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Technical note

High-throughput real-time PCR and melt curve analysis for sexing Southern Ocean seabirds using fecal samples

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ABSTRACT

Sex identification of birds is of great interest in ecological studies, however this can be very difficult in many species because their external features are almost monomorphic between the sexes. Molecular methodology has simplified this process but limitations still occur with widely accepted methods using polymerase chain reaction and gel electrophoresis, especially when applied to degraded DNA. Real-time polymerase chain reaction assays are emerging as a more efficient, sensitive, and higher throughput means of identification, but there are very few techniques validated using fecal samples and small target sizes. We present a real-time melt curve analysis assay targeting a small region of the *CHD-1* gene allowing for high-throughput, sensitive, specific, and easy-to-interpret sexing results for a variety of Southern Ocean seabirds using fecal and tissue samples.

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1. Introduction

The sex of many bird species as both neonates and adults is not obvious morphologically. This presents a challenge for demographic and ecological studies, where sex ratio and behavioral differences between sexes is of

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determine their gender. The discovery of sequence variation between the sex-linked chromodomain helicase DNA-binding protein 1 (*CHD1*) W and Z alleles has allowed the application of molecular techniques to determine avian gender. The adoption of the *CHD1* gene polymorphism as a sex marker has lead to DNA-based sex identification being validated for multiple specimen types including blood, tissue, feathers, and buccal swabs [1–3]. However, the collection of these samples can impact the study species [4,5]. Many bird species are protected for conservation or cultural reasons, so minimizing the impact of research on them is desirable. Some of the limitations encountered with the use of invasive sample collection methods can be vancements in molecular technology have shown the benefit of using this sample type for study [6–8].

Universal primer sets have been developed that amplify the *CHD1-W* and *Z* alleles simultaneously. Results are based on the interpretation of bands on electrophoretic gels where there are differences in the length of the W and Z amplicons. The use of band size as the detection method often produces results that are difficult to interpret because of the low resolving power of agarose gels or small differences in W and Z amplicon size in a given bird species [9]. Other studies have shown the potential problems with interpretation of banding patterns in gels when polymorphisms or preferential amplification of one gene occurs [10]. Previous studies using fecal samples in avian sexing with gel electrophoresis methods have shown preferential

Data accessibility: All data are presented in this article.

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amplification of one allele leading to incorrect interpretation of results [11]. Degradation and fragmentation of DNA may also negatively impact on the use of traditional methods of *CHD*-based sexing assays because of the length of the target region required. Smaller amplification targets (<200–300 bp) will have higher success rates than larger regions when amplifying from the degraded DNA found in feces [12]. Low quantity and quality of DNA and poor extract quality (e.g., inhibitors present) can limit the success and reliability of noninvasive samples such as feces [13].

The advantages of real-time polymerase chain reaction (PCR) over traditional amplification and agarose gel visualization are numerous. Real-time PCR has been shown to be more sensitive, cost effective, require less labor, and give higher throughput [14,15]. It is a closed system of detection, thus reducing risk of contamination and yet, it has only been relatively recent that modification to avian sexing methods and targeting of new sites for real-time PCR have begun to emerge [16–18].

Direct conversion of gel methods to real-time analysis is not possible without gene-specific fluorescent-labeled probes or the use of melt curve analysis (MCA). Real-time PCR generates data as accumulated amplification and would not differentiate between genes. Melt curve analysis uses the dissociation characteristics of double-stranded DNA to provide a melt point at which at least 50% of the DNA is denatured. The conversion to real-time platforms of avian sexing methods has been discussed in previous publications [16,19,20]. These studies have shown that in some species, the melt point in MCA is not differentiated between *CHD1-W* and *CHD1-Z* genes.

In this study, we present the use of fecal samples, real-time PCR, and MCA to determine sex in multiple avian species of the Southern Ocean. This method will assist in addressing controversial issues of invasive collection, nuisances with interpretation of results, and inherent problems involved with molecular analysis.

2. Materials and methods

2.1. Specimen extraction

Adélie Penguin (*Pygoscelis adeliae*) and Emperor penguin (*Aptenodytes forsteri*) fecal samples were collected from East Antarctica; Black browed albatross (*Thalassarche melanophrys*), Royal penguin (*Eudyptes schlegeli*), and King penguin (*Aptenodytes patagonicus*) fecal samples were collected from Macquarie Island, Tasmania; and Shy Albatross (*Thalassarche cauta)* fecal samples from Albatross Island, Tasmania. Northern and Southern Giant Petrel (*Macronectes hellis and Macronectes giganteus*), Rockhopper (*Eudyptes chrysocome*), Gentoo (*Pygoscelis papua*), Adélie, Royal, and King penguin flesh samples collected from deceased animals found on Macquarie Island were also extracted for validation. Samples were stored in 70% ethanol at −70 °C until extraction. DNA was extracted using Maxwell 16 Tissue DNA Purification kit (Promega) and eluted into 300 μL elution buffer. OneStep PCR Inhibitor Removal Kit (Zymo Research) was then used. DNA was stored at −70 °C until testing.

2.2. PCR and gel electrophoresis

A final volume of 10 μL consisted of 1× Phusion High-Fidelity PCR Master Mix with HF buffer (New England Biolabs), 5× BSA (New England Biolabs), 1 μM of both forward and reverse primer (2550F/2718R, P8/P2, and M5/P8) with the following cycling conditions: activation 98 °C for 2 minutes, 40 cycles of 98 °C for 5 seconds, 54 °C for 20 seconds, 72 °C for 20 seconds with a final 72 °C extension for 1 minute and holding at 4 °C. A 3% agarose gel was used to visualize products and to interpret bands.

2.3. Primer design

Previously published *CHD1* gene sequences for both Z and W alleles were aligned using BioEdit Sequence Alignment Editor (GU451235.1, GU451232.1, GU451233.1, GU451234.1, GU451239.1, GU451237.1, GU451238.1, and GU451236). Primers were designed within a region widely used for avian sexing of blood and feather samples [1]. The design spans a region containing three single nucleotide polymorphisms from alleles W and Z of penguins (Fig. 1). Modifications were made to the M5 primer [2] including making it a forward primer, PengF1 (this also could be classified as a modification of PR primer [9]). Design for a reverse primer also correlated with being a modification to CHD-W-F [16] including making it a reverse primer, PengR1 (Fig. 2). Analysis of secondary structures was performed using OligoAnalyzer 3.1 on the Integrated DNA Technologies website.

2.4. Real-time PCR method

Polymerase chain reaction mix containing 1× LightCycler 480 Probes Master (Roche), 5× BSA (New England Biolabs), Forward primer and reverse primer (PengF1/PengR1) 1 μM, 1× EvaGreen (Biotium). Amplification and MCA were performed on the LightCycler 480 (Roche) conditions were as follows: Activation at 95 °C for 5 minutes, 40 cycles of 95 °C 10 seconds, 60 °C for 30 seconds with signal acquisition and an extension at 72 °C for 10 seconds. Melt curve conditions were 55 °C to 95 °C at a ramp rate of 2.2 °C/s with five acquisitions per degree. An annealing temperature as low as 52 °C was also tested to determine if base variations in different species could also be detected.

2.5. Real-time assay result interpretation

Known female and male samples were used as positive controls. Negative controls were added to every run. For each sample and/or positive control, a typing result was considered valid where; (1) an amplification curve with a crossing point of less than 35 cycles was observed upon quantification analysis, and (2) melting peaks of 78 °C and/or 81 °C were observed during MCA. Sample results outside these criteria were deemed negative. Samples were tested in duplicate and results were accepted only after two positive amplifications. Any samples that produced only one of two positive amplifications in initial testing were repeated a third time to confirm the result.

GU451235.1 *Spheniscus magellanicus* CHD1Z CAGATCAGCTTTAATGGAAGTGAAGGGAGGCGCAGTAGGAGCAGAAGATACTCTGGATCTGATAGTGACTCCAT

GU451232.1 *Aptenodytes patagonicus* CHD1Z CAGATCAGCTTTAATGGAAGTGAAGGGAGGCGCAGTAGGAGCAGAAGATACTCTGGATCTGATAGTGACTCCAT

GU451233.1 *Eudyptes chrysocome* CHD1Z CAGATCAGCTTTAATGGAAGTGAAGGGAGGCGCAGTAGGAGCAGAAGATACTCTGGATCTGATAGTGACTCCAT

GU451234.1 *Pygoscelis papua* CHD1Z CAGATCAGCTTTAATGGAAGTGAAGGGAGGCGCAGTAGGAGCAGAAGATACTC**G**GGATCTGATAGTGACTCCAT

GU451235.1 *Spheniscus magellanicus* CHD1W CAGATCAGCTTTAATGGAAGTGAAGGGA**AA**CGCAGTAGGAGCAGAAGATA**T**TCTGGATCTGATAGTGACTCCAT

GU451232.1 *Aptenodytes patagonicus* CHD1W CAGATCAGCTTTAATGGAAGTGAAGGGA**AA**CGCAGTAGGAGCAGAAGATA**T**TCTGGATCTGATAGTGACTCCAT

GU451233.1 *Eudyptes chrysocome* CHD1W CAGATCAGCTTTAATGGAAGTGAAGGGA**AA**CGCAGTAGGAGCAGAAGATA**T**TCTGGATCTGATAGTGACTCCAT

GU451234.1 *Pygoscelis papua* CHD1ZW CAGATCAGCTTTAATGGAAGTGAAGGGA**AA**CGCAGTAGGAGCAGAAGATA**T**TCTGGATCTGATAGTGACTCCAT

Forward primer (PenF1) CAGCTTTAATGGAAGTGAAGG

Reverse primer (PenR1) GGATCTGATAGTGACTCC

Fig. 1. Alignment of W and Z allele of chromodomain helicase DNA-binding protein (*CHD1*) sequences showing primer location and single-nucleotide polymorphism location.

3. Results

3.1. Sex identification with M5/P8 and gel electrophoresis

Female Adélie penguin tissue DNA amplified using primer sets (2550F/2718R, P2/P8, and M5/P8) in our hands showed P2/P8 and M5/P8 amplified a product but only M5/P8 resolved the band size differences clearly using agarose gel electrophoresis.

Royal penguin tissue did not amplify and Gentoo tissue amplicon resulted in a single band on a gel. This band presented at the correct size for the female (W) amplicon but did not show the corresponding male amplicon. Initial testing with fecal samples showed a

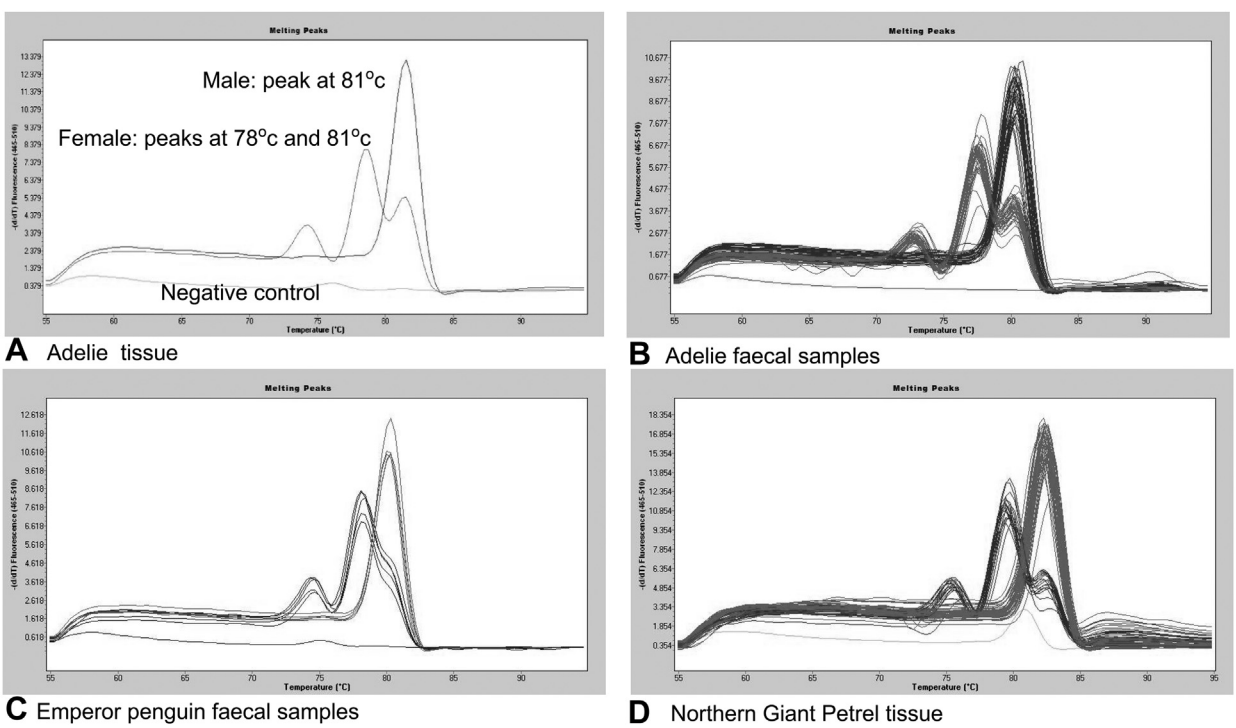

Fig. 2. LightCycler 480 melt curve peak results for (A) Adelie penguin tissue, (B) Adélie penguin fecal samples, (C) Emperor penguin fecal samples, and (D) Northern Giant Petrel tissue.

Table 1
M5/P8 gel electrophoresis and real-time melt curve analysis results for Southern Ocean seabird tissue and fecal samples.

Species	Number of samples tested	M5/P8 results			Real-time and MCA results		
		Male	Female	Neg	Male	Female	Neg
Tissue sample							
Pygoscelis adeliae (Adélie)	2	1	1		1	1	
Eudyptes chrysocome (Rockhopper)	1		1			1	
Pygoscelis papua (Gentoo)	1		1			1	
Eudyptes schlegeli (Royal)	1			1	1		
Aptenodytes patagonicus (King)	1	1			1		
Macronectes hallis (Northern Giant Petrel)	91	74	18		74	18	0
Macronectes giganteus (Southern Giant Petrel)	3	2		1	2	1	
Fecal sample							
Pygoscelis adeliae (Adélie)	58	5	14	39	22	27	9
Aptenodytes forsteri (Emperor)	17	8	6	3	9	8	0
Eudyptes schlegeli (Royal)	6				2	2	2
Aptenodytes patagonicus (King)	6	1	3	2	2	2	2
Thalassarche cauta (Shy albatross)	34				10	6	18
Thalassarche melanophrys (Black browed albatross)	60				8	13	39

Abbreviation: MCA, melting curve analysis.

low positive rate of amplification with M5/P8 primers (Table 1).

3.2. Redesign of primers and validation of real-time and melt curve sex identification assay

Available penguin *CHD1* sequences on Genbank were aligned and examined for conserved regions less than 300 bp in length containing base changes between *CHD1-Z* and *W*. A region containing up to four single-nucleotide polymorphisms between Bantock's M5 primer and Chang's chd-w-f primer (Fig. 1) was targeted. Minor modifications and reversing the direction of both primers to amplify the 67 bp region provided a base for a real-time assay with melt curve capabilities. This design was tested on penguin tissue and produced successful results (see Fig. 2). Male tissue amplified with a crossing point less than 35 cycles and had a melt curve peak at 81 °C, female tissue also amplifying at a crossing point less than 35 cycles gave a multiple melt curve profile with major peaks at 78 °C and 81 °C. Adélie fecal samples amplified with M5/P8 primers resulted in 33% successfully sexed. Our assay resulted in 84% successfully sexed.

Northern and Southern Giant Petrel tissue DNA extracts previously analyzed for sex determination using M5/P8 primers (unpublished data) correlated with results using our real-time MCA assay (Table 1). Preliminary results were also successfully obtained for Rockhopper, Gentoo, Royal, and King penguin tissue and also Emperor, Royal, and King fecal extracts. Shy and Black browed albatross fecal extracts showed promising results using this real-time and MCA assay (Table 1). No positive amplifications produced conflicting results.

A random selection of samples that did not perform well on the first round of amplification using M5/P8 gel method were diluted 1:10 and 1:100 and retested using both gel method and real-time PCR assays. Both methods performed well when diluted, indicating inhibitors were present in these samples affecting the M5/P8 method. The P2/P8 primers have shown that polymorphisms occur in certain species.

4. Discussion

Initial evaluation of widely used primers for avian sexing determined M5/P8 primer set to be the most appropriate assay to use on our sample set. Bantock, et al. [2] illustrated a significant improvement in obtaining a result using their M5/P8 primers over P2/P8 primers. Targeting a smaller amplicon on a degraded sample is supported in other work [12]. Because of the difficulty in gaging initial DNA quality and quantity in fecal samples and that these samples contain target DNA and prey DNA, quantity cannot be adjusted to account for variances in the sample and obtain consistency in evaluation of bands. During validation of our assay, a number of female samples showed only one band on analysis with M5/P8 primers. This band was at the band size for a female (W) but it is an indication of potential risk associated with interpretation of gels.

Inconsistencies within species can make it difficult to interpret banding patterns [10]. Previous authors have stated that varying the annealing temperature, cycle time, reagent, and gel method all contribute to the successful amplification of difficult samples. This is not feasible when dealing with hundreds of samples on a limited budget when sexing, although critical, is only a component of the overall study. As many unsuccessful or false results are not published [21], it is difficult to ascertain the robustness of each sexing method.

Real-time MCA requires each species to be evaluated initially but the inherent increase in sensitivity of this method should produce more consistent positive samples from degraded, low quality samples than that of PCR and gel visualization. Our assay increased the number of results from 33% using M5/P8 primers to 84% on Adélie fecal samples. This is a significant improvement in sensitivity, specificity, cost, and time. Real-time MCA is not without its limitations but real-time platforms provide software that, once programmed, relies less on skilled interpretation of results.

The methods of using generic bird sexing primers on the feces of birds of prey could become unreliable because of the ability to detect the predator and prey DNA.

Further study would be required to elucidate the broad range of species this real-time assay could cover.

4.1. Conclusions

In conclusion, this study successfully sexed 10 different avian species with minimal optimization. Sensitivity, speed, and cost were improved with this method over that of traditional sexing techniques. Tighter regulation of acceptable results is easier with this technology than that of gel electrophoresis allowing for high-throughput, reproducible, sensitive, and specific results to be obtained in the hands of researchers less experienced with molecular techniques.

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Author contributions: Cassandra Faux performed assay design, sample processing, laboratory work and wrote the manuscript. Julie McInnes performed sample collection, processing, laboratory work, and contributed to the manuscript. Simon Jarman contributed to the design consultation and manuscript preparation.

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