Sexing Greater Flamingo Chicks from Feather Bulb DNA

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Abstract.—Adult Greater Flamingos (*Phoenicopterus roseus*) are sexually dimorphic, with males being on average larger and heavier than females. However, there is no practical way to sex the chicks by their morphology. Here we describe a method relying on quick and easy DNA extraction from feathers. A PCR test employing primers to amplify introns whose lengths usually differ between the CHD-W and the CHD-Z genes allow sex discrimination. This method is thus a fast, accurate and inexpensive protocol to sex flamingo chicks from feathers bulbs sampled in the field. *Received 19 July 2006, accepted 12 December 2006.*

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Because sex is known to influence many demographic aspects in birds, its identification is important when marking individuals for behavioral or ecological studies (Greenwood 1980; Short and Balaban 1994; Barbraud *et al.* 2003). Greater Flamingo (*Phoenicopterus roseus*) is a sexually dimorphic species with males being on average larger and heavier than females (Johnson *et al.* 1993). Thus it is possible to determine the sex of individuals when they are observed as immature or adult, yet with some risk of error. On the other hand, at the chick stage, sex determination requires more information than just external morphology.

Bertault *et al.* (1999) developed the first molecular technique enabling sex determination of Greater Flamingo chicks. Their technique involved DNA extraction from blood samples. However, in field conditions, collecting blood samples and preserving them under proper conditions is tedious. On the other hand, extracting DNA from feather samples is becoming widespread because feathers are easy to collect and preserve in the field (Duan and Fuerst 2001; Grant 2001; Malago *et al.* 2002; Sacchi *et al.* 2004). In this paper, we present a protocol to extract DNA from feather bulb samples of Greater Flamingo chicks in order to sex them easily.

The identification of the chromo-helicase-DNA binding gene (CHD), which is highly conserved among all non-ratite birds (Griffiths and Tiwari 1995; Ellegren 1996), has greatly facilitated sex determination in birds. One copy of the CHD gene exists on each of the sex chromosomes, W and Z. While the CHD-W gene is unique to females, which is the heterogametic sex (Ellegren 1996; Griffiths *et al.* 1996), the CHD-Z gene is common to both sexes (Griffiths and Korn 1997; Fridolfsson and Ellegren 1999). Sex determination relies on distinguishing the CHD-W and CHD-Z genes from each other.

A common approach to achieve this is to use the PCR-RFLP method (see Griffiths and Tiwari 1995; Griffiths *et al.* 1996). This method was employed by Bertault *et al.* (1999) to sex flamingo chicks from blood samples. It relies on the amplification of a particular part of the CHD genes using PCR primers. Follow-

ing the amplification, specific restriction enzymes are used to digest only one of the two genes (Griffiths and Tiwari 1995; Griffiths *et al.* 1996; Sacchi *et al.* 2004). Another approach is the single strand conformation polymorphism (SSCP) analysis (see Ellegren 1996), an electrophoretic protocol that permits distinguishing the slight sequence differences between PCR fragments of the same length. However, because both approaches present some technical difficulties, we chose to use a more recently developed and straightforward PCR technique that was proved successful in various species (see Griffiths *et al.* 1998; Fridolffson and Ellegren 1999). In this approach, the CHD-W and CHD-Z genes are distinguished by the amplification of an intron present in both genes. Because the introns are noncoding, they are less conserved and thus their lengths usually differ between the two genes. As a result, the PCR products on the gel electrophoresis vary in size revealing one band in males at the CHD-Z and two bands in females corresponding to both the CHD-W and CHD-Z. In contrast to the previous method, this approach does not require the use of a restriction enzyme to separate the PCR products after the amplification. This technique had been shown to work on blood samples of flamingo chicks (Dano, unpubl. data). In this paper, we present the adaptation of this latter approach required to sex flamingo chicks using feather bulb samples.

METHODS

Sampling and DNA Extraction

We used feather samples collected during banding operations of 2003 and 2004 in France and in 2004 in Turkey. Feathers (2-7 cm length) were taken from the neck or the back of the chicks or from the edge of their wings. The samples were placed in plastic bags or paper envelopes without touching their tips. Once the banding operation was terminated, either silica gel beads were added directly inside the samples, or samples were placed in new plastic bags containing silica. Silica gel absorbs moisture from its surrounding environment, helping to lower the humidity that can in turn degrade the DNA (Taberlet *et al.* 1999). Thus, using silica gel as desiccating agent allows preserving feather samples at room temperature. Samples were regularly controlled to see if the silica gel beads changed color (when they are saturated with water) and if necessary they were replaced with new beads.

DNA templates were obtained from the feather bulbs using the alkaline extraction method (for details see Wang *et al.* 1993; Rudbek and Dissing 1998). We tried different concentrations and volumes of chemicals and especially we considered the final concentration of NaOH in the PCR as an important determinant of the extraction success. Finally the physical condition of the feather samples were considered, i.e. in one set of experiments, ethanol (70°) was added on the samples to humidify the driest feathers before extraction. Precautions against cross contaminations were taken through sterilizing the apparatus with alcohol and DNA AWAY (Molecular Bio Products).

DNA Amplification

The amplification was performed in 20 μl of final volume and we tried different volumes of template DNA between 2 μl and 10 μl (completed with H_20). The reaction contained 0.4 μM of each primer (2550F: 5'-GTTACTGATTCGTCTACGAGA-3' and 2718R: 5'-ATTGAAATGATCCAGTGCTTG-3'; Fridolfsson and Ellegren 1999), 0.2 mM of each dNTP, 0.5 unit *Taq* polymerase (Sigma) in 1× reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl) and 1.5 mM $MgCl_2$. Amplification was performed on an Eppendorf Thermal cycler, with an initial denaturation at 94°C for 2 min, followed by a touch-down scheme where the annealing temperature was lowered 1°C per cycle, starting from 50°C until a temperature of 42°C is reached. Then 30 additional cycles were run at a constant annealing temperature of 42°C. Denaturation was at 94°C for 30 s, annealing for 30 s and extension at 72°C for 1 min. A final extension step of 5 min was added after the last cycle. Negative controls (water instead of DNA) were run with every PCR, and standard precautions were taken to avoid contamination. DNA isolated from blood samples of two Greater flamingos and two Wandering albatrosses (*Diomedea exulans*) (one male and one female) were used as control to assess the PCR success. The PCR products were run on 1.8% agarose-gel with standard TBE buffer, stained with ethidium bromide. After electrophoresis at 100 V for approximately 45 minutes, gels were examined and photographed under UV light.

RESULTS

DNA Extraction Protocol from Greater Flamingo Feathers

Among the different concentrations and volumes of chemicals tried, the following method gave the highest success: (i) depending on the size of the feathers, two to three bulbs were chosen (Fig. 1) and cut vertically into two to create an area of exposure, (ii) the samples were then transferred into 1.5 ml Eppendorf containing 40 μl NaOH 0.2 N, (iii) following vortex, the samples were placed in water bath (bain-marie) at 75°C for 20 min, (iv) following vortex, they were placed into ice, and finally (v) 160 μl Tris-

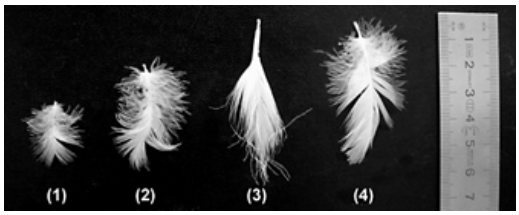

Figure 1. The size spectrum of feather samples used in DNA extraction. Number of feathers taken for the extraction was correlated with the size of the feathers; from very small and small feathers (1 and 2 in order) three samples were taken, whereas from middle sized feathers (3 and 4) two samples were taken.

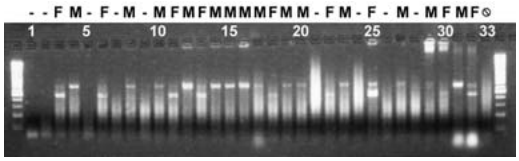

Figure 2. PCR results on 1.8% agarose TBE gel. The determined sex for each individual is mentioned above the gel (F-female, M-male) and the absence of successful amplification was denoted with "-". First and last lanes contain molecular weight marker (Gene Ruler 100 bp DNA Ladder plus-Euromedex). Lanes 29 to 32 contain the four controls: one male and female from both Greater flamingo and Wandering albatross (in order). Finally the last lane (⊘) contains the negative control (water instead of DNA).

HCl 0.04 N pH 8 (4°C) was added to the samples. After this step, the DNA extract can be conserved at -20°C. Using ethanol to humidify the driest samples before extraction did not enhance the results, and therefore this approach was not retained. Finally, using 2 µl of the template DNA gave the highest success at the PCR step.

DISCUSSION

The extraction protocol yielded rather good quality DNA, and intron size differences between the CHD-W and the CHD-Z genes enabled the sex determination of Greater Flamingo chicks. In this approach, females are expected to have two bands and males only one (Griffiths *et al.* 1998; Fridolffsson and Ellegren 1999). However, in our results, more than half of the females (57%) had only one clear band at the CHD-W. In other words, while the CHD-Z product was clear in males, it was either absent or very weak in females due to the PCR yield. Similar results were obtained from the amplification products of the control samples, where female Greater flamingo had only one band at the CHD-W (lane 30, Fig. 2). There exists several species where the CHD-Z can not be observed due to the CHD-W amplification outclassing that of the CHD-Z when both templates are present as targets for PCR (see Fridolfsson and Ellegren 1999). However in our case it did not happen on all females and there were individuals where two clear bands could be observed (see lanes 3, 13 and 25, Fig. 2). This might be due to variations in the quality of the DNA at the start of the amplification. However, because the size difference between the CHD-W and CHD-Z products were recognizable (with the CHD-Z being larger), even when females missed the CHD-Z product, the presence of the band at the CHD-W enabled the sex determination.

This protocol allowed us to extract DNA from a variety of feathers with different sizes. Yet, some samples of very small size did not yield enough DNA. We thus believe that small to medium sized feathers are the ideal target samples to extract DNA.

In conclusion, the DNA extraction protocol and the sexing technique we present in this study is an innovative tool that enables sex determination of Greater flamingo chicks from feathers bulbs. Both the sample collection and preservation, and the DNA extraction steps from feather samples are faster, easier and less expensive in comparison to that from blood samples. Finally, our sample collection protocol would not inflict any damage to the growing feathers; we avoided samples from the tail or the wing section of the chicks and collected small to middle sized feathers.

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