Sex Identification of Four Penguin Species Using Locus-Specific PCR

Peijun Zhang,1,2,3 Jiabo Han,1 Quansheng Liu,4 Junxin Zhang,4 and Xianfeng Zhang2,3∗

1Liaoning Ocean and Fisheries Science Research Institute, Department of Conservation Biology of Endangered Animals, Dalian, China
2 Institute of Hydrobiology, Chinese Academy of Sciences, Research Group of Conservation Biology of Aquatic Animals, Wuhan, China
3Laboratory of Biodiversity and Conservation of Aquatic Organisms, Chinese Academy of Sciences, Wuhan, China
4Hangzhou Polar Ocean Park, Center of Aquatic Animals, Hangzhou, China

Traditional methods for sex identification are not applicable to sexually monomorphic species, leading to difficulties in the management of their breeding programs. To identify sex in sexually monomorphic birds, molecular methods have been established. Two established primer pairs (2550F/2718R and p8/p2) amplify the CHD1 gene region from both the Z and W chromosomes. Here, we evaluated the use of these primers for sex identification in four sexually monomorphic penguin species: king penguins (Aptenodytes patagonicus), rockhopper penguins (Eudyptes chrysocome), gentoo penguins (Pygoscelis papua), and Magellanic penguins (Spheniscus magellanicus). For all species except rockhopper penguins, primer pair 2550F/2718R resulted in two distinct CHD1Z and CHD1W PCR bands, allowing for sex identification. For rockhopper penguins, only primer pair p8/p2 yielded different CHD1Z and CHD1W bands, which were faint and similar in size making them difficult to distinguish. As a result, we designed a new primer pair (PL/PR) that efficiently determined the gender of individuals from all four penguin species. Sequencing of the PCR products confirmed that they were from the CHD1 gene region. Primer pair PL/PR can be evaluated for use in sexing other penguin species, which will be crucial for the management of new penguin breeding programs. Zoo Biol. 32:257–261, 2013. © 2012 Wiley Periodicals, Inc.

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INTRODUCTION

Penguins are globally popular in zoos and aquariums [Andrews et al., 2008; Ghiron et al., 2008; Mann and Mann, 2008; Yamaguchi et al., 2008; Yu et al., 2008]. To maintain penguin colonies at these educational institutions, it is necessary to manage captive breeding programs. However, the success of these programs is limited by correct gender assignment of these sexually monomorphic birds [Bi and Zhao, 2006; Ma and Jia, 2006; Yu et al., 2006; Yu et al., 2008]. In other sexually monomorphic birds, the problem of sex identification is solved in two ways. Traditional methods, such as cloacal examination, biochemical and cytogenetic analysis, and sound discrimination, are time-consuming methods and can stress the animal [Bermúdez-Humarán et al., 2002; Quinn et al., 1990]. Alternatively, a molecular method for sex identification relies on the amplification of the chromo-helicase-DNA-binding 1 (CHD1) gene found on the sex chromosomes. Assuming the length of the CHD1 region differs between the two chromosomes, males will have a single product (CHD1Z) and females for two (CHD1W and CHD1Z) [Ellegren and Sheldon, 1997; Griffiths et al., 1996; Kahn et al., 1996; Sacharczuk et al., 2002] that can be distinguished by gel electrophoresis. In some species, primer pair preferentially amplifies one CHD1 gene, leading to undetectable amounts of the other one [Fridolfsson and Grant sponsor: Ocean Park Conservation Foundation, Hong Kong; Grant number: IHB/CN/2007065.

∗Correspondence to: Xianfeng Zhang, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China. E-mail: zhangx@ihb.ac.cn
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Ellegren, 1999; Griffiths et al., 1998]. When this happens, PCR failure of \( \text{CHD1Z} \) fragment results to a detection of only the \( \text{CHD1W} \) gene in females, otherwise, results to the sex failure in females. However, a previous popular study of molecular method focused on birds of Carinatae, while the validity for Impennes were seldom related [Fridolfsson and Ellegren, 1999; Griffiths et al., 1998].

In this study, we evaluate the performance of two established \( \text{CHD1} \) primers in the sex identification of juvenile penguins from four species: king penguins (\( \text{Aptenodytes patagonicus} \), KP), rockhopper penguins (\( \text{Eudyptes chryso-come} \), RP), gentoo penguins (\( \text{Pygoscelis papua} \), GP), and Magellanian penguins (\( \text{Spheniscus magellanicus} \), MP). Detailed biology information of the four penguin species is shown in Table 1.

**MATERIAL AND METHODS**

We sampled juvenile penguins from the Hangzhou Polar Ocean Park in China. Our samples included two KPs, two RPs, eight GPs, and 30 MPs. From each individual, we used venipuncture to collect a blood sample. We mixed 1-μl blood with 100-μl anhydrous alcohol and stored the mixture at room temperature. We extracted genomic DNA from the blood samples using a Wizard Genomic DNA Purification Kit (SBS, Shanghai, China) according to the manufacturer’s instructions.

We used two established primer pairs, 2550F (5′-GTT ACT TCG TCT ACG AGA-3′)/2718R (5′-ATT GAA ATG ATC CAG TGC TTG-3′) [Fridolfsson and Ellegren, 1999] and p8 (5′-CTC CCA AGG ATG AGR AAY TG-3′)/p2 (5′-TCT GCA TCG CTA AAT CCT TT-3′) [Griffiths et al., 1998], to amplify \( \text{CHD1} \) from the four penguin species. Using Primer3 V0.4.0 [Steve and Helen, 2000] on the sequences amplified by p8/p2, we designed a new primer pair PL (5′-CCC AAG GAT GAT AAA TTG TGC-3′)/PR (5′-CAC TTC CAT TAA AGC TGA TCT GG-3′) to amplify \( \text{CHD1} \) from penguins.

All PCR reactions were conducted on a Techne® TC-5000 (Bibby Scientific, Chelmsford, UK) machine with a final volume of 100 μl containing 1 × PCR buffer (10 mM Tris-Cl, 50 mM KCl, pH 8.3), 0.5 μM of each primer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.25U Taq DNA polymerase (Biostar), and 10–100-ng genomic DNA. For all PCRs, an initial denaturation (94°C/5 min) was followed by 36 cycles of denaturation (94°C/30 sec), annealing (2550F/2718R and PL/PR: 55°C/45 sec, p8/p2: 48.5°C/45 sec), and extension (72°C/45 sec), and ended with a final extension (72°C/10 min). Here, we used 100-μl PCR reactions considering the following DNA sequencing, otherwise, 10 μl was enough. All PCR products were separated on 3% agarose gel (Sangon, Shanghai, China), and purified with the DNA Purification Kit (Biostar, Shanghai, China) according to the manufacturer’s instructions when sequencing.

Purified PCR products of p8/p2 and PL/PR were cloned into pMD18-T vectors (Takara, Dalian, China) and transformed into DH5α competent cells following the manufacturer’s recommendation. After incubation at 37°C on LB

**TABLE 1. Detailed biology information for KPs, GPs, RPs, and MPs**

<table>
<thead>
<tr>
<th>Species</th>
<th>Distribution</th>
<th>IUCN criterion</th>
<th>Threats</th>
<th>Mean age at maturity (Y)</th>
<th>Fledging period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP</td>
<td>Subantarctic</td>
<td>LC</td>
<td>Climate change, SST warming</td>
<td>5–6</td>
<td>310–350</td>
</tr>
<tr>
<td>GP</td>
<td>Subantarctic</td>
<td>NT</td>
<td>Climate change, tourism, pollution, fishing, SST warming</td>
<td>3–4</td>
<td>80–105</td>
</tr>
<tr>
<td>MP</td>
<td>Subantarctic</td>
<td>NT</td>
<td>Climate change, fishing, marine oil pollution, and garbage</td>
<td>4–5</td>
<td>60–85</td>
</tr>
<tr>
<td>RP</td>
<td>Subantarctic</td>
<td>YU</td>
<td>Climate change, land predators, ecotourism pollution, fishing, SST warming</td>
<td>≥4</td>
<td>60–70</td>
</tr>
</tbody>
</table>

agar-ampicillin plates overnight, at least 15 clones per band were checked for an insert by PCR. Between five and 10 insert-positive clones per band were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Carlsbad, CA) with the M13 forward primer on an ABI 3730 automated DNA sequencer. Purified PCR products of 2550F/2718R were sequenced directly using the same sequencing system. The sequences were aligned with ClustalX 1.81 [Thompson et al., 1997]. A CHD1 origin for the PCR products was confirmed by doing a BLAST search against the GenBank nr database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

We used a molecular method for sex identification to determine the sex ratio in captive breeding programs of four penguin species. Using the primer pair 2550F/2718R, we amplified a 665-bp fragment of CHD1Z and a 747-bp fragment of CHD1W (Fig. 1). When the PCR products were separated by gel electrophoresis, the presence of both bands indicated that an individual was female, while only the shorter band indicated that the individual was male. We amplified two bands from three of the four species (Fig. 2). The amplification of a single band from the two RP samples indicated that either both samples are male or the PCR failed for CHD1W. To determine if these results indicate PCR failure, we used primers p8/p2 and amplified a 374-bp fragment of CHD1Z and a 392-bp fragment of CHD1W (Fig. 3) from all four species. Compared to primers 2550F/2718R, p8/p2 was less powerful for sex identification in penguins. In all cases, the bands were faint and the small size differential between the CHD1Z and CHD1W fragments made it difficult to distinguish the gender of RPs, GPs, and MPs by 3% agarose gel electrophoresis (Fig. 4).

In addition to fragment size, we distinguished between CHD1Z and CHD1W by sequencing. We cloned and sequenced the products amplified with 2550F/2718R and p8/p2 (Figs. 1 and 3). The sequences were submitted to GenBank (accession numbers: GU451225-GU451239). For primer pair 2550F/2718R, we detected CHD1Z and CHD1W fragments from KPs, GPs, and MPs while we only detected CHD1Z fragments from RPs. These fragments corresponded to the observed bands (Fig. 2). Both primer pairs gave the same sex identification results for KPs, GPs, and MPs. However, for RPs, we detected only a CHD1W fragment from one RP and only a CHD1Z fragment from the other one, suggesting that the first RP is female and the second RP is male (Fig. 4). Additionally, these results confirmed that primer pair 2550F/2718R failed for sex identification of RPs. Based on the gel electrophoresis results and sequencing results, we determined the sex ratios (F:M) for juvenile KPs (2:0), RPs (1:1), GPs (3:5), and MPs (17:13).

Because the existing sex identification primers either failed to amplify one of the CHD1 bands or hard to resolve different bands through 3% agarose gel in a species, we designed a new pair of sex identification primers (PL/PR) from the fragments amplified by primer pair p8/p2. This primer pair amplified distinguishable 276-bp CHD1Z and 294-bp CHD1W bands for all four penguin species (see in Fig. 3). For KPs, GPs, and MPs, the presence of both bands indicated that an individual was female, while only the shorter band indicated that the individual was male. For RPs, the presence of a shorter band indicated an individual was male while only a longer one for the female (Fig. 5). Sequencing confirmed these results.

DISCUSSION

Right run time can get a clearer image and maximize band differentiation in agarose gel electrophoresis. In this study a time of 50–60 min is recommended with a voltage of 6–8 V/cm. Primer pairs 2550F/2718R and p8/p2 are powerful tools for the sex determination of most species of birds [Fridolfsson and Ellegren, 1999; Griffiths et al., 1998]. However, our study showed that they did not work for all penguin species.

Fig. 1. Alignment of CHD1Z and CHD1W fragments amplified using 2550F/2718R from four species of penguins. KP = king penguins, RP = rock penguins, GP = gentoo penguins, and MP = Magellanic penguins.
species. When we combined our results from both primer pairs, we could identify the sex of individuals from four penguin species. But, the limited resolution between bands amplified by p8/p2 and PCR failures with 2550F/2718R in RPs showed that these primers were not ideal for sex identification in penguins. As a result, we designed primer pair PL/PR, which accurately identified the sex of the four penguin species.

Known limitations of the primer pairs may have led to their failure in penguins. In some species, primer pair 2550F/2718R preferentially amplifies the shorter fragment, leading to undetectable amounts of the longer fragment [Fridolfsson and Ellegren, 1999]. Likewise, for RPs, we observed that 2550F/2718R only amplified the shorter CHD1Z and not CHD1W. Primer pair p8/p2 is known to
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Fig. 5. Gel electrophoresis of CHD1Z and CHD1W fragments amplified by PL/PR. Lane 1: KP. Lanes 2 and 3: RPs. Lane 4: ladder. Lanes 5 and 6: GPs. Lanes 7 and 8: MPs. KP = king penguins, RP = rock penguins, GP = gentoo penguins, and MP = Magellanic penguins.

CONCLUSIONS

1. Primer pair 2550F/2718R can identify the sex of KPs, GPs, and MPs very well, except RPs, through PCR and agarose gel electrophoresis method.
2. Primer pair p8/p2 gives special PCR products for KPs, RPs, GPs, and MPs, of different gender, respectively; while only products of KPs can be clearly separated using agarose gel electrophoresis.
3. The new established primer pair PL/PR can identify the sex of four penguins easily and it is expected to be a valuable primer pair for sexing other penguin species.

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REFERENCES


