, staff named the crane "Karma" and housed it temporarily at the BNCVC.

Karma could be a founder individual for ex-situ conservation of BNCs. Before providing a mate, Karma's sex must be identified, but BNCs cannot be sexed based on their external morphology. In the past 20 years, effective methods for molecular sexing of birds have been developed, but these techniques have not been applied to BNCs. The most preferred method for initially sexing a new bird species is based on the chromo-helicase-DNA binding region (CHD) and polymerase chain reaction (PCR) (Dubiec & Zagalska-Neubauer, 2006). To determine Karma's sex, several methods were used, starting with the simplest.

Material and methods

All genetic work was performed at the DNA laboratory of Center for Molecular Biodiversity Research, National Museum of Nature and Science, Tokyo. A molted feather (150 mm in length) was collected from Karma's enclosure in Phobijkha on July 25, 2019 (Fig. 1). The base of the shaft was used as the source of DNA. The shaft was cut horizontally into pieces < 3mm in size and DNA was extracted using an UltraClean Tissue & Cells DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. DNA yield was measured using a Qubit 4.0 Fluorometer with Qubit dsDNA HS Assay Kits (Thermo Fisher Scientific, Waltham, MA, USA).

First, the method of Ellegren (1996) was used, wherein three PCR primers (945F, 3224R, and cfR) were employed to amplify a 210 bp fragment from the CHD of the W chromosome (CHD-W) and a 630 bp fragment from the CHD of the Z chromosome (CHD-Z). CHD sex-linked genes were amplified in 20-µl reactions using 1.0 µl of template DNA, 2.0 µl of 10× buffer, 0.20 mM of dNTPs, 0.40 µM of 2945F primer, 0.20 µM each of 3224R and cfR primers, and 0.5U of Taq polymerase (Takara Ex-Taq; Takara Bio, Shiga, Japan) under the following thermal cycler (Takara Dice; Takara Bio) program: denaturing at 94°C for 3 min, followed by 40 cycles of denaturing at 94°C for 30 s, annealing at 48°C for 30 s, a chain extension at 72°C for 45 s, and a final extension at 72°C for 5 min. Sex-linked genes were separated by electrophoresis with a 1.5% agarose gel at 100 V for 20 min (Mupid 2-plus; Takara Bio), with a 100 bp ladder used as a size marker(stained with Midori Green Direct; Fast Gene, Nippon Genetics Europe, Dueren, Germany).

Figure 1. The molted feather used for DNA extraction.

Next, the method described by Fridolfsson & Ellegren (1999) was used. This method employs a PCR primer set (2550F and 2718R) to amplify a ca. 400 bp fragment of CHD-W and a ca. 600 bp of CHD-Z. CHD sex-linked genes were amplified under the same conditions described above, with a few exceptions: more primer was used (0.4 µM of both primers), annealing was conducted at

50°C, chain extension was run for 45 s, and electrophoresis was run with a 4% agarose gel for 30 min.

Third, the method described by Griffiths et al. (1998) was employed, whose primer set, P2 and P8, amplifies a region of the CHD genes that includes the intron. In most bird species, the size of the amplified fragment differs between the Z and W chromosomes, which can be distinguished by electrophoresis using a 3–4% agarose gel. PCR amplification was conducted under the same conditions as in the Fridolfsson & Ellegren (1999) method, but annealing was conducted at 48°C and electrophoresis was run with a 4% agarose gel for 50 min.

Finally, the method described by Griffiths et al. (1996) using a primer set consisting of P2 and P3 was used. In this method, the amplified fragment of 110 bp was digested with restriction endonuclease, where the *DdeI* site is unique to CHD-W and the *HaeIII*, *MboII* and *XhoI* sites are unique to CHD-Z. PCR amplification was conducted under the conditions described by Griffiths et al. (1998), but annealing was conducted at 55°C for 15 s and chain extension was conducted for 15 s. Prior to electrophoresis (4% agarose gel for 15 min), the PCR product was digested with each of the four restriction enzymes in 10-µl reactions of 8.5 µl of PCR product, 1.0 µl of 10× buffer, and 0.5 µl (5U) of enzyme (*DdeI, HaeIII*, *MboII* or *XhoI*; Takara), and incubated at 37°C for 60 min.

PCR was conducted on the DNA collected from Karma, with three positive controls. Control DNA samples from two females and one male were obtained from the National Museum of Science and Nature in Tokyo (NSMT-DNA 3931, 52239, and 9709, respectively). The sex of all three control individuals had been determined by examining the appearance of their sex organs after death.

Results

The final DNA yield was 1.45 ng/µl. The method of Ellegren (1996) successfully amplified a 210 bp fragment on CHD-W for both female controls (Z/W), and one 630 bp fragment on CHD-Z for the male control (Z/Z, Figure 2). This suggests that this sexing method is effective for BNCs, although, unexpectedly, DNA from the second female specimen did not amplify on the Z chromosome. Unfortunately, no fragment was amplified from Karma using this method (Figure 2).

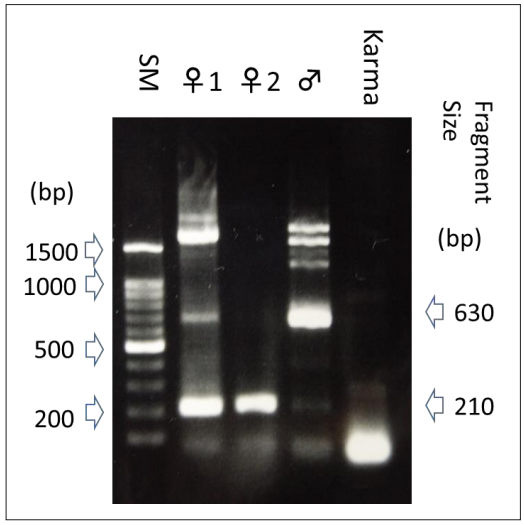

Figure 2. Electrophoresis image obtained using the method of Ellegren (1996). Lanes: SM is the 100 bp ladder size marker; ♀1 and ♀2 are control females (NSMT-DNA 3931 and 52239, respectively); ♂ is the control male (NSMT-DNA 9709). Marker sizes are shown on the left and fragment sizes of target chromo-helicase-DNA binding (CHD) genes are shown on the right.

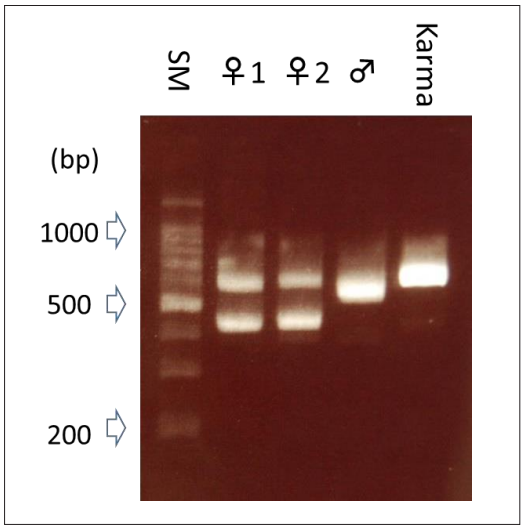

Figure 3. Electrophoresis image obtained using the method of Fridolfsson & Ellegren (1999). Abbreviations follow Figure 2.

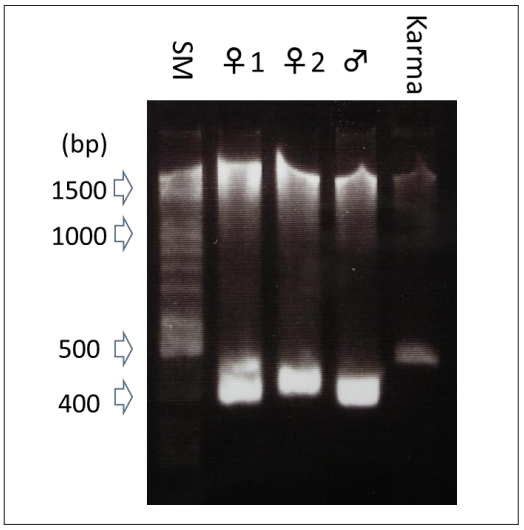

Figure 4. Electrophoresis image obtained using the method of Griffiths et al. (1998). Abbreviations follow Figure 2.

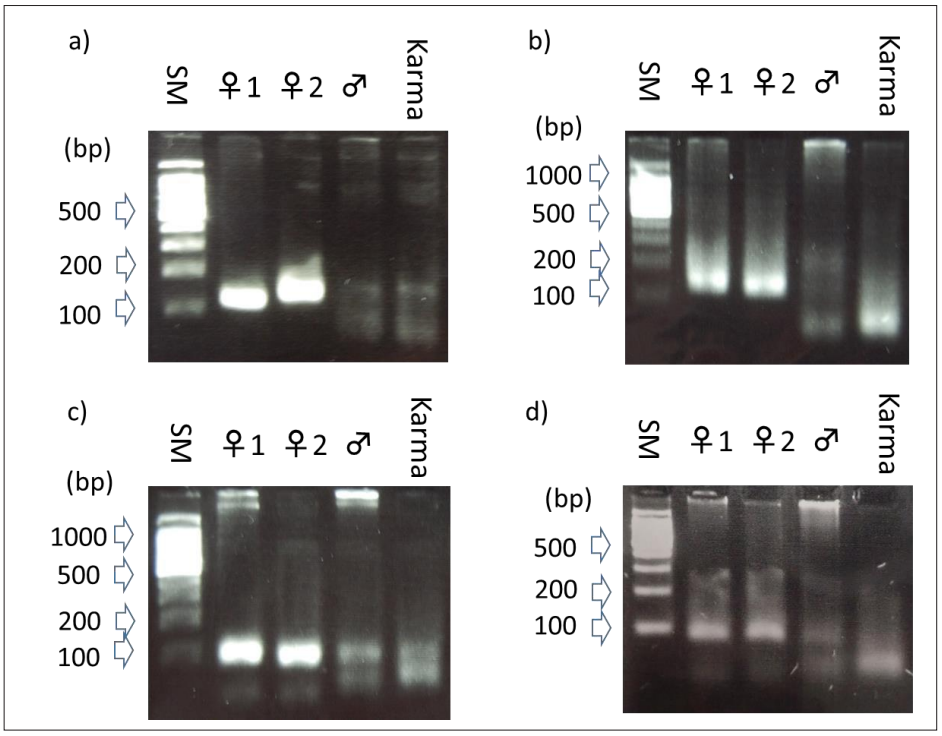

Figure 5. Electrophoresis image obtained using the method of Griffiths et al. (1996). Abbreviations follow Figure 2. The polymerase chain reaction (PCR) products were digested with a) *HaeIII*, b) *MboII*, c) *XhoI*, or d) *DdeI* prior to electrophoresis.

The method of Fridolfsson & Ellegren (1999) successfully amplified both a ca. 400 bp fragment of CHD-W and a ca. 600 bp fragment of CHD-Z for the two control females, and a ca. 600 bp fragment for the male control, as expected (Figure 3). Karma was shown to have a 600 bp fragment on the Z chromosome but no 400 bp fragment on the W chromosome, suggesting that Karma is male. The method of Griffiths et al. (1998) amplified a 400–500 bp fragment for each individual, but no differences were observed between the sexes (Figure 4). The method of Griffiths et al. (1996), using the primer set P2 and P3, successfully amplified a 110 bp fragment for both sexes. After the *HaeIII*, *MboII*, or *XhoI* cut, the two control females retained this fragment, but the male did not (Figure 5a–c). The sample from Karma showed the same pattern as the control male, again suggesting that Karma is male. Notably, the same fragment patterns were observed under the *DdeI* cut as with the three other enzymes, yet the *DdeI* site was expected to only cut CHD-W in females (Figure 5d).

Discussion

Three of the four methods tested were effective for determining sex in BNC (Table 1), although the method of Ellegren (1996) showed an unexpected fragment pattern. Romanov et al. (2019) tested five sexing methods, including a new method specific for Galliformes, and found that at least one method was successful in sexing 84 of 88 tested species across 13 avian orders. Our findings show that BNC can be sexed using three methods, i.e., those of Ellegren (1996), Fridolfsson & Ellegren (1999), and Griffiths et al. (1996). Determining the techniques that are effective for accurately sexing birds is highly valuable for both wild and captive bird studies (D'Aloia & Eastham, 2000).

Although the methods of Ellegren (1996) and Griffiths et al. (1998) failed to identify the sex of Karma, those of Fridolfsson & Ellegren (1999) and Griffiths et al. (1996) suggested that Karma is male. Griffiths & Tiwari (1995) examined the sex of the last wild individual of Spix's Macaw (*Cyanopsitta spixii*), which was the world's most endangered bird species at that time, and identified it as male. Later, a female Spix's Macaw was released as a prospective mate. Karma represents an opportunity for the establishment of an ex-situ conservation effort for BNCs in Bhutan, where the next step will be to introduce Karma to a prospective female mate.

Table 1. Results of molecular sexing methodologies applied to black-necked crane specimens. The control individuals were two females and one male obtained from the National Museum of Nature and Science, Tokyo.

	Ellegren (1996)	Fridolfsson & Ellegren (1999)	Griffiths et al. (1998)	Griffiths et al. (1996)			
				HaeIII	*MboII*	*XhoI*	*DdeI*
Control individuals	△	○	×	○	○	○	△
Karma	×	0 ale	×	0 ale	0 ale	0 ale	0 ale?

○= successful, △= successful but unexpected fragment pattern, × = failure.

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