NOTES

Sex Determination in Greater Flamingo Chicks Through DNA Analysis

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Abstract.—In many bird species, sexing individuals accurately is impossible if no sexual dimorphism occurs. This is often particularly true for chicks. However, genomic differences between sexes exist, and molecular techniques make it possible to determine the sex at any stage, provided that a specifically-designed protocol exists. Such a protocol for the Greater Flamingo (*Phoenicopterus ruber roseus* Pallas) is presented here, together with results from Greater Flamingos in the wild. *Received 3 March 1999, accepted 27 March 1999.*

Key words.—Greater Flamingo, *Phoenicopterus ruber roseus*, PCR-based protocol, sex determination, sex ratio.

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For the majority of bird species, determining the sex of nestlings has not been possible without dissection, until the advent of molecular techniques. As the accuracy of sexing techniques increased, determination has become easier, and birds can now be readily sexed, regardless of age or size, and with little disturbance.

All DNA-based sex discriminating techniques rely on the genomic difference between male and female cells: in birds, females are heterogametic with both W and Z chromosomes, whereas males have only two Z chromosomes. Apart from use of flow cytometry to detect differences in the amount of DNA in the cells (provided that there are significant size differences between the W and Z chromosomes), all methods are PCR-based: any sequence that is only present on the W chromosome can theoretically be used as a marker of the female sex. However, most of that chromosome is made of highly-variable elements, usually preventing use of a method on more than one species (Griffiths *et al.* 1992; Ogawa *et al.* 1997; Lessells and Mateman 1998). The sequence of the chromo-helicase-DNA (*CHD*) gene, in contrast, is highly conserved among all non-ratite birds (Griffiths and Tiwari 1995; Ellegren 1996). Since there are two independent copies of the gene, one on each of the sex chromosomes, any difference between the two copies can be exploited to identify an individual's sex.

Sex has thus become easy to sort out, by two main means: fragment-specific PCR (Ellegren 1996) or PCR-RFLP (Griffiths and Tiwari 1995; Griffiths *et al.* 1996; Lessells *et al.* 1996). More recently, Griffiths *et al.* (1998) found that in most species a single pair of primers could be used to determine the sex of an individual bird through the amplification of an intron, the size of which differs between the W- and Z- linked copies.

This last method proved inapplicable for sex determination in the Greater Flamingo, the profile of females did not clearly differ from that of males on a three percent agarose gel. Thus, a PCR-RFLP-type protocol was developed instead.

We sampled the blood of twenty-five captive adults (sexed both according to a clear size dimorphism and to behavior) to develop the protocol (see Studer-Thiersch 1986 and Richter and Bourne 1990 for a description of the morphological method of sex determination in adult flamingos). This protocol was subsequently used to determine the sex of 133 chicks sampled during banding at the Camargue colony (France) in 1996. About 30 µl of blood were sampled with capillaries and directly transferred into one ml of Queen's buffer. Blood was stored at 4°C and DNA was extracted by precipitation with 5/2

v/v isopropanol and 1/10 v/v sodium acetate (3M, pH 8) after 15 minutes of incubation at 55°C with 400 µg of Proteinase K (Sigma). Following the protocol of Griffiths *et al.* (1996), the 111 bp region of interest was amplified using ten pmol of each of the P2 and P3 primers, as defined by Griffiths and Tiwari (1995), 100 µM of each dNTP, 1 unit of *Taq* polymerase (Eurogentec) in a 1× reaction buffer (75 mM Tris-HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01%(w/v) Tween 20), 20 ng of genomic DNA, 2 mM $MgCl_2$, in a total reaction volume of 20 µl. The PCR was run on a PTC100 thermocycler (MJ Research) with a denaturing step at 95°C for five minutes, followed by 35 cycles of 95°C for 35 s, 49°C for 35 s, and 40 s at 72°C, and a final step of ten minutes at 72°C.

Fragments from an adult individual of each sex were amplified, purified (DNA Purification System, Promega) and sequenced with a Thermosequenase Cycle Sequencing kit (Amersham). The sequences revealed two ambiguous nucleotidic sites, which proved to be due to differences between the autosomal and W-linked copies of the gene. One of these differences was found to correspond to an *Mbo* II restriction site only present in the autosomal sequence (see Fig. 1a).

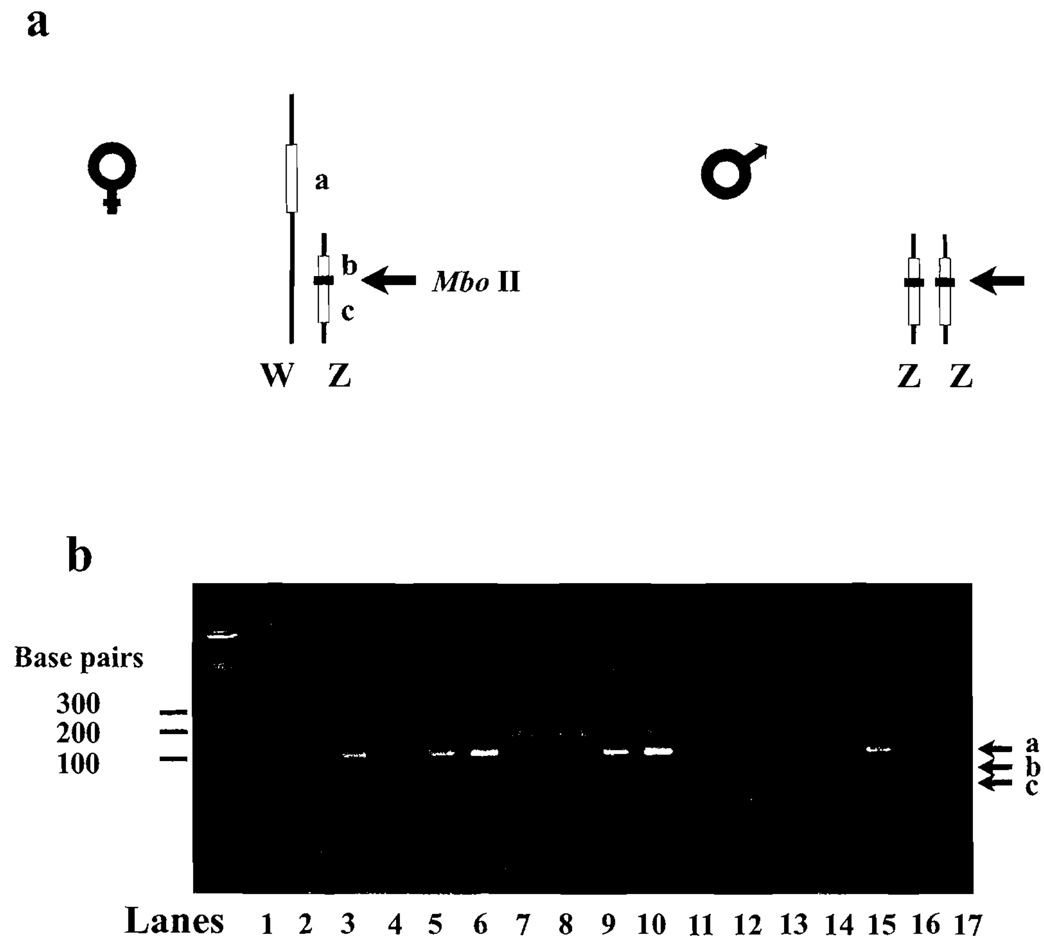

Figure 1. (a) The arrow indicates the *Mbo* II restriction site and the fragments are named as follows. a: total amplified fragment (111 bp), b and c: digestion products (70 and 41 bp, respectively). Only the Z-linked copy presents the restriction site, leading to fragments b and c, whereas the W-linked copy remains uncut, leading to fragment a; (b) results for 16 individuals run on a three percent agarose gel: females (lanes 3, 5, 6, 9, 10, 15 and 17) display both the complete band and bands corresponding to the restricted fragments (b and c), and males (lanes 1, 2, 4, 7, 8, 11 to 14 and 16) show only the bands of digested fragments. The first lane is a molecular weight marker.

For the sexing protocol, the 111 bp fragment was amplified as described above, and ten µl of the PCR product were then digested by five units of the *Mbo* II enzyme (Eurogentec) in its one × reaction buffer (3.3 mM Tris-acetate, pH 7.9, 1.0 mM Mg-acetate, 6.6 mM K-acetate; 0.01 mg/ml BSA) at 37°C for one hour. The digestion products were separated on a three-percent agarose gel stained with ethidium bromide and viewed under U.V. light (see Fig. 1b for an example). Tested on the 25 morphologically-sexed adults, the method gave a 100% concordance (8 males and 17 females).

Among the 133 chicks sexed, this simple protocol yielded 65 males and 68 females, corresponding to a ratio of males of 0.489, not significantly different from the value of 0.50 expected under the null hypothesis of a balanced sex ratio (binomial exact test, $P > 0.8$, see Siegel and Castellan 1988).

This technique was also tested on the Chilean Flamingo (*Phoenicopterus chilensis*), and correctly sexed four adults (three males and a female, sexed the same way as the Greater Flamingo adults). Its applicability to the other four flamingo species or sub-species remains to be tested.

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