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Molecular sexing of unusually large numbers of *Spheniscus magellanicus* (Spheniscidae) washed ashore along the Brazilian coast in 2008

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ABSTRACT. There have been few studies on Magellanic penguins (*Spheniscus magellanicus*). In 2008, these penguins washed ashore along the Brazilian coast in unusually high numbers, some reaching as far as northeast Brazil. As Magellanic penguins show little sexual dimorphism, sex determination by morphological features is not accurate. Here, we tested a molecular procedure for sexing specimens of *S. magellanicus* washed ashore along the coasts of Sergipe, Rio

de Janeiro and Rio Grande do Sul in 2008, comparing the sex ratio between these localities. Tissue samples were collected from 135 dead, beached specimens. We carried out total genomic DNA extraction and CHD-Z/CHD-W gene amplification by PCR using P2 and P8 primers. Amplicons were separated by 12% acrylamide gel electrophoresis. We found a greater proportion of females (70%). Sex could be determined because females have two intronic regions of CHD gene of different size in the sex chromosomes, visualized as two bands on the gel (380 and 400 bp approximately), while males have only one (400 bp). Therefore, this method proved to be effective and sensitive for sex determination of *S. magellanicus* individuals. Data on sex ratios are useful for understanding the dynamics and ecology of Magellanic penguin populations.

Key words: DNA sexing; Sex determination; Sex chromosomes; CHD introns; Magellanic penguin

INTRODUCTION

Morphological identification of sex in birds can often be difficult, and thus, the innovation of molecular sexing techniques is crucial for investigating many subjects in ecology and evolution (Ellegren and Sheldon, 1997; Dubiec and Zagalska-Neubauer, 2006; Shizuka and Lyon, 2008). The molecular methods most frequently used for sexing birds are based on the size differences between introns of the CHD gene on the Z and W sex chromosomes (Griffiths et al., 1998; Fridolfsson and Ellegren, 1999). Heterogametic females (ZW) are expected to have two different-sized introns, while homogametic males (ZZ) should show only one (Shizuka and Lyon, 2008).

The Magellanic penguin, *Spheniscus magellanicus* (Forster, 1781), is the most abundant species of penguins in South America, with breeding colonies along the Pacific and Atlantic coasts (Chile and Argentina), including the Falkland Islands (Scolaro, 1987a; Stokes and Boersma, 1999; Yorio et al., 2001). On the Atlantic coast, the species disperses northward after the breeding season, usually reaching the southern coast of Brazil (Sick, 1997; García-Borboroglu et al., 2006). *S. magellanicus* is the most abundant bird species stranding annually along the Brazilian coast, mainly during austral winter and spring (Sick, 1997; Petry et al., 2004; Vega et al., 2009; Mäder et al., 2010). In 2008, Magellanic penguins reached the Brazilian coast at an unusual frequency, including reports of stranded specimens in Northeast Brazil, an extremely rare event (García-Borboroglu et al., 2010).

This species shows little sexual dimorphism (Martínez, 1992; Agnew and Kerry, 1995) and is generally sexed using biometric data (Amat et al., 1993; Renner et al., 1998), copulation observation (Scolaro et al., 1990), cloacal examination (Renner et al., 1998; Renner and Davies, 1999), or dissection (Scolaro et al., 1983, 1987b). However, these approaches present limitations, and just molecular procedures have proved to be useful for positive sexing of individuals at any life stage (Bertellotti et al., 2002). Although some genetic procedures have been applied to penguin species (Dubach, 1996; Costantini et al., 2008; Lee et al., 2010), these techniques were applied just once for Magellanic penguins (see Bertellotti et al., 2002).

In the present study, we tested a molecular procedure for sex determination of 135 samples of dead *S. magellanicus* stranded along the Sergipe, Rio de Janeiro and Rio Grande do Sul coasts in 2008, comparing the sex ratio between these localities.

MATERIAL AND METHODS

GEMM-Lagos (Grupo de Estudos de Mamíferos Marinhos da Região dos Lagos) staff and collaborators made regular beach monitoring along the Brazilian coast in 2008, recovering stranded marine mammals, sea turtles and seabirds (Figure 1). A total of 135 samples were collected from dead specimens of *S. magellanicus* found in Sergipe (N = 12), Rio de Janeiro (N = 106) and Rio Grande do Sul (N = 17). Most of the individuals were immature (75 specimens from a total of 79 classified according to life stage), since they lacked the clearly defined banding of the adults (Sick, 1997). Samples of liver and/or breast muscle were collected and stored in 70% Ethanol. The sex of some fresh individuals was determined by dissection (N = 10, six females and four males), and they were used as positive controls for polymerase chain reaction (PCR).

Figure 1. Collection sites and number (N) of stranded Magellanic penguin (*Spheniscus magellanicus*) samples gathered along the Brazilian coast in 2008.

Total genomic DNA extraction was carried out according to a modified protocol from Damato and Corach (1996), using a lysis buffer containing 2% cetyl trimethyl ammonium bromide (CTAB), 100 mM Tris-HCl pH 8.0, 20 mM EDTA and 1.4 M NaCl. CHD-Z and CHD-W introns were amplified by PCR using P2 (5'-TCT GCA TCG CTA AAT CCT TT-3') and P8 (5'-CTC CCA AGG ATG AGR AAY TG-3') primers (Griffiths et al., 1998). Each 10-µL reaction mixture contained about 10 ng genomic DNA, 1 µM of each primer and 0.25 U *Taq* DNA polymerase (Biotools), also including 2 mM $MgCl_2$ and 200 µM dNTPs. The PCR amplifica-

tion profile was 94°C for 2 min, followed by 40 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 45 s, and a final extension step of 72°C for 5 min. All reactions were carried out including positive and negative controls (template-free reactions) in order to test for contamination and to assure the fidelity of the PCR amplifications.

Each 10 μL of amplified fragment were mixed with 2 μL of loading buffer (30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol). This mixture was loaded on a non-denaturing 12% acrylamide gel (99:1 acrylamide:bis-acrylamide) (Sambrook et al., 1989), and run for 3 h at 140 V. Afterwards, the gels were stained with silver nitrate according to Bassam et al. (1991) and dried, and the image was digitized.

After sexing *S. magellanicus* from Sergipe, Rio de Janeiro and Rio Grande do Sul, a chi-square test was used to compare the sex ratio in these three sampled sites.

RESULTS

CHD-W and CHD-Z introns of Magellanic penguins showed about 400 bp and 380 bp, respectively, when amplified with P2 and P8 primers (Figure 2). Such small differences between CHD-W and CHD-Z amplified fragments (20 bp) required runs on 12% acrylamide gels to better separate the bands, since acrylamide provides better resolution compared to agarose (Dubiec and Zagalska-Neubauer, 2006). However, similar fragment lengths were found by Bertellotti et al. (2002), separating fragments on 3% agarose gel. In the present study, the ten individuals (six females and four males) of *S. magellanicus* used as controls for PCR showed 100% correlation between molecular and morphological sex determination.

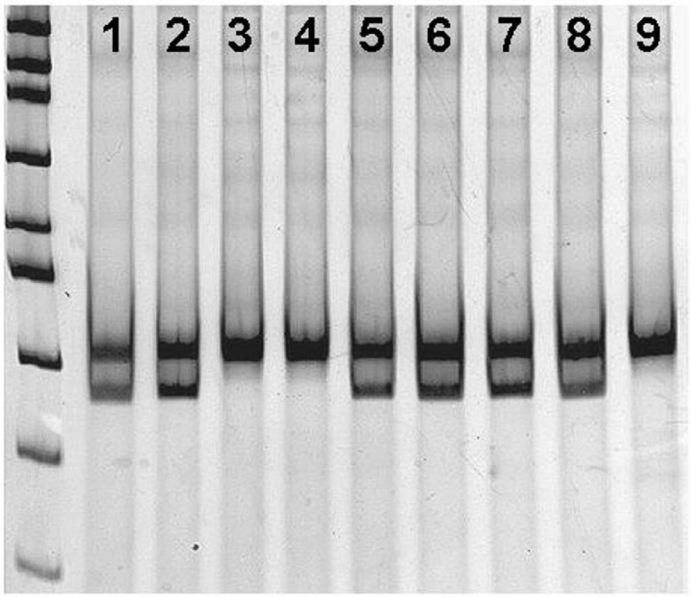

Figure 2. Amplicons of CHD-Z and CHD-W introns of Magellanic penguins (*Spheniscus magellanicus*) separated by 12% acrylamide gel electrophoresis and stained with silver nitrate. *Lanes 1*,*2* and *5*-*8* = heterogametic females (ZW), *lanes 3*,*4* and *9* = homogametic males (ZZ). At the left, Gene Ruler™ 100 bp DNA ladder (Fermentas - Life Sciences) with the following visible fragments in base pairs: 1031, 900, 800, 700, 600, 500, 400, 300 and 200.

Five individuals (three from Rio de Janeiro and two from Rio Grande do Sul) could not be sexed due to their advanced state of decomposition. Sex determination of 130 samples of *S. magellanicus* stranded along the Brazilian coast in 2008 indicated a greater proportion of females (70%). Magellanic penguins from Rio de Janeiro (N = 103) showed 70% females, more than double the number of males (30%). Sergipe and Rio Grande do Sul, even with smaller sample sizes (N = 12 and N = 15, respectively), showed similar ratios (Figure 3). A chi-square test demonstrated that there were no differences in *S. magellanicus* sex ratio between Sergipe, Rio de Janeiro and Rio Grande do Sul ($\chi^2 = 1.62$; df = 2; $P \approx 0.5$).

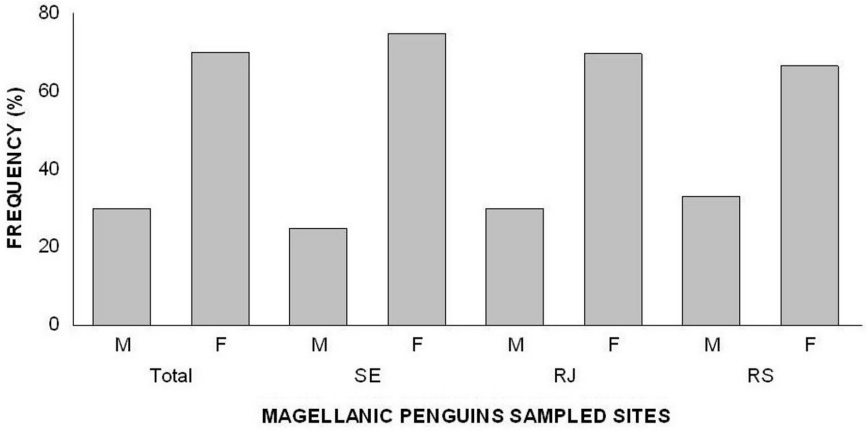

Figure 3. Sex ratio of Magellanic penguins (*Spheniscus magellanicus*) stranded along the Brazilian coast in 2008. M = male; F = female; SE = Sergipe; RJ = Rio de Janeiro, and RS = Rio Grande do Sul.

DISCUSSION

The molecular method used in the present study proved to be effective and sensitive for sex determination of *S. magellanicus* individuals. Heterogametic females (ZW) could be distinguished on polyacrylamide gel electrophoresis (PAGE) by two bands, while homogametic males (ZZ) showed only one. Bertellotti et al. (2002) correlated molecular and biometric approaches, and concluded that only molecular procedures proved appropriate for accurately sexing both adults and chicks of Magellanic penguins. Dubiec and Zagalska-Neubauer (2006) still highlighted that sex determination based on the amplification of CHD genes is the most rapid, inexpensive and reliable technique. Nonetheless, some studies have pointed to the existence of polymorphisms in CHD-Z introns of several bird species, which complicates the determination of sex since heterozygote males will also have two different-sized introns (Dawson et al., 2001; Lee et al., 2002; Robertson and Gemmell, 2006). It is still unknown how many species possess polymorphism in the CHD-Z gene, which will only be detected with adequate sampling of known sexed individuals (Shizuka and Lyon, 2008).

Our results indicated a greater proportion of females, which could be explained if colonies of Magellanic penguins have a female-biased sex ratio. However, in many species of penguins, the sex ratio is markedly biased towards males (Williams, 1996). Another hypothesis to explain this greater proportion of stranded females along the Brazilian coast is that dispersal forays of females could begin earlier in their lives, consequently increasing female mortality. As females begin breeding before males (Scolaro, 1987a), it is also possible that females also start their migration first. In

fact, in the present study, 95% of the specimens sampled that were classified according to life stage were juveniles. The predominance of juveniles of *S. magellanicus* along the Brazilian coast was also noticed in previous studies (e.g., García-Borboroglu et al., 2006; 2010; Mäder et al., 2010). Satellite telemetry studies have already demonstrated extensive foraging ranges among Magellanic penguins (Stokes and Boersma, 1999; Pütz et al., 2000; Boersma et al., 2009). By mid-April, all adults have completed their molt and start their annual pelagic migration, with many individuals reaching the Southern coast of Brazil (Scolaro, 1987a; García-Borboroglu et al., 2010).

Despite being probably by far the most abundant bird species stranded along the Brazilian coast, Magellanic penguins are still poorly studied. Hence, the continuity of this kind of study and related ones is very important. Data on sex determination will be important for better understanding the dynamics and ecology of Magellanic penguin populations, including sex ratio development, sexual selection, parental care strategies, migration, demography and conservation (Shizuka and Lyon, 2008).

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