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Advances in molecular sexing of birds: a high-resolution melting-curve analysis based on *CHD1* gene applied to *Coturnix* spp.

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Sex identification in birds through molecular methods is an important tool for the management and preservation of species. Advances in real-time PCR-based techniques overcome some limitations of the more classical molecular analysis methodologies. Here, we describe a new approach, based on high-resolution melting (HRM) curve analysis of the *CHD1* gene, for avian gender identification. This method was successfully applied to carry out sexual differentiation based on melting curve patterns in common quail (*Coturnix c. coturnix*) and Japanese quail (*Coturnix c. japonica*). We clearly demonstrate the efficacy of a simple HRM assay for a rapid and efficient gender differentiation of these subspecies and propose this methodology as a valuable addition to expand the applicability of real-time PCR-based technology in avian molecular sexing.

Introduction

In addition to behavioural studies and breeding programs, application of molecular methods for sex identification in birds is an important tool for wildlife conservation projects. Molecular-analysis approach becomes important particularly in case of birds that have no sexual dimorphism, and for sexing of young birds. These methods may be used as a basis or a complement to population, behavioural and forensic studies; analysis of the species mating systems; and improvement of avifauna preservation programs, as captive breeding projects (Fridolfsson & Ellegren 1999, Ito *et al*. 2003, An *et al*. 2007). Additionally, the improvements of genetic resources involv-

ing the common quail are important tools for the current management program developed by the European Union (Natura 2000 2009). Sex determination in birds follows a ZW gametic system, where females are heterogametic (ZW) and males are homogametic (ZZ). The gene encoding chromodomain helicase DNA binding 1 (*CHD1*) is highly conserved but shows some intronic variations between chromosome Z and W (*CHDZ* and *CHDW*) (Griffiths *et al*. 1996). The size differences of *CHD1* introns enabled the design of specific primers, which were then successfully used, for gender determination in many bird species (Griffiths *et al*. 1998, Kahn *et al*. 1998, Fridolfsson & Ellegren 1999). The standard methodology is based on amplification by PCR of *CHDZ* and *CHDW* alleles using universal *CHD1* primers, and a subsequent electrophoresis. In theory, the amplification products should migrate as a single band in males (*CHDZ*) and two bands in females (*CHDZ* and *CHDW*); however, the polymorphic nature of the intron length, along with the variability in electrophoresis resolution power, does not allow for accurate sex identification in some bird species (Griffiths *et al*. 1998, Kahn *et al*. 1998, Wang *et al*. 2007). To overcome this limitation, several alternative methodologies have been developed for bird sexing, such as amplified fragment length polymorphism (AFLP) (Griffiths & Orr 1999), restriction fragment length polymorphism (RFLP) (Sacchi *et al*. 2004), single strand conformation polymorphism (SSCP) (Ramos *et al*. 2009, Morinha *et al*. 2011), microsatellites (Nesje & Røed 2000) and oligonucleotide microarrays (Wang *et al*. 2008). However, these are costly and time-consuming techniques with limited applicability as a routine methodology.

Over the past few years, new methodologies have been proposed for high-throughput avian molecular sexing, such as capillary electrophoresis (Lee *et al*. 2010), real-time PCR using TaqMan probes (Chou *et al*. 2010, Rosenthal *et al*. 2010) and real-time PCR combined with melting curve analysis (Chang *et al*. 2008, Brubaker *et al*. 2010, Huang *et al*. 2011). Recently, the high-resolution melting (HRM) analysis started to be used in combination with real-time PCR technology. The HRM analysis is a simple and rapid PCR-based technique for detecting DNA sequence variations (Montgomery *et al*. 2007) through changes in the melting temperature of a DNA duplex (Taylor 2009). The length of the amplicons, their GC content, and nucleotide sequence may influence the melting of the double-stranded DNA molecules (Ririe *et al*. 1997), thus allowing for the detection of single-nucleotide polymorphisms and small insertions and deletions by real-time HRM assay. High sensitivity and specificity, associated with low cost when compared with traditional methods that require electrophoresis and techniques using labelled probes, make HRM a suitable cost-effective approach for high-throughput applications (Montgomery *et al*. 2007, Taylor 2009, Vossen *et al*. 2009).

We have previously analysed and characterized the differences in the *CHDZ* (385 bp) and *CHDW* (379 bp) fragments in the common and Japanese quails (GenBank ID: HQ175995, HQ175996, HQ175997 and HQ175998) using primers P2 and P8 (Morinha *et al*. 2011). This six-nucleotide difference could not be observed using the protocols described before (Chang *et al*. 2008, Brubaker *et al*. 2010, Huang *et al*. 2011) and primers P2 and P8, because only one melting peak is detected in both males and females. The small difference between the *CHD1* amplicons allows the study of a novel approach using HRM for gender determination in these subspecies. In this work, we developed a protocol based on HRM analysis that improves the direct avian sexing by real-time PCR, using universal primers P2/P8 (Griffiths *et al*. 1998).

Material and methods

We used four animals of both common quail and Japanese quail, each group with two females and two males. DNA was extracted from muscle tissue collected from the wings using the QuickGene DNA tissue kit S (Fujifilm) according to manufacturer's instructions, with some modifications. Briefly, 20 mg of muscle was triturated with a scalpel and then incubated at 55 °C for 4 hours, with vortexing every 40 minutes. DNA was collected by two sequential elutions with 200 μl followed by 100 μl of H_2O.

The fragments analysed by HRM were amplified using the universal primers described before:

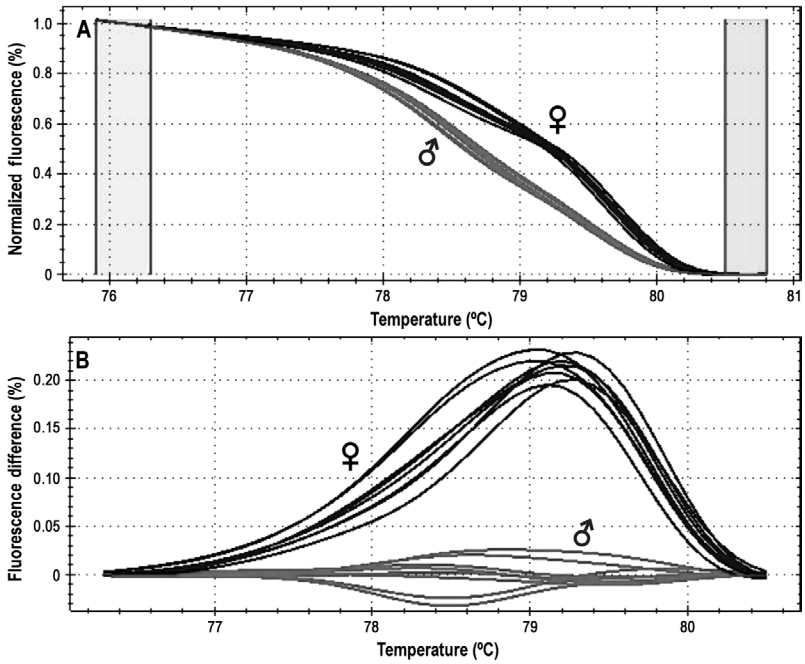

Fig. 1. HRM analysis of PCR products amplified with *CHD1* universal primer pair (P2/P8). (**A**) normalized and temperature-shifted melting curves, and (**B**) fluorescence differences for sex genotyping of common and Japanese quails. Males (♂) and females (♀) from both subspecies showed similar melting curve patterns.

P2 (5´-TCTGCATCGCTAAATCCTTT-3´) and P8 (5´-CTCCCAAGGATGAGRAAYTG-3´) (Griffiths *et al*. 1998). PCR analysis of each sample (10 μl) was carried out in duplicate, with 5 μl of SsoFast EvaGreen Supermix (Bio-Rad), 7.7 μM of each primer, and 20 ng of genomic DNA. PCR cycling and HRM analysis were performed using the CFX96 real-time PCR detection system (Bio-Rad) as follows: DNA was denatured at 98 °C for 2 min and then amplified with 40 cycles at 98 °C for 5 s and 55 °C for 5 s, followed by one cycle at 95 °C for 1 min and 55 °C for 5 min; The amplified amplicons were then melted using a 65 °C to 95 °C temperature range, with increments of 0.2 °C s^{-1}.

The melting curve data were analysed with the CFX Manager™ (ver. 2.0, Bio-Rad) and Precision Melt Analysis™ (ver. 1.1, Bio-Rad). Melting profiles were normalized by selecting the pre- and post-denaturation transition regions, which were defined as 100% and 0%, respectively. To eliminate slight temperature errors and/or buffer differences between wells, the normalized melting curves were temperature-overlaid by selecting a low fluorescence interval (5% to 10% fluorescence) and shifting each curve along the temperature axis (*x*-axis) to the point where the entire double-stranded DNA is completely denatured, to best overlay samples within this range. Samples were clustered into groups with similar melting profiles and displayed as fluorescence *versus* temperature plots.

Results and discussion

We successfully used HRM to identify sex in common and Japanese quails. The melting curve profiles showed the expected differences in normalized temperature-shifted data between males and females, allowing for an accurate gender distinction of all samples (Fig. 1). The melting temperatures ranged from 78.6 °C to 78.8 °C for male samples and 79.6 °C for female samples.

We believe this work presents, for the first time, a validated molecular approach based on the direct application of HRM analysis for sex differentiation in birds. Previous studies have combined real-time PCR and melting-curve analysis for avian molecular sexing, but such protocols required primer pairs that amplified fragments with significant length differences between *CHDZ* and *CHDW* (Chang *et al*. 2008, Brubaker *et al*. 2010, Huang *et al*. 2011). These

differences allowed for the detection of one melting peak in males (ZZ) and two melting peaks in females (ZW). When the differences in the intron length are very small, as is the case in the common and Japanese quails (6 bp) (Morinha *et al*. 2011) the previously described protocols do not work. The alternative is to redesign the sex-specific primers (Chang *et al*. 2008, Huang *et al*. 2011), but this approach is time-consuming and more expensive when compared with the direct HRM analysis. The HRM assay presented here overcomes this limitation and is a significant improvement for carrying out avian gender differentiation using a real-time PCR approach with the universal primers P2/P8. This methodology can be a powerful tool to be used also for other bird species, but the genetic variability in the sequence and introns length between *CHDZ* and *CHDW,* and/or factors such as DNA purity, fluorescent dye and real-time equipment performance are influencing parameters, and therefore, downstream optimizations should be considered.

The technique described in this paper represents an improved approach for rapid and efficient avian molecular sexing, increasing the direct applicability of real-time PCR technology. The simplicity, low cost, efficiency and high sensitivity/specificity make this method a versatile genotyping tool for complement ecological and conservation studies, as well as for current and future bird management programs.

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