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Using PCR-RFLP for sexing of the endangered Galápagos petrel (*Pterodroma phaeopygia*)

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ABSTRACT. *Pterodroma phaeopygia* is a critically endangered avian species of the Galápagos Islands. This bird is sexually monomorphic, making it difficult to identify the sex. This information, however, is relevant to studies of behavior, ecology, and management of wild or captive populations. Here, we aimed to implement a molecular approach for determining sex in this petrel. DNA was extracted from the blood and the feathers of 24 adult *P. phaeopygia*, with samples from a female and a male *Gallus gallus* for comparison. We amplified the cromo-helicase DNA binding protein 1 (*CHD-1*) gene by PCR, using primers P2 and P8. Allele *CHD-1*W is unique to females and *CHD-1Z* occurs in both sexes. We then digested these PCR products using the restriction enzyme *Hae*III. The PCR amplified a 400-bp product for both alleles. The digestion of the *G. gallus*, amplicons split the *CHD-1Z* allele into two fragments (of 320 and 80 bp), while *CHD-1*W

remained intact. Thus, the male exhibited two bands (digested *CHD-1*Z) and the female three bands (undigested *CHD-1*W and digested *CHD-1*Z). Applying this RFLP method on DNA derived from blood, 9 of the 24 petrels were found to be male, while 15 were females. The same results were obtained using feathers as the source of DNA. To our knowledge, this is the first report of molecular method for sexing this species. The potential of sexing this petrel from feathers is remarkable as it minimizes blood sampling induced stress. This method could be used to reinforce the conservation efforts for this bird, to investigate population sex ratios and to develop new conservation strategies.

Key words: *Pterodroma phaeopygia*; Bird sexing; Galapágos petrel; Avian management; Avian conservation

INTRODUCTION

The Galápagos petrel, *Pterodroma phaeopygia* (Procellaridae), is an endemic bird from the Galápagos Archipelago, where it is known to breed on five islands: Santa Cruz, Floreana, Santiago, San Cristóbal, and Isabela (Friesen et al., 2006). They nest in burrows that are mostly built in the soft soil of water gullies or in deep holes in the slopes of craters. They are annual breeders and do not replace their single egg if it is damaged or lost (Harris, 1970). The Galápagos petrel reached a dramatically low population size in the 1990s, mainly as a result of depredation by introduced alien species (Cruz and Cruz, 1990). Currently, this bird is ranked as critically endangered by the IUCN (IUCN, 2012).

To guarantee the conservation of *P. phaeopygia*, the Galápagos National Park Directorate (GNPD) has been carrying out management actions (Coulter et al., 1985; Cruz and Cruz, 1987). Efforts are focused on eradication of introduced predators (Cruz et al., 2005) and studies have been carried out into the distribution, diet (Harris, 1970), genetics (Friesen et al., 2006), reproductive success and breeding characteristics of the species (Harris, 1970; Tomkins, 1985; Cruz-Delgado et al., 2010). However, there is little information relating to gender identification of this petrel. *P. phaeopygia* is sexually monomorphic for over 50% of the birds. For avian species, this feature is unfavorable for studies of sex ratios, population viability analysis (Gilpin and Soulé, 1986), breeding and nestling care strategies, or for implementing management programs, such as assisted reproduction in captivity or translocation of nestlings (Helander et al., 2007; García et al., 2009; Naim et al., 2011).

Early attempts in sexing Galápagos petrels included cloacal examination, laparotomy of dead birds (Harris, 1970), morphometry (Cruz and Cruz, 1990) and analysis of vocalizations (Tomkins, 1985). Unfortunately, these techniques have drawbacks that limit their routine use. Cloacal examination is applicable only to certain adults, specifically females that have recently laid an egg, and their respective mates (Boersma and Davis, 1987). Surgical procedures should be avoided in live endangered birds (Miyaki et al., 1998). Morphometry requires multivariate analysis (Cruz and Cruz, 1990) and vocalization records can take some years to generate sufficient resolution (Genevois and Bretagnolle, 1995).

Currently, several studies have demonstrated genetic-based techniques as a simple, safe, and effective approach to sexing birds, in both field and captive breeding projects (Bermúdez-Humarán et al., 2002). The genetic method relies on DNA sequence differences in

the sex chromosomes of the birds. In avian species, females are heterogametic (ZW), while males are homogametic (ZZ). Therefore, it is expected that some DNA sequences will occur in females but not in males (Smith and Sinclair, 2001). Although the gene encoding chromohelicase-DNA binding protein 1 (*CHD-1*) is found on both Z and W chromosomes, their alleles are not identical, providing an opportunity to distinguish avian sex (Ellegren, 1996).

PCR with a single pair of primers, P2 and P8, has commonly been used to amplify a length of DNA spanning an intron in the *CHD-1* gene. Frequently, the intron differs in size between the two sex chromosomes resulting in PCR products that, on the basis of agarose gel electrophoresis, separate into two bands for females (the *CHD-1*Z and *CHD-1*W alleles) and a single band for males (the *CHD-1*Z allele) (Griffiths et al., 1998). In some species, the intron size is similar, but an RFLP assay of the PCR products facilitates identification of the alleles. The *Hae*III restriction site has been shown to be highly conserved in the *CHD-1Z* allele of many avian species but does not occur on the *CHD-1W* allele, making it useful for gender determination (Boutette et al., 2002).

The importance of this PCR based method has been emphasized in conservation projects of different avian species including petrels, e.g., the Antarctic petrel *Thalassoica antarctica*, and the endangered *P. magentae*, which is endemic to the Chatham Islands in New Zealand (Bertault et al., 1999; Jarvi and Banko, 2000; Svein-Håkon et al., 2000; Russello and Amato, 2001; Lawrence et al., 2008). In this context, we aimed at sexing a population of *P. phaeopygia* by using this approach.

MATERIAL AND METHODS

Samples

Blood and feather quill samples from a colony of *P. phaeopygia* were collected over a period of four days, in May 2005, taking advantage of the annual petrel census carried out by the GNPD on Santa Cruz Island. Previously marked nests were localized using global positioning system (GPS) data. Each petrel was caught by hand, measured, weighed, and checked for an existing tag, or ring tagged if this was absent. Blood samples (a volume less than 1% of the bird's weight) were extracted from the brachial vein, using a hypodermic syringe and a 21G needle, then transferred to 0.5 mL EDTA tubes. Feather quills were extracted from the region surrounding the cloaca using sterilized forceps, and were placed in a microtube containing a buffer solution (50 mM Tris HCl, pH 8.0, 20 mM EDTA, pH 8, 2% SDS) as described by Bello et al. (2001). All samples were kept at 4°C until transported to the laboratory where blood was stored at -20°C and feather tips at 4°C. Blood samples from one female and one male *G. gallus* were also collected to act as a positive control. The sampling procedures were approved by the GNPD, were performed by their veterinary staff, and all efforts were made to minimize the stress of handling and sampling the birds.

DNA isolation

Whole blood DNA isolation was performed according to Bermúdez-Humarán et al. (2002) with few modifications. Briefly: 25 µL of blood sample was added to 500 µL lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0) and 100 µg/mL

proteinase K was incubated at 55°C overnight. A standard PCI (phenol: chloroform: isoamyl alcohol 25:24:1) procedure was followed for DNA purification (Sambrook and Russell, 2001). Feather quill DNA was isolated according to the method of Bello et al. (2001), except with an incubation temperature for lysis of 37°C.

PCR-RFLP

Standardized PCRs were performed on DNA from all samples using a PTC-200 thermal cycler (MJ Research). The PCRs were carried out in a volume of 50 µL per sample, containing 1X buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (each) (Invitrogen), 20 pmol of each primer [P8: 5'-TCCCAAGGATGAGRAAYTG-3' and P2: 5'-CTGCATCGCTAAATCCTTT-3'] (Griffiths et al., 1998) and 1.5 U *Taq* polymerase (Promega M166A). The thermal profile for amplification was 94°C for 5 min; 40 cycles of 94°C for 45 s, 49°C for 42 s, and 72°C for 1 min; with a final step at 72°C for 7 min. PCR products were visualized by electrophoresis in 3% agarose gels containing ethidium bromide, and run at 70 V for 60 min in 1X TBE buffer. Alternatively, products were first subjected to digestion with restriction endonuclease *Hae*III. Each digestion was performed in a volume of 30 µL containing 27 µL of PCR product, 0.5 µL restriction endonuclease (Invitrogen cat. 15205-016) and 1X of specific buffer. The incubation was carried out at 37°C for 2 hours following the manufacturer protocol.

RESULTS

Two hundred nests recorded in a previous census were located by GPS data. Only 22 burrows were occupied at the time of the visit, 20 hosted one individual and 2 hosted couples. Blood and feather quill samples were obtained from all 24 individuals, together with the parameters required for the census. Forty-eight samples (24 from blood and 24 from quills) were processed for DNA isolation and subsequent PCR.

Using the primers P2 and P8, we obtained amplicons of 400 bp from all the samples analyzed, with no discrimination between males and females (Figure 1). Similar results were obtained using the DNA isolated from the blood of *G. gallus*.

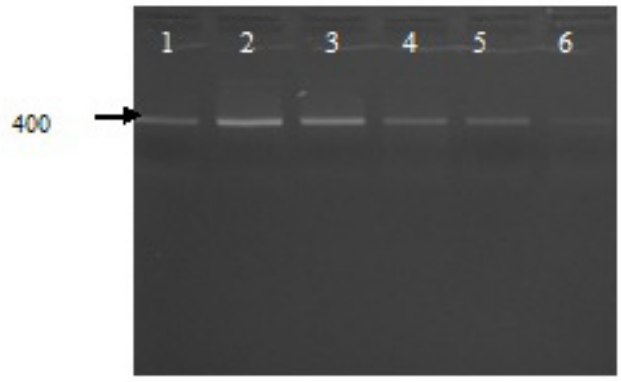

Figure 1. PCR product obtained with primers P2-P8 from *Pterodroma phaeopygia* blood samples and analyzed by electrophoresis on a 3% agarose gel. *Lanes 1-4 = P. phaeopygia*, *lanes 5-6* internal controls (*Gallus gallus*).

The subsequent digestion of the amplicons with *Hae*III led to two different restriction profiles corresponding to the digestion of the allele *CHD-1Z* (Figure 2). As can be seen from the control, the male (*CHD-1Z*/*CHD-1Z*) exhibited two bands as a result of the digestion of the PCR product into two fragments of 320 and 80 bp, while the female (*CHD-1Z*/*CHD-1W*) displayed three bands; two corresponding to the digested fragments of the *CHD-1Z* allele and one corresponding to the undigested *CHD-1*W allele (400 bp).

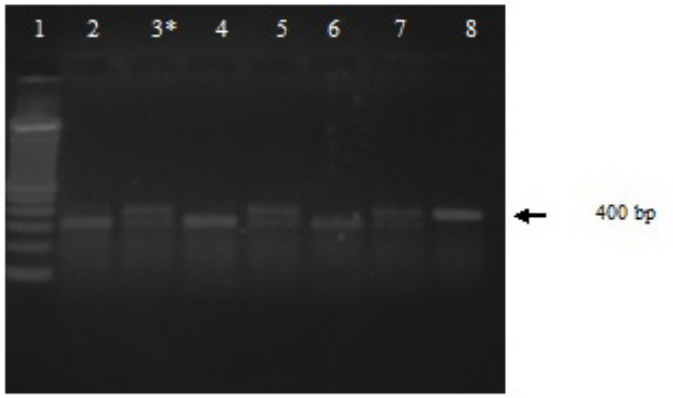

Figure 2. Restriction patterns of CHD amplicons after digestion with *Hae*III. *Lane 1* = MPM of 100 bp; *lanes 2-3* = internal controls: male and female, respectively; *lanes 4-7 = Pterodroma phaeopygia*; *lane 8* = internal control without digestion. *Note that after restriction with *Hae*III two bands are generated for *CHD-1Z* alleles. On the agarose gel, we observe one as the smaller (80 bp) migrate at the position of primers.

After PCR-RFLP, nine had DNA profiles identical to the female *G. gallus* control and fifteen had DNA profiles identical to the male control. For the petrels found as couples, one individual from each burrow bore the restriction profile of a female and the other of a male (not shown). Female and male petrels as identified by PCR-RFLP did not show any differences in the morphologic characters registered during census.

DISCUSSION

Accurate identification of sex in birds is important for the management and conservation of avian wildlife in several ways, i.e., clarifying behavioral and ecological studies, and improvement of *in situ* or *ex situ* conservation programs (Donald, 2007). This information is even more critical for studying sex ratio in threatened bird populations, which is often heavily skewed and increases the risk of extinction (Donald, 2007; Lambertucci et al., 2012). *P. phaeopygia* is at the center of important conservation efforts by the GNPD, and so a reliable method for obtaining and including gender data to evaluate their conservation status or guarantee its management is essential.

In this study, the evaluation of a simple PCR did not permit gender diagnosis of the Galápagos petrel. However, this was achieved by combining the PCR with digestion with a restriction enzyme. The enzyme *Hae*III generated (RFLP) restriction profiles similar to those obtained from various orders of birds (Boutette et al., 2002), including petrels (Lawrence et al., 2008).

The petrel genders diagnosed from blood samples were reproducible using feathers. This method is subject to technical failure, resulting from polymorphism in the hybridization sites of primers, preferential amplification or polymorphism of Z chromosome (Dawson et al., 2001; Robertson and Gemmell, 2006). An additional molecular test, to evaluate the incidence of this technical failure in detecting sex by PCR, could not be performed within the scope of this study. Future work is required to assess this issue.

Given the reproducibility observed, both blood and feathers proved to be a reliable source of DNA and the method proved to be an efficient and effective technique for genetic sexing Galápagos petrels. The potential for sexing petrels from DNA obtained from feathers is relevant, as feather sampling simplifies and minimizes handling, reducing stress to the animal, particularly when sexing chicks or other fragile individuals (Griffiths et al., 1998; Bello et al., 2001).

Female and male petrels as identified by PCR-RFLP did not show any differences by morphometry, however the sample size was small and future studies could be oriented towards identifying morphological differences between the sexes based on the molecular markers. For example, in other studies comparing molecular and morphological characters, sexual plumage dichromatism and iris color differences were identified and validated as reliable traits for sex identification (Igic et al., 2010; Nogueira and Alves, 2008).

To our knowledge, the PCR-RFLP reported here is the first application of molecular sexing in *P. phaeopygia*. While further promising DNA sexing methods are being developed (see Morinha et al., 2012 for a review), this can already be integrated as a conservation tool for this species.

The use of a PCR-RFLP for sexing petrels is illustrated in *T. antarctica* and *P. magenta*. For the first species, the molecular method led to the identification of a pair of female nestlings (Svein-Håkon et al., 2000). For the second, the method led to the identification of biased sex ratios in chicks and adults. Furthermore, for *P. magenta* the sexing approach is being applied in a management program involving the translocation of male chicks to artificial nests in predator-free zones. The goal of this translocation is to guarantee the survival of the chicks and establish new reproductive colonies at sites where it is expected; considering the philopatric tendency of these species, it is likely that they will return when the reach sexual maturity (Lawrence et al., 2008).

CONCLUSION

A PCR-RFLP method for sexing *P. phaeopygia* was optimized. It was found that the PCR RFLP method from feather samples alone was sufficient for reliable and rapid sexing of individuals of this species, without any additional risk. This molecular method, in combination with procedures that support its accuracy, can be used to reinforce the efforts to conserve this bird. This technology is available for the GNPD and can be validated for other Galápagos avian species.

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