# A Cheap and Quick Method for DNA-based Sexing of Birds

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**Abstract.**—Although the collection of air-dried blood samples on filter paper has been used for field work as a routine, it has required the use of special and expensive types of paper. In the present manuscript the use of common filter paper to collect and store air-dried bird blood samples is validated for its use in DNA based sexing techniques with a very simple method avoiding DNA purification for PCR. This method not only simplifies the laboratory work but also does not add any additional cost for DNA-based sexing techniques. The method was tested and successfully employed for sex determination by molecular techniques on blood samples taken on common filter paper during different field trips from four seabird species. *Received 16 May 2007, Accepted 01 February 2008.*

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Since the pioneer studies on gender identification of monomorphic birds using molecular biology techniques (Quinn *et al.* 1990; Longmire *et al.* 1993), different sexing techniques have been developed taking advantage of differences in length of introns in two chromo-helicase-DNA-binding genes (*CHD1*) located in avian sex chromosomes in all nonratite birds (Ellegren 1996; Griffiths *et al.* 1996, 1998; Lessells and Mateman 1996; Ellegren and Sheldon 1997; Kahn *et al.* 1998; Sheldon 1998; Fridolfsson and Ellegren 1999). These DNA-based techniques have been widely and successfully applied by field ornithologists to determine sex of adults and chicks, and constitute a remarkably useful methodological tool for ecology, evolutionary biology, breeding and conservation issues (Jodice *et al.* 2000; Bertellotti *et al.* 2002; Redman *et al.* 2002; Quintana *et al.* 2003; Copello *et al.* 2006; Svagelj and Quintana 2007).

The genetic sex determination in birds has been performed using genomic DNA extracted from feathers (Mundy *et al.* 1997; Eguchi and Eguchi 2000; Bello *et al.* 2001), tissue (Kahn *et al.* 1998; Fridolfsson and Ellegren 1999; Arnold *et al.* 2003), urine (Nota and Takenaka 1999) and blood samples (Ellegren 1996; Griffiths *et al.* 1996; Jodice *et al.* 2000; Redman *et al.* 2002; Tomasulo *et al.* 2002). Although the blood is a very good source of genomic DNA, both the preservation and transport of samples from the field to the laboratory may have some disadvantages. Usually the samples are stored in aqueous solutions, and some times they must be kept at low temperature before processing. For that reason, preservation buffers have been established (Seutin *et al.* 1991) and other researchers have applied the use of FTA® (Gutiérrez-Corchero *et al.* 2002) and IsoCode® cards (Fraga *et al.*, pers. comm.) for field collection and long term preservation of air dried blood samples to be used for DNA extraction.

Although the use of these cards makes DNA extraction easier, their availability and cost may be also inconvenient for field work. In the present manuscript, the use of common filter paper to collect and store air-dried bird blood samples is described and validated for its use in DNA-based sexing techniques in combination with a very simple method avoiding DNA purification for PCR. The method was validated for sex determination in four seabird species.

## STUDY AREA AND METHODS

### Study Species

Samples were taken from birds of known sex as determined by necropsy (gonadal inspection) from the following species: White-chinned Petrel (*Procellaria aequinoctiallis*) (six males and four females), Black-browed Albatross (*Thalassarche melanophrys*) (four males and one female) and Southern Giant Petrel (*Macronectes gi-*

*ganteus*) (one female). All these birds were sampled after death by incidental capture in the Argentine longliner fleet, and the heart was taken and preserved in ethanol 96% until DNA extraction. Furthermore, nine samples from Imperial Cormorant (*Phalacrocorax atriceps*) (four males, five females) were also sexed by behavioral methods, taking into consideration that, as in other "blue-eyed cormorant" species, males and females of this species can be distinguished by their vocalizations (Bernstein and Maxson 1982; Brothers 1985; Malacalza and Hall 1988; Green 1997; Casaux and Baroni 2000).

Blood samples from these last cormorants and from other individuals belonging to the other three species were obtained from living birds by pricking the basilic vein or by venipuncture of the jugular vein, using 22 or 23G × 2.5 cm needles. Three or four drops of blood were placed on a small (50 × 20 mm) piece of commercial filter paper (either common laboratory paper or a piece of a commercial cone type paper normally used for coffee filters). The piece of paper with the blood sample was air-dried and then each paper was stored separately in a small sealed plastic bag to avoid any contamination until analysis.

### Genetic Sex Determination

Once at the laboratory the samples, papers with a dried blood sample and tissues in ethanol were cut into small pieces, using sterile scissors and subjected to two different procedures. The same samples were treated in two different ways: (i) using proteinase K/phenol:chloroform based DNA extraction method with minor modifications (Sambrook *et al.* 1989) and (ii) adapting Tomasulo *et al.* (2002) method in order to avoid the traditional proteinase K/phenol:chloroform based DNA extraction procedure.

i) Small pieces of air-dried paper samples or tissue preserved in ethanol were placed in a lysis buffer containing 0.1M Tris, 0.05M sodium EDTA, 0.05 M NaCl, and 1% SDS at pH = 8. A proteinase K digestion was then performed for 3.5 h at 56°C, followed by two extractions of phenol:chloroform. The DNA was then precipitated by the addition of 7.5 M ammonium acetate and ethanol 100%, washed with ethanol 70%, dried and finally resuspended in TE buffer. Ethanol preserved samples from birds of known sex were first washed three times in lysis buffer to then proceed to proteinase K digestion and phenol:chloroform extraction.

ii) The samples were placed in a plastic 1.5 ml tubes and 200 µl of NaOH 50 mM was added. The tubes were heated for ten min in a boiling water bath and finally 100 µl were taken and neutralized with an equal volume of Tris-HCl 1M pH = 8, diluted 1:1 in TE buffer pH = 7.6 (TE), and stored at 4°C until PCR analysis.

The different size of an intron within the highly conserved *CHD1* gene was used to screen birds for sex differentiation using one pair of primers to amplify the *CHD1-W* and *CHD1-Z* genes located on the avian sex chromosomes as designed by Fridolfsson and Ellegren (1999):

Forward primer (2550F): 5' GTT ACT GAT TCG TCT ACG AGA 3'

Reverse primer (2718R): 5' ATT GAA ATG ATC CAG TGC TTG 3'.

In brief, PCR amplifications were performed in a total volume of 25 µl using either: (a) 1 µl of a 1:10 or 1:20 dilutions of each sample as described in (i) or (b) between 50-200 ng (1-5 µl) of purified genomic DNA as described in (ii), 2.5 U of Taq polymerase (Promega), 1 µl of stock solutions of each primer (25 µM), 0.5 µl of a dNTPs solution (10 µM) and a final concentration of 1.5 mM of $MgCl_2$. An initial denaturing step of five min at 95°C was then followed by 35 cycles of 30 s at 95°C, 45 s at 47°C and 30 s at 72°C, followed by five min at 72°C.

PCR products were separated in 1.8% agarose gels (Biodynamics, Argentina), following electrophoresis in standard TBE buffer and visualized by ethidium bromide staining to reveal the presence of one or two bands representing a male or a female pattern respectively.

The samples were analyzed between one month and five years after storage with no differences on the results.

## RESULTS AND DISCUSSION

### Comparison among Morphological, Behavioral and Genomic Sex

There was no mismatch among behavioral, morphological, and genomic sex. For example, the nine Imperial Cormorants sexed by both behavioral and genetic techniques were classified to be the same by both methods (100% in agreement). Furthermore, the morphological characteristics of the gonads of three different bird species (see above) were compared to the PCR profile obtained using 2550F and 2718R primers. Independently of the species, all samples belonging to females gave two PCR fragments (*CHD1-Z* and *CHD1-W*) meanwhile, all male samples gave only one (*CHD1-Z*). In all samples examined 2550F and 2718R, designed in order to amplify both copies of the gene revealed a difference of approximately 200 bp; *CHD1-Z* (650 bp) and *CHD1-W* (450 bp) as already reported by Fridolfsson and Ellegren (1999).

### Genetic DNA Sexing from DNA Obtained with Two Different Extraction Methods

PCR analyses were performed from template DNAs obtained from the same animal blood sample by different methods. Each air-dried bird blood sample preserved on common filter paper and then DNA extraction was performed either avoiding proteinase K/phenol:chloroform based methods (adapting Tomasulo *et al.* 2002) or using standard DNA extraction procedures and gave consistently the same results.

Independent of the extraction method used, the samples from the same bird gave two PCR fragments in case of females and

only one in the case of males. However, in some cases a preferential amplification of *CHD1-W* was evident in samples taken from females. As the *CHD1-Z* is evident in males, it was easy to distinguish the differential pattern (data not shown). It is important to note that this pattern was always seen when the samples were subjected to standard proteinase K/phenol:chloroform protocol but never when DNA was extracted using the method adapted from Tomasulo *et al.* (2002) even in the case when the same sample was subjected to PCR after different DNA extraction methods. At present, we have not a clear explanation for such a pattern.

Once the blood samples were dried on filter paper, in the laboratory the two DNA extraction methods described were performed and the samples subjected to PCR for molecular sex determination. It is important to note that the two extraction methods were equally successful to perform molecular sex determination. Some examples are given in Figure 1 where the same samples were subjected to different DNA extraction methods (compare lines 1 to 5; 2-6; 3-7 and 4-8).

Two different studies have already reported the use of specially-coated cards for long term preservation and easy isolation of high molecular weight DNA (FTA®, Gutiérrez-Corchuero *et al.* 2002; IsoCode® cards, Fraga *et al.,* pers. comm.). Both reports stressed the advantages for the use of these cards against blood preserved with different storage buffers or kept at low temperatures until the researchers got back to the laboratory. These advantages include: (i) a very small volume of blood can be taken from the bird (two drops are usually enough); (ii) the DNA is protected from environmental and microbiological degradation; (iii) DNA is directly extracted from the paper matrix with no need of standard DNA extraction protocols; (iv) the sample storage is easy and cheap. It is also easier to handle the cards compared to a set of tubes in the field; (v) samples can be sent everywhere with no problems using regular mail and (vi) samples can be stored for long time at room temperature and even longer at the freezer when they arrive to the laboratory. The use of IsoCode® cards is also easier than FTA® because there is no need of specific reagents and it takes only one h compared to the six h extraction method of the FTA® cards (Fraga *et al.*, pers. comm.).

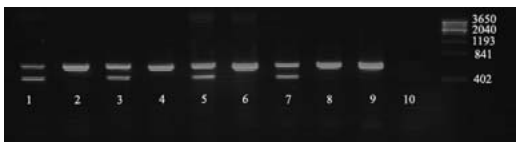


**Figure 1. DNA sex identification using PCR with 2550F and 2718R primers. From left to right, Lane 1: Female *Thalassarche melanophrys*. Simple DNA extraction. Lane 2: Male. *Thalassarchee melanophrys*. Simple DNA extraction. Lane 3: Female *Procellaria aequinoctiallis*. Simple DNA extraction. Lane 4: Male *Procellaria aequinoctiallis*. Simple DNA extraction. Lane 5: Female *Thalassarche melanophrys*. Sample subjected to standard proteinase K based DNA extraction. Lane 6: Male. *Thalassarche melanophrys*. Sample subjected to standard proteinase K based DNA extraction. Lane 7: Female. *Procellaria aequinoctiallis*. Sample subjected to standard proteinase K based DNA extraction. Lane 8: Male. *Procellaria aequinoctiallis*. Sample subjected to standard proteinase K based DNA extraction. Lane 9: Positive control. Lane 10: Negative control.**

The method reported in this paper shares these advantages and also adds three more: (i) it is much cheaper; (ii) cone type paper for coffee filters can be obtained at any home store, a point that is particularly important when field work has to be done far away from chemical stores and (iii) the extraction of material for PCR takes 30 min at the most being the results as good as the ones obtained using purified DNA.

Finally, the common filter-based system with the use of a very simple method for extracting material for PCR not only simplifies the laboratory work but also does not add any additional cost for DNA-based sexing techniques. Furthermore, it is a very simple way to avoid the use of tubes and refrigerated transport in the field and the use of any special kind of paper difficult to obtain in field work if any problem occurs.

In summary, the common filter-based preservation system is a good way to preserved blood samples for PCR based studies. This method is not only easy for filed studies but also can be used to preserve the material for a long time (at least for five years). In addition, PCR based bird sexing can be easily performed with a simple method with no DNA purification.

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