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HIGH-THROUGHPUT GENDER IDENTIFICATION OF PENGUIN SPECIES USING MELTING CURVE ANALYSIS

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Most species of penguins are sexual monomorphic and therefore it is difficult to visually identify their genders for monitoring population stability in terms of sex ratio analysis. In this study, we evaluated the suitability using melting curve analysis (MCA) for high-throughput gender identification of penguins. Preliminary test indicated that the Griffiths's P2lP8 primers were not suitable for MCA analysis. Based on sequence alignment of Chromo-Helicase-DNA binding protein (*CHD*)-W and CHD-Z genes from four species of penguins (*Pygoscelis papua, Aptenodytes patagonicus, Spheniscus magellanicus, *and* Eudyptes chrysocome*), we redesigned forward primers for the CHD-W/CHD-Z-common region (PGU-ZW2) and the CHD-W-specific region (PGU-W2) to be used in combination with the reverse Griffiths's P2 primer. When tested with P. papua samples, PCR using P2l PGU-ZW2 and P2lPGU-W2 primer sets generated two amplicons of 148- and 356-bp, respectively, which were easily resolved in 1.5% agarose gels. MCA analysis indicated the melting temperature (Tm) values for P2lPGU-ZW2 and P2lPGU-W2 amplicons of P. papua samples were 79.75° C–80.5° C and 81.0° C–81.5° C, respectively. Females displayed both ZW-common and W-specific Tm peaks, whereas male was positive only for ZW-common peak. Taken together, our redesigned primers coupled with MCA analysis

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allows precise high throughput gender identification for* P. papua*, and potentially for other penguin species such as* A. patagonicus, S. magellanicus*, and* E. chrysocome *as well.

Keywords: High-throughput; Melting curve analysis; Molecular gender identification; Penguins; Real-time PCR

INTRODUCTION

Penguins comprising the bird family *Spheniscidae* (order Sphenisciformes) are sensitive to the ecological environment of oceans (1). Monitoring the population stability of penguins can reflect the influence of global warming (2, 3). Gender and reproductive status of penguins may affect their feeding and migrating behavior and, therefore, influence their migration and survival (4, 5). Accordingly, the precise sex ratio of the population is a pivotal index to monitor the stability of penguin populations (6) and to establish a breeding program. However, most species of penguins are sexual monomorphic. Although the males are usually larger than females, gender identification of penguins by morphologic observation is difficult, especially for the chicks (1, 7–9).

To date, many DNA-based techniques of gender identification have been developed to improve the avian gender determination (10–19). In general, the principle of most methods is based on differences in intron length between the chromo-helicase-DNA binding protein (*CHD*)-*Z* and *CHD-W* genes (20, 21), that is, ZW for female and ZZ for male. For the gender identification of penguins, four sets of PCR primers such as P2/P8 (7, 22), 1237L/1272H (23), 2550F/2718R (24), and 2917F/3088R (20) have been reported.

However, these primer sets each has its limitations and works in preferred species. For example, when using P2/P8 primers (21), gender identification was inconclusive in Humboldt penguins (*Spheniscus humboldti*) (22, 25), King penguins (*Aptenodytes patagonicus*), Gentoo penguins (*Pygoscelis papua*), and Magellanic penguins (*S. magellanicus*) (26) in 3% agarose gel electrophoresis. While the 2550F/2718R primers (24) can provide the distinct bands for gender identification for *A. patagonicus, P. papua*, and *S. magellanicus* but not for the Rockhopper penguins (*Eudyptes chrysocome*). In contrast, P2/P8 primers (21) have the opposite results (26). Therefore, development of the universal primers for multiple species of penguins in gender identification is still challenging.

In order to find the universal primers of gender identification for several penguin species, a strategy to redesign primers from the aligned *CHD-Z* and *CHD-W* sequences of tested species have been proposed (26, 27). For example, the new PL/PR primers in locus-specific PCR (26) have been redesigned for universal gender identification of four penguin species, that is, *A. patagonicus, P. papua, S. magellanicus*, and *E. chrysocome*. In our previous work (27), the new PGU-ZW1/PGU-W1 primers were redesigned for three penguin species such as *P. papua, S. magellanicus*, and *E. chrysocome*.

In ecological research, the gender identification of large numbers of penguins may be required. For example, 135 specimens of *S. magellanicus* were subjected to PCR with P2/P8 primers and the PCR amplicons were separated by 12% acrylamide gel electrophoresis (28). Even the gender identification using the PL/PR primers (26)

and the PGU-ZW1/PGU-W1 primers (27) as previously mentioned still require the gel electrophoresis step and are low throughput. Therefore, the high throughput method for gender identification of penguins remains challenge.

Recently, we applied the melting curve analysis (MCA) for high throughput gender identification to several non-penguin species of birds such as *Spilornis cheela hoya* (29), *Columba livia, Columba pulchricollis*, and *Streptopelia tranquebarica* (30), as well as blue-breasted quail and chicken (31). For MCA analysis, PCR amplicons with different lengths often display different melting temperature (Tm) values. Therefore, we proposed that MCA may be ideal for gender identification of some penguin species such as *P. papua, A. patagonicus, S. magellanicus*, and *E. chrysocome*.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Four blood samples from banded *P. papua* with known gender by anatomical inspection (females: G304, G404, and G508; male: AD7564) were provided by the Hi-Scene World Enterprise Co. LTD., Taiwan. Blood DNA was purified by a Qiagen blood kit as previously described (32).

P2/P8 Primers-Based Gender Identification Using Melting Curve Analysis (MCA)

The P2/P8 primers (21) for molecular gender identification of birds were used. The PCR reaction mixture contained 1X PCR buffer, 0.16 μM primers, 0.2 mM dNTPs, 0.7U Platinum-Taq enzyme (Invitrogen Inc., Sao Paulo, SP, Brazil), 1.5 mM $MgCl_2$, SYBR Green I (1:2000; Invitrogen), and 10–20 ng DNA (total volume, 10 μL). The PCR program was as follows: 94°C (4 min); 5 cycles of 94°C (30 s), 47°C (30 s), 72°C (30 s); 50 cycles of 94°C for (30 s), 47°C (20 s), 72°C (20 s); and 72°C (5 min). Subsequently, the melting curve was analyzed with the default program of iQ^{TM} 5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA, USA).

Sequence Alignment Analysis

The sequence alignment of *CHD-Z* and *CHD-W* genes for *P. papua, A. patagonicus, S. magellanicus*, and *E. chrysocome* were performed using the Biology Workbench 3.2 (http://workbench.sdsc.edu/). Their accession nos. were listed as follows: GU451234 and GU451238 for *P. papua,* GU451232 and GU451236 for *A. patagonicus*, GU451235 and GU451239 for *S. magellanicus*, and GU451233 and GU451237 for *E. chrysocome*.

Redesigning Primers-Based Gender Identification Using MCA

The forward primers located in the *CHD-ZW*-common and *CHD-W*-specific regions were designed from the sequence alignment result of four penguin species. These forward primers share the same reverse primer P2 (21) for both *CHD-ZW*-common and *CHD-W*-specific PCR reactions. The PCR program was as follows: denaturation

for 3 min at 95°C followed by 50 cycles of denaturation at 95°C for 30 s; annealing at 61°C for 30 s; and extension at 72°C for 7 s. Subsequently, the melting curves were analyzed as previously described. The presence and purity of PCR products were examined with 1.5% agarose gel electrophoresis.

Identifying the Bird Species with Homologous Sequences of the Redesigned Primers

The sequences of the redesigned primers were input to the blastn program in BLAST tool (33) in NCBI with the settings of "nucleotide collection (nr/nt)" in "others" database, and "optimize for somewhat similar sequences" for program selection.

RESULTS

P2/P8 Primers-Based MCA

The aim of the present study was to examine the suitability of MCA for the high-throughput gender identification of penguin species using the P2/P8 primers (21) and our newly redesigned universal primers. Previously, we found that the PCR products of *CHD-Z* and *CHD-W* genes for *P. papua* were resolved in 3% agarose gel electrophoresis (27). It was reproducible in the current study (data not shown). Because the samples of *A. patagonicus, S. magellanicus*, and *E. chrysocome* were unavailable to us, the *P. papua* samples were used as the example in this study. MCA of the PCR products of *CHD-Z* and *CHD-W* genes for three females and one male of *P. papua* were examined and only one Tm peak at approximately 82°C was visible for these *P. papua* (Fig. 1). Therefore, the MCA analysis for PCR products amplified by P2/P8 primers was unsuitable to determine the gender of *P. papua*.

Sequence Alignment and Primer Redesigning for Gender Identification

The sequence alignment of *CHD-Z* and *CHD-W* genes for *P. papua, A. patagonicus, S. magellanicus*, and *E. chrysocome* were demonstrated (Fig. 2). To

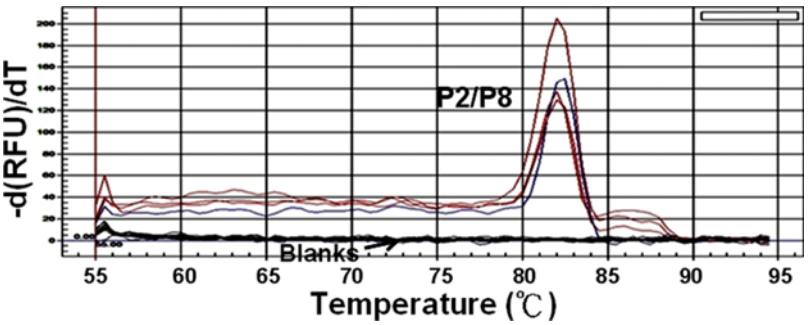

Figure 1 Representative MCA curves for PCR amplicons using the P2/P8 primer set in four individuals of *P. papua*. Tm values of P2/P8 amplicons of both three females (G304, G404, and G508) and one male (AD7564) for *P. papua* were similar. RFU, Relative fluorescence unit.

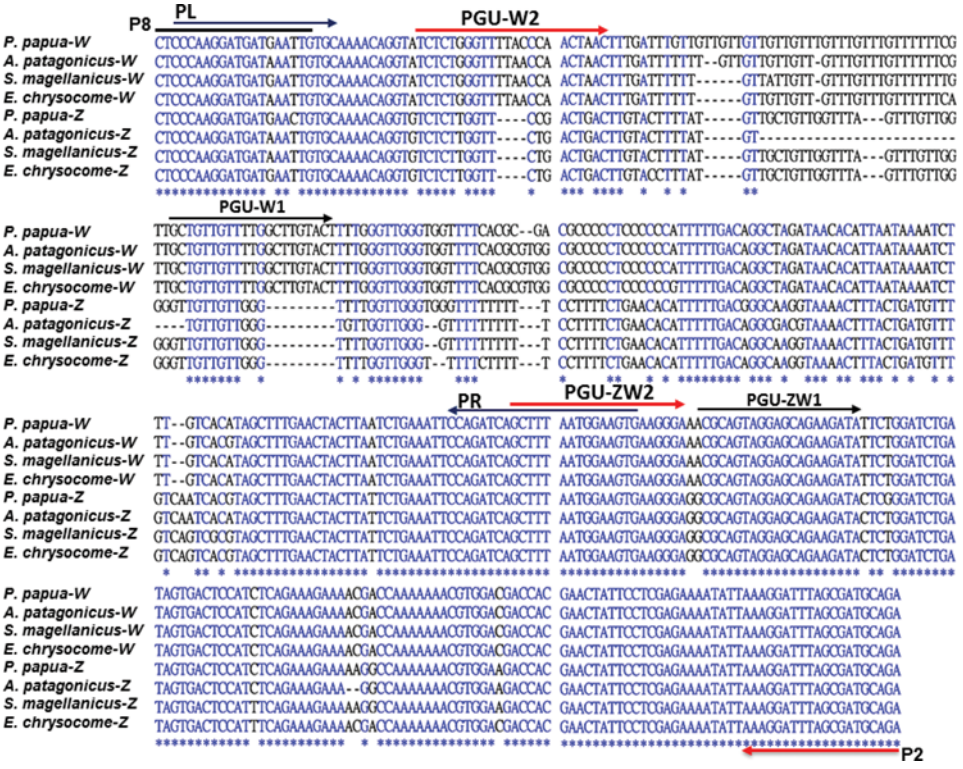

Figure 2 Sequence alignment of *CHD-W* and *CHD-Z* genes of four penguin species. The accession nos. of these species were described in Materials and methods. The bars indicate the regions for the redesigned primers in this study (PGU-ZW2/PGU-W2) and in literatures [PL/PR (26) and PGU-ZW1/PGU-W1 (31)]. Stars and dashed lines indicate the conserved and deleted regions between *CHD-Z* and *CHD-W* sequences, respectively.

improve the MCA performance for gender identification using P2/P8 PCR, it is essential to extend the length difference between the PCR amplicons involving the *CHD-Z* and *CHD-W* genes. After manual inspection, the forward primers for CHD-ZW-common and CHD-W-specific regions were redesigned as follows: PGU-ZW-2: 5-GCTTTAATGGAAGTGAAGGGA-3 and PGU-W2: 5-TCTCTG GGTTTTAMCCAACT-3. Using the word count function in "Microsoft Word," the lengths of PCR products amplified by the P2/PGU-ZW2 and P2/PGU-W2 primer sets were 148- and 356-bp, respectively. The P2/PGU-ZW2 primer set was used as the positive PCR control for both females and males, whereas the P2/PGU-W2 was female-specific. The other primers [PL/PR (26) and PGU-ZW1/PGU-W1 (31)] as indicated in Fig. 2 will be discussed later.

Examination of Common Gender Primer and Gender-Specific Primer Using Gel Electrophoresis

To examine the performance of our redesigned primers, PCR was amplified with P2/PGU-ZW2-common and P2/PGU-W2-specific primer sets in different

PCR wells, and PCR amplicons were able to be resolved in 1.5% agarose gel electrophoresis (Figs. 3A and 3B, respectively). In the example of all females and male of *P. papua*, they shared the same PCR amplicon length for the positive control for *CHD-ZW*-common PCR reaction using the PGU-ZW2-common primer coupled with P2 (the right side of Fig. 3A). *CHD-W*-specific PCR using P2/PGU-W2-specific primers was successfully amplified in female birds (G304, G404, and G508), but not in a male bird (AD7564).

High-Throughput Gender Identification: MCA

The MCA analyses for the same PCR reaction amplified with P2/PGU-ZW2-common and P2/PGU-W2-specific primer sets (Fig. 3A) were performed (Figs. 3B and 3C). Female individuals of these penguin species were identified because two distinct Tm peaks were visualized; one is the Tm value for the *CHD-Z/CHD-W*-common PCR amplicons using P2/PGU-ZW2 primers (79.75–80.5°C; ZW) and the other is the *CHD-W*-specific PCR amplicons using P2/PGU-W2 primers (81.0–81.5°C; W; Fig. 3B). In contrast, male individual was identified because it only displayed a single Tm peak (79.75–80.5°C; ZW; Fig. 3C) for the PCR amplicon using P2/*CHD-ZW2* primers.

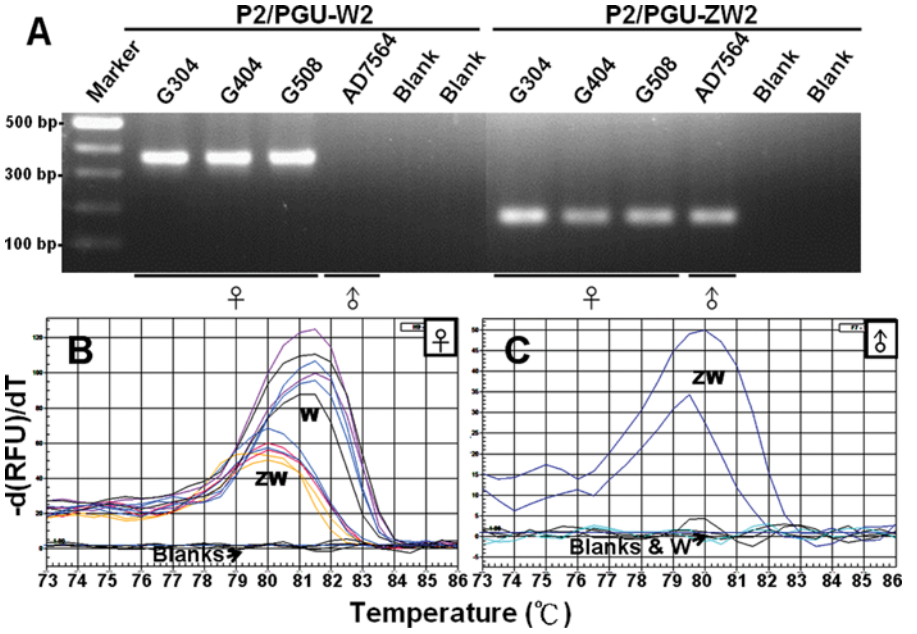

Figure 3 Gel electrophoresis and MCA using the redesigned PGU-ZW2 and PGU-W2 primers for four individuals of *P. papua*. The female-specific PCR and common gender PCR were amplified using (A) P2/PGU-W2 and P2/PGU-ZW2 primers and performed in different PCR wells to facilitate subsequent separation in 1.5% agarose gel. (B, C) MCA curves for high-throughput molecular gender identification of samples in *P. papua*. (B) Three females (G304, G404, and G508) and (C) one male (AD7564) of *P. papua* were included. ZW and W indicate the primer mixtures for P2/ PGU-ZW-common (148-bp) and P2/PGU-W-specific (356-bp). Tm values for ZW and W as indicated were 79.75–80.5 and 81.0–81.5, respectively. The MCA experiments were duplicated.

DISCUSSION

In the molecular gender identification of penguins, several methods such as PCR-restriction fragment of length polymorphism (RFLP) (22) and PCR with redesigned primers (26, 31) have been established to overcome the short intron length difference between *CHD-Z* and *CHD-W* genes. However, PCR-RFLP requires the enzyme digestion step (34) and, therefore, needs extra time and money. Moreover, the strategy using redesigned primers (26, 31) did not demonstrate high throughput gender identification of penguins because the electrophoresis step was still needed.

Both PL/PR primers (26) were located in the ZW-common regions as indicated (Fig. 2). The length differences of PCR amplicons using PL/PR primers were 18-bp, that is, PL/PR PCR amplicons of *CHD-Z* and *CHD-W* genes were 276- and 294-bp, respectively. Alternatively, in our previous work (31) and the current study, the primers were redesigned on the *CHD-Z*/*CHD-W* common region for PCR positive control and on the *CHD-W*-specific region for gender identification of female. Moreover, the length differences of PCR amplicons using redesigned primers for *CHD-Z*/*CHD-W* common region and *CHD-W*-specific region become more extended, i.e., 161-bp for P2/PGU-ZW1 (126-bp) and P2/PGU-W1 amplicons (287-bp); 209-bp for P2/PGU-ZW2 (148-bp), and P2/PGU-W*2* amplicons (356-bp).

Because the length differences have been highly extended in the present study, it allows gender detection based on different curves in MCA using redesigned primers of P2/PGU-ZW2 and P2/PGU-W*2* (Fig. 3). For example, Tm values for P2/PGU-ZW2 and P2/PGU-W*2* amplicons were 79.75–80.5 and 81.0–81.5, respectively. Due to the different Tm values, Tm peak numbers can be automatically counted using the built-in software in the real-time PCR machine. In contrast, the methods involved gel electrophoresis was labor-intensive and low throughput. Therefore, the MCA-based gender identification was more efficient.

BLAST analysis indicated the sequence of the female-specific primer (PGU-W2) was highly conserved (23/24 matched) within the *CHD-W* gene of the Familiy Accipitridae, such as *Accipiter trivirgatus* (FJ896035) and *Pernis ptilorhynchus* (FJ896029). The sequence of the common gender primer (PGU-ZW2) was fully conserved within both the *CHD-W* and *CHD-Z* genes of the Familiy Accipitridae, such as *A. trivirgatus* (FJ896035 and FJ896035) and *P. ptilorhynchus* (FJ896029 and FJ896028), respectively. This BLAST result suggests that our proposed redesigned primers of penguin gender identification are the potential candidate primers for other non-penguin species and it warrants for proof.

Recently, the PCR melting curve-based high-resolution melting (HRM) analysis was applied to the gender identification of birds such as common quail and Japanese quail (35) using the primers P2/P8. However, this method requires a more expensive next-generation real-time PCR platform combined with specific HRM software rather than the traditional real-time PCR machine.

Taken together, the MCA-based real-time PCR method is an effective and high-throughput method to identify the genders of *P. papua*. Our redesigned primers for MCA also have a potential to apply to other penguin species such as *A.patagonicus, S. magellanicus*, and *E. chrysocome* due to the prediction of sequence alignment of *CHD-Z* and *CHD-W* genes. To our knowledge, this is the first high-throughput gender identification for penguins.

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