

GENETICS AND MOLECULAR BIOLOGY

Sexing chickens (*Gallus gallus domesticus*) with high-resolution melting analysis using feather crude DNA

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ABSTRACT Identification of sex in broiler chickens allows researchers to reduce the level of variation in an experiment caused by the sex effect. Broiler breeds commonly used in research are no longer feather sexable because of the change in their genetics. Other alternate sexing methods are costly and difficult to apply on a large scale. Therefore, a sexing method is required that is both cost effective and highly sensitive as well as having the ability to offer high throughput genotyping. In this study, high-resolution melting (**HRM**) analysis was used to detect DNA variations present in the gene chromo-domain helicase DNA binding 1 protein (**CHD1**) on the Z and W chromosomes (**CHD1Z** and **CHD1W**, respectively) of chickens. In addition, a simplified DNA

extraction protocol, which made use of the basal part of chicken feathers, was developed to speed up the sexing procedure. Three pairs of primers, that is, CHD1U-NEHRM1F/R, CHD1UNEHRM2F/R, and CHD1U-NEHRM3F/R, flanking the polymorphic regions between **CHD1Z** and **CHD1W** were used to differentiate male and female chickens via distinct melting curves, typical of homozygous or heterozygous genotypes. The assay was validated by the HRM-sexing of 1,318 broiler chicks and verified by examining the sex of the birds after dissection. This method allows for the sexing of birds within a couple of days, which makes it applicable for use on a large scale such as in nutritional experiments.

Key words: sexing, *CHD1*, high-resolution melting, chicken

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INTRODUCTION

The ability to source sexed broiler chicks enables researchers to improve pen uniformity in the experiment either by using same sex birds or equal proportions of both the sexes in all treatments (Gous, 2017). Currently, researchers have difficulty sourcing sexed d-old broiler chicks, particularly in Australia, because of the change from slow-feathering to fast feathering parent stock of the current commercial broiler breeds (Greg Hargreave, Baiada, personal communication). This has resulted in feather sexing no longer being an available option to sex d-old chicks. This has forced many researchers to use parent lines or as-hatched commercial broilers in nutritional studies (Da Costa et al., 2017). However, issues arise as to whether parent lines are a true representation of commercial broilers in terms of body growth

and feed conversion efficiency and if they can be used as alternatives to commercial broilers in nutritional studies. The use of as-hatched birds may introduce increased variation resulting in a lower power to detect the responses of the birds.

Vent sexing, which is fast and accurate, is being used as an alternative method to feather sexing. However, it requires well-trained and experienced personnel (Otsuka et al., 2016), and most hatcheries are reluctant to allow vent sexers to sex a small number of birds on a regular basis for researchers because of biosecurity risks. Vent sexing also involves manual handling of the birds, and the resulting stress can increase early chick mortality by up to 1% (Phelps et al., 2003). Other sexing methods include *in ovo* sexing such as near-infrared fluorescence and Raman spectroscopy (Galli et al., 2016), estrogen radioimmune assays (Phelps et al., 2003), and genetic engineering modification (Doran et al., 2018). These methods are technically complex and not feasible for large-scale use such as in nutritional trials.

Molecular sexing methods have been developed based on the fact that female birds have sex chromosomes which are heterogametic (ZW), and males have sex chromosomes which are homogametic (ZZ). The gene

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encoding chromodomain helicase DNA binding 1 protein (*CHD1*), which is located on the sex chromosomes, has nucleotide and size variations between the intronic regions of the Z (*CHD1Z*) and W (*CHD1W*) chromosomes (Griffiths et al., 1996; Morinha et al., 2012). This allows for the design of several specific primers based on these variations between *CHD1Z* and *CHD1W*. A primer pair, P2 and P8, has been developed for sexing different avian species (Griffiths et al., 1998; Wang et al., 2011). Once the extracted DNA has undergone PCR, the PCR products of different sizes from the W and Z chromosomes are resolved by agarose gel electrophoresis, and the sexes can be distinguished with males showing a single band and females showing 2 bands (Dubiec and Zagalska-Neubauer, 2006). Other primers have also been designed and tested for a range of avian species (Çakmak et al., 2017; Koshiishi and Wada, 2018; Sulandart and Zein, 2012). However, this is a long process which involves post-PCR manipulation of the samples, and such a procedure does not allow for high-throughput sexing of birds needed at a flock scale or for animal trials involving hundreds of birds. Real-time PCR techniques combined with melting curve analysis offer a simpler, more cost-effective, and higher throughput method for DNA sexing of birds (Chang et al., 2008; Chen et al., 2012). However, the need to amplify a single sample in 2 reactions, poor sensitivity to differentiate amplicons with small size differences, and a relatively long process to extract DNA from blood or tissues still cannot satisfy the requirement for fast, economical, and large-scale sexing of chickens. More recently, a qPCR assay to determine the mRNA expression ratio of CHD-ZW/CHD-Z in embryonic gonads and other tissues from posthatching birds was developed to identify the sex of chickens (Wan et al., 2017). This method is useful to determine RNA or cDNA samples with unknown sex origin. However, this approach is not practical for sexing birds *per se* because of its invasive sampling from animal tissues and complex laboratory procedures.

High-resolution melting curve (HRM) analysis has been developed to detect DNA variations, especially single or multiple nucleotide mutations present in small PCR amplicons (Liew et al., 2004; Hoffmann et al., 2007; Wu et al., 2008). This technology makes use of an intercalating dye, such as Syto 9 green fluorescent nucleic acid stain, which can bind to the double-stranded DNA following PCR. The gradual increase in temperature will denature or melt the PCR amplicons. This will release the dye and cause a decrease in the fluorescence signal. Melting data are then collected in real-time by special equipment or a real-time PCR machine with HRM functions. As heterogeneous DNA molecules in different samples give rise to different melting curves, the distinctive melting curve profiles are then used to detect single or multiple nucleotide variations in the sequences under investigation. This method does not require any additional post-PCR handling (Montgomery et al., 2007; Reed et al., 2007; Taylor, 2009), and along with the automated genotyping calling

function integrated within the software, hundreds of samples can be processed within a short period of time. Morinha et al. (2011) was able to successfully identify the sex of certain quail species using HRM analysis of the CHD1 gene with the P2 and P8 primers (Griffiths et al., 1998). In addition, Morinha et al. (2013) was able to apply the same procedure to successfully sex a further 14 different bird species despite the small differences in intron sizes between the CHD1W and CHD1Z chromosomes. Although this method demonstrates the ability to make use of the common P2/P8 primers to sex a variety of bird species, the stringent DNA quality that is required for this assay to be successful may limit the ability to sex birds on a large scale using feather crude DNA. This method was also used to genotype only 1 male and female in some species (Morinha et al., 2013) which may put doubt on the reproducibility of this method for certain bird species. It also needs to be taken into account that the P8 primer has 2 degenerate bases (R and Y) at 3'-end of the sequence which is not ideal for HRM analysis, as the primer would possibly produce heterologous PCR products even with a homozygous genotype. Such heterologous PCR products may produce melting curves as if a heterozygous genotype was present because of high sensitivity of HRM to heterologous DNA molecules. This is shown in Figure 1 of Morinha et al. (2011), where clear melting transitions in the male birds were present indicating heterologous PCR products. However, when PCR primers P2 and P8 are used, the presence of polymorphisms in the CHD-Z gene may occur. Thus, heterogametic males can be erroneously genotyped as females thereby reducing the accuracy of sexing (Dawson et al., 2001).

MATERIALS AND METHODS

In the present study, HRM analysis incorporated with a fast DNA extraction protocol was developed to genotype chickens to determine their sex accurately. In this protocol, feathers from broiler chickens were used as the samples, and a quick DNA extraction method was developed along with optimized real-time PCR and HRM procedures to shorten the time required for the analysis. The reagents used for the DNA extraction as well as those used for the PCR and HRM analysis were also evaluated to ensure the most economical ones were used making the protocol cost-effective.

Feathers were collected from 45-day-old chicks for the initial development of the assay, and a further 1,318 birds were used for validation of the method. All animal trials in which these birds were used were approved by the Animal Ethics Committee of the University of New England. A wing feather was pulled from each broiler chick, and each bird was tagged with a specific number. The feathers for each bird were placed in individual zip-lock bags labeled with the corresponding bird number and then stored at 4°C until use.

The content from the basal part of each feather was squeezed out and placed in labeled 2 mL Eppendorf tubes. 500 µL Tris HCl (20 mmol, pH 7.5) was then

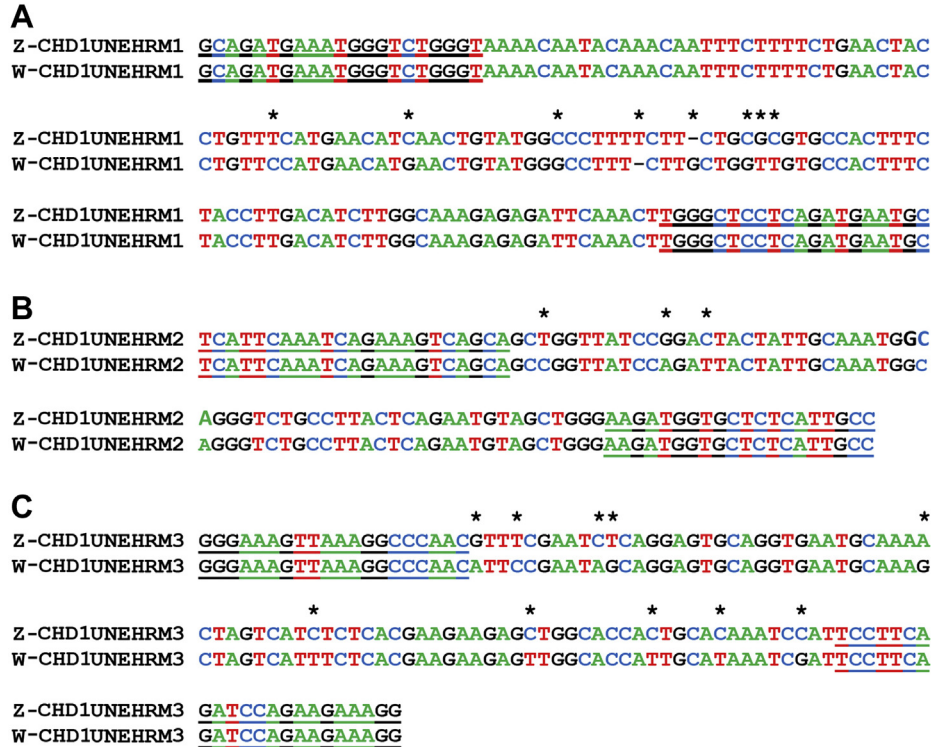


Figure 1. Alignment of the PCR amplicon sequences amplified by primer pairs a. CHD1UNEHRM1F and CHD1UNEHRM1R; b. CHD1UNEHRM2F and CHD1UNEHRM2R; and c. CHD1UNEHRM3F and CHD1UNEHRM3R. Z-CHD1UNEHRM1, Z-CHD1UNEHRM2 and Z-CHD1UNEHRM3 are the sequences of the corresponding amplicons on the Z chromosome, and W-CHD1UNEHRM1, W-CHD1UNEHRM2 and W-CHD1UNEHRM3 are the sequences of the corresponding amplicons on the W chromosome. The underlined text shows the sequences where the primers are located, and * indicates the nucleotide variations between the genomic DNA of the Z and W chromosomes.

added to each tube followed by 20 μ L Proteinase K (200 μ g/mL). The samples were incubated in the Multi-Therm shaker (Benchmark Scientific, Inc., Edison, NJ) set to 56°C for 15 min at 400 rpm followed by further incubation for 5 min at 90°C to inactivate the Proteinase K. The lysate was then centrifuged at full speed (16,000 $\times g$) for 2 min, and the supernatant was transferred into new labeled 1.5 mL Eppendorf tubes. The solution was diluted 5 times using nuclease-free water and stored in -20°C until used as a DNA template.

The DNA sequences of the *CHD1* gene located on chromosome Z (*CHD1Z*, NM_204941) and W (*CHD1W*, AF181826) of *Gallus gallus* were aligned using the NCBI blast tool by selecting “Align two or more sequences” option (<https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi>). The sequences were subjected to the BLAT function of UCSC Genome Browser (<https://genome.ucsc.edu/>) to determine the exon-exon junctions of the gene, so that the appropriate primers could be designed for genomic DNA amplification. Three pairs of primers located on the Z chromosome (chrZ: 51381960-51382120; 51379847-51379949; and 51395174-51395296) and W chromosome (chrW: 5026134-5026294; 5031763-5031865; and 5001259-5001381) were designed. Each primer flanked the nucleotide variations between the sequences of the *CHD1Z* and *CHD1W* genes using Primer 3 (Rozen and Skaletsky, 2000) and analyzed using NetPrimer to detect possible secondary structures (<http://www.premierbiosoft.com/netprimer/netprimer.html>,

[Premier Biosoft International, Palo Alto, CA](http://www.premierbiosoft.com/netprimer/netprimer.html)). In addition, all primers, except primer CHD1UNEHRM2F, were designed in the conservative regions of the Z and W chromosomes, that is, primer regions which have no nucleotide variations between *CHD1Z* and *CHD1W* genes. Primer CHD1UNEHRM2F, however, was designed in a region where nucleotide variations were present between *CHD1Z* and *CHD1W* genes, that is, with A/G and G/A polymorphisms present. To ensure proper annealing of the primer to the genomic DNA sequences of both Z and W copies and to make sure no heterologous PCR products were produced, an extended sequence in the polymorphic site of the primer was designed. This resulted in a single molecule primer TCATTCAAATCAGAAAGTCAGCA rather than a degenerate primer TCATTCAAATCARAARTCAGCA as one would do for a PCR assay other than HRM analysis. The sequences and related information of the designed primers are shown in Table 1. In this table, the names of the primers were defined as first pair: CHD1UNEHRM1F and CHD1UNEHRM1R; second pair: CHD1UNEHRM2F and CHD1UNEHRM2R; and third pair: CHD1UNEHRM3F and CHD1UNEHRM3R. Their corresponding amplicons derived from PCR templates amplified with the different primer pairs were defined as following: Z-CHD1UNEHRM1 from Z and W-CHD1UNEHRM1 from W chromosomes amplified using the first primer pair; Z-CHD1UNEHRM2 from Z and W-CHD1UNEHRM2 from W chromosomes amplified with the

Table 1. Sequences and amplification information of the primers used in the gene chromodomain helicase DNA binding protein 1 (*CHD1*) for the determination of the sex of chickens using high resolution melting curve analysis.

Primers	Sequences (5' - 3')	Amplicon size (bp)	Ta (C°)	Gene	Amplicon location in the genome	Reference sequence accession number
CHD1UNEHRM1F	GCAGATGAAATGGGTCTGGGT	161	60	<i>CHD1</i>	chrZ: 51381960-51382120	NM_204941
CHD1UNEHRM1R	GCATTCATCTGAGGAGCCCA				chrW: 5026134-5026294	AF181826
CHD1UNEHRM2F	TCATTCAAATCAGAAAGTCAGCA	104	60	<i>CHD1</i>	chrZ: 51379847-51379949	NM_204941
CHD1UNEHRM2R	GGCAATGAGAGCACCATCTT				chrW: 5031763-5031865	AF181826
CHD1UNEHRM3F	GGGAAAGTTAAAGGCCCAAC	123	60	<i>CHD1</i>	chrZ: 51395174-51395296	NM_204941
CHD1UNEHRM3R	CCTTTCTTGGATCTGAAGGA				chrW: 5001259-5001381	AF181826

second primer pair; and Z-CHD1UNEHRM3 from Z and W-CHD1UNEHRM3 from W chromosomes amplified with the third primer pair.

The Master Mix used for real-time PCR amplification was prepared on ice and consisted of the following: 10 μ L nuclease free water, 4 μ L 5 \times MyTaq reaction buffer (Bioline, Sydney, Australia), 300 nM primers, 1.5 μ M Syto 9 green fluorescent nucleic acid stain (Molecular Probes Inc., Eugene, OR), and 1 U MyTaq DNA polymerase (Bioline, Sydney, Australia). The Master Mix was prepared fresh before each analysis. Sixteen μ L of Master Mix followed by 4 μ L of sample was loaded into a PCR tube using the automated liquid handling robotics QIAgility (Qiagen, Hilden, Germany). High-resolution melting analysis following real-time PCR amplification was performed using the Rotor-Gene Q real-time PCR cycler (Qiagen). The PCR conditions were as follows: denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 10 seconds followed by annealing and extension at 60°C for 10 seconds. The PCR products were first annealed at 50°C for 30 s and then melted from 70°C to 85°C with an increase of 0.1°C every 2 s. The run took approximately 70 min to complete.

Fluorescence data were obtained at the end of each annealing/extension step during PCR amplification and each step of the HRM. Automatic gain optimization was set for fluorescence level acquirement. High-resolution melting analysis was implemented using the HRM module of Rotor-Gene Q software (Qiagen). The fluorescence levels for HRM were normalized for all the samples analyzed by adjusting their start and end fluorescence signals. By analyzing the data using the difference graph function, difference plots were achieved. This function calculates the normalized fluorescence differences of all the genotypes by deducting them from the target genotype. Genotypes ZZ or ZW of the birds were scored automatically by the software and verified manually. The melting data were also analyzed using the Melt analysis module of the software. In this analysis, negative first-derivative melting curves were produced from the fluorescence vs. temperature plots and genotypes were identified by examining these derivative melt plots.

RESULTS

Sequence Alignment and the Identification of Nucleotide Variations

The alignment of the *CHD1Z* and *CHD1W* DNA sequences identified 252 single nucleotide polymorphisms out of 2,944 bp aligned sequence with 91% identity (data not shown). Three exons were chosen for primer design, and the resulting predicted amplicons from respective chromosomes are shown in Figure 1. In the CHD1UNEHRM1 amplicon, 8 nucleotide variations were found with TCCT-CGC as a haplotype on the Z chromosome and CGG-GGTT as a haplotype on the W chromosome. In the CHD1UNEHRM2 amplicon, 3

nucleotide variations were found with TGC as a haplotype on the Z chromosome and CAT as a haplotype on the W chromosome, and in the CHD1UNEHRM3 amplicon, 10 nucleotide variations were found with GTCTACCCCC as a haplotype on the Z chromosome and ACAGGTTTTG as a haplotype on the W chromosome.

Confirmation of PCR Amplicons

All 3 pairs of primers amplified DNA samples from male and female chickens, and the expected sizes of the amplicons were produced (Figure 2). PCR fragments at the sizes of 161 bp, 104 bp, and 123 bp were produced by the primer pairs of CHD1UNEHRM1F/R, CHD1UNEHRM2F/R, and CHD1UNEHRM3F/R, respectively. The results showed that primer pair CHD1UNEHRM3F/R produced more abundant PCR products and amplification was more specific, whereas the primer pair CHD1UNEHRM1F/R had 2 faint nonspecific bands for the female chickens. Primer pair CHD1UNEHRM2F/R was specific, but the intensity of its PCR product was relatively low.

High-Resolution Melt Analysis for Genotyping of Male and Female Birds

HRM analysis of CHD1UNEHRM1 revealed that male birds that possess ZZ chromosomes with homozygous genotype (TCC-CGC/TCC-CGC) showed a single transition of the fluorescent signal in the melting curve, that is, single melting phase in a temperature range of 74.0°C and 80.5°C. This confirms the presence of homologous DNA molecules in the PCR products of male birds. Female birds which possess ZW chromosomes with heterozygous genotype (TCC-CGC/CGG-GGTT) had 2 transitions in the melting curve, that is, 2 melting

phases, indicating the presence of heterologous DNA molecules in the PCR products (Figure 3A). High-resolution melting analysis of CHD1UNEHRM2 from male and female birds with respective homozygous (TGC/TGC) and heterozygous (haplotype TGC/CAT) genotypes showed single and double transitions in the melting curves, that is, single and 2 melting phases respectively in a temperature range of 75.0°C and 81.0°C (Figure 3B) confirming the presence of homologous and heterologous DNA molecules in the PCR products. Similarly, HRM analysis of CHD1UNEHRM3 from male and female birds with respective homozygous (GTCTACCCCC/GTCTACCCCC) and heterozygous (GTCTACCCCC/ACAGGTTTTG) genotypes showed single and double transitions in the melting curves, that is, single and 2 melting phases respectively in a temperature range of 77.0°C and 81.5°C (Figure 3C) also confirming the presence of homologous and heterologous DNA molecules in the PCR products.

Difference plots produced by the normalized fluorescence level of all the genotypes minus that of a ZW genotype showed a clear differentiation between male and female birds as demonstrated with all 3 primer pairs (Figures 3D–3F). In addition, melt curve analysis showed that male birds homozygous for the amplicons produced from CHD1UNEHRM1, CHD1UNEHRM2 and CHD1UNEHRM3 had single peaks in their derivative melting curves, peaked at 79.0°C, 80.0°C, and 80.4°C, respectively, confirming the homozygosity in male birds. In contrast, female birds had 2 peaks at 75.5°C and 79.2°C for CHD1UNEHRM1, 77.6°C and 80.2°C for CHD1UNEHRM2, and 79.4°C and 80.4°C for CHD1UNEHRM3 (Figures 3H–3J), confirming the heterozygosity of female birds. In comparison among the melting curves (HRM, difference plot, and melt) of the 3 primer pairs, CHD1UNEHRM3 had a better visual separation between male and female samples and

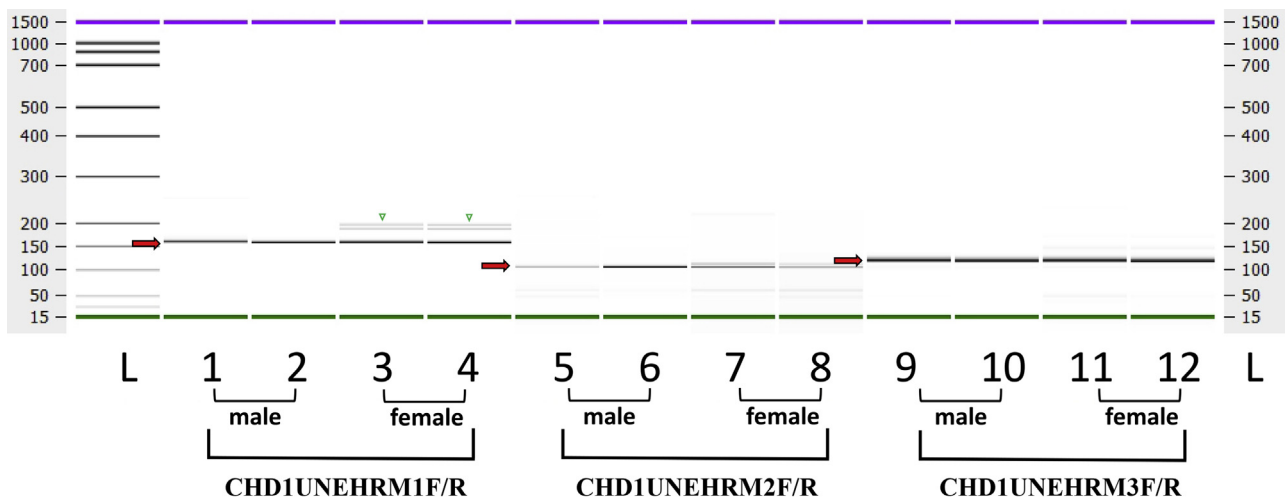


Figure 2. The PCR products amplified from male and female chickens with the primer pairs CHD1UNEHRM1F/R, CHD1UNEHRM2F/R and CHD1UNEHRM3F/R analyzed with Agilent Bioanalyzer 2100. L, DNA ladder; 1-4, PCR products amplified by primer pair CHD1UNEHRM1F/R; 5-8, by primer pair CHD1UNEHRM2F/R and 9-12 by primer pair CHD1UNEHRM3F/R. DNA templates were from feathers of male and female chickens as shown. The upper (purple) and lower (green) markers act as internal standards and are used to align the lanes for a consistent length of DNA in all the lanes. Arrows show the fragments sized 161, 104, and 123 bp that were amplified by the respective primer pairs. Triangles indicate nonspecific bands amplified by primer pair CHD1UNEHRM1F/R.

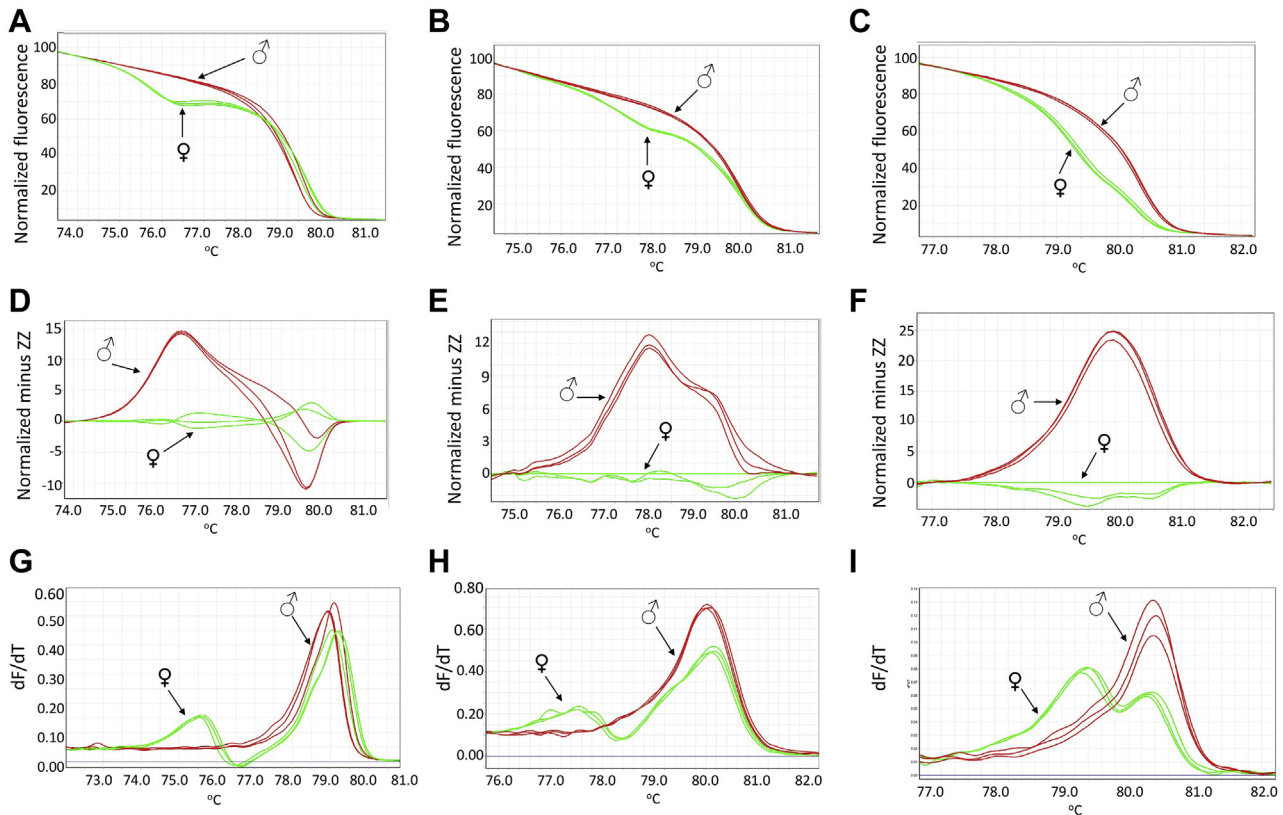


Figure 3. High-resolution melting (HRM) curve profiles of amplicons from primer pairs CHD1ZUNEHRM1F/R (A, D, and G), CHD1ZUNEHRM2 F/R (B, E, and H), and CHD1ZUNEHRM3F/R (C, F, and I). Figures A, B, and C are normalized HRM curves—melting data were normalized by adjusting the start and end fluorescence signals; D, E, and F difference plot—the normalized fluorescence level minus the level of a single ZW genotype; G, H, and I derivative plot—derivative melting curves produced from the fluorescence plotted against temperature plots. ♂: male chickens (red); ♀: female chickens (green).

showed more consistent melting curves within the replicates.

Validation of the HRM genotyping in a population of d-old chickens

The current assay successfully determined the sex of a group of 45 broiler chicks at d of hatch using the crude DNA extracted from feather tips. High-resolution melting curves of the male birds showed 2 melting phases, whereas the curves from female chickens showed a single melting phase. This was distinguished for all 3 amplicons amplified by their respective primer pairs (Figure 4). The sex of the birds was confirmed by dissecting the birds to examine the presence of testes following the completion of the trial. The sexes of all the birds were consistent between testis observation and feather DNA sexing followed by HRM analysis.

In addition to the 45 broiler chicks used to validate HRM genotyping at 1-d-old, a further 1,318 birds were DNA sexed using the same HRM protocol resulting in 600 females and 718 males from 3 other studies. The birds were dissected at the end of the trials, and the results revealed that all birds were sexed correctly using HRM genotyping confirming the accuracy of this method.

DISCUSSION

Owing to the current inability to feather sex many commercial breeds of broiler chickens, alternative sexing methods need to be developed for research purposes. While vent sexing is fast and accurate, many hatcheries are reluctant to allow vent sexers to sex a small number of birds for research purposes because of biosecurity risks. Furthermore, vent sexing can increase early chick mortality by up to 1% due to the stress associated with manual handling of the birds (Phelps et al., 2003). Recently, *in ovo* sexing methods have been reported in the literature such as fluorescence and Raman spectroscopy (Galli et al. 2016; Galli et al., 2017; Galli et al., 2018), chemical biomarker assays (estrone sulphate) (Weissmann et al., 2013), and DNA determination (Clinton et al., 2016). However, these technologies are not feasible for the industry or for poultry research because of the requirement of sophisticated equipment and/or procedures. Genetic engineering methods have been implemented for the production of female-only birds for egg layers (Doran et al. 2018); however, such modification for single-sex broilers has yet to be realized. Therefore, a simple, economic, and high throughput sexing method is needed to aid poultry researchers in obtaining sexed broilers within a few days posthatch, before experimental treatments are applied.

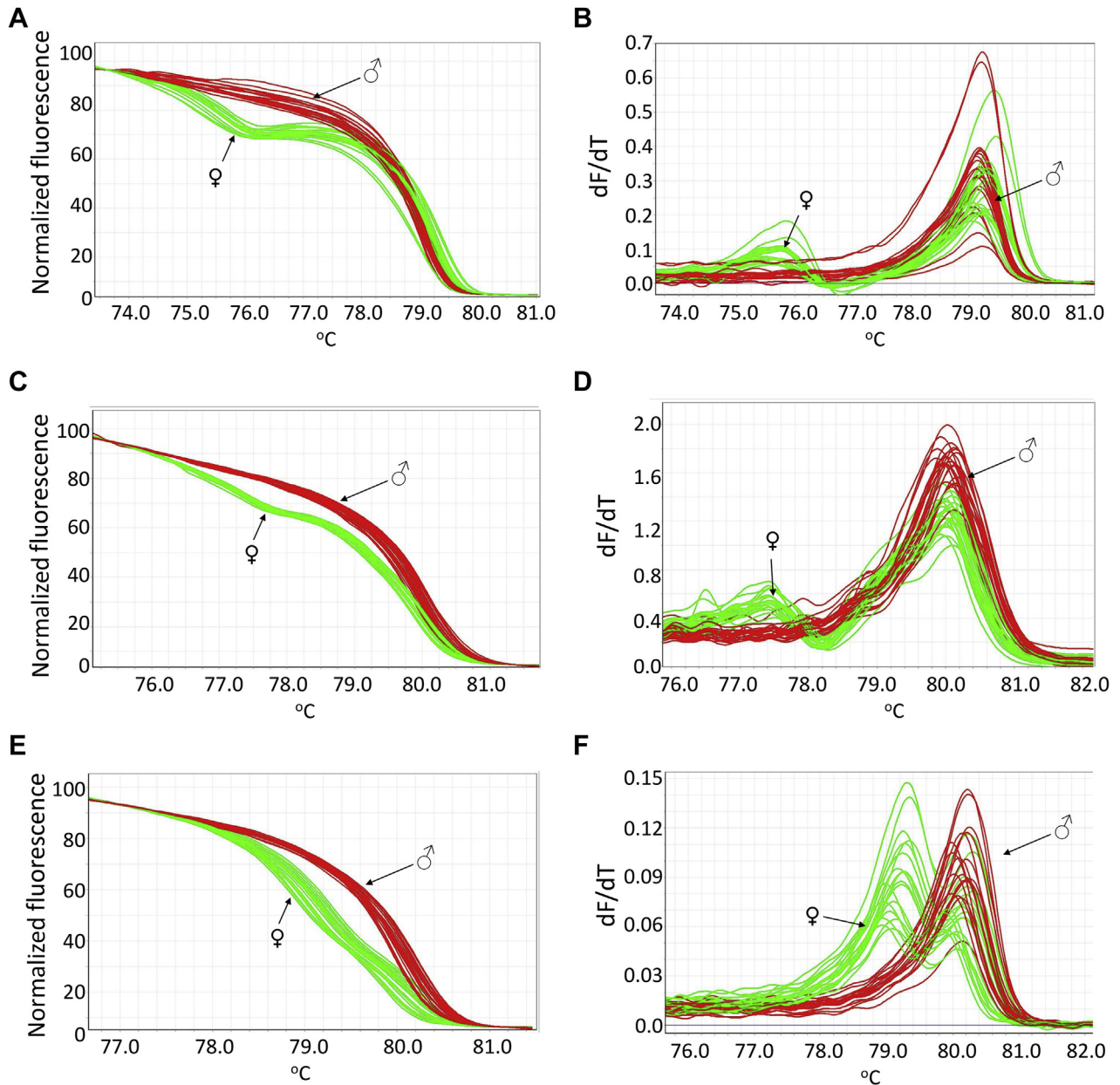


Figure 4. Validation of the high-resolution melting (HRM) curve analysis using primer pairs CHD1ZUNEHRM1F/R (A and B), CHD1ZUNEHRM2 F/R (C and D), and CHD1ZUNEHRM3 F/R (E and F) in a population of 45 birds. It was shown that 24 birds were male (♂, red), and 21 were female (♀, green). A, C, and E show normalized HRM curves; B, D, and F show derivative plots.

In the present study, HRM analysis was successfully used to determine the gender of d-old broiler chicks in combination with a simple, fast and economical DNA extraction method from feathers. It is estimated that 500 to 800 birds can be DNA sexed within a day using the procedure described in this article, making it appropriate to be used in broiler nutrition research that may require hundreds of birds. The ability to sex broilers within the first couple of days can ensure an even distribution of male and female birds in all experimental units, that is, pens/cages. This is beneficial as evenly distributed male and female birds can minimize pen-to-pen variations caused by the sex effect (Da Costa et al., 2017). Furthermore, the cost of the DNA sexing with HRM

analysis was also minimal with an estimate of approximately \$0.70 (AUD) per bird for reagents and lab consumables. An additional \$40 (AUD) per h for labor should also be considered for lab and data analysis and sample collection. This amount is deemed affordable for research purposes.

Previous studies have reported the use of DNA sexing of birds using electrophoresis (Griffiths et al., 1998; Wang et al., 2011), melting curve-based PCR methods (Chang et al., 2008; Huang et al., 2011; Chen et al., 2012), multiplex PCR (Koshiishi and Wada, 2018), and real-time qPCR on mRNA expression (Wan et al., 2017). The electrophoresis-based PCR method involves post-PCR manipulations (Griffiths et al., 1998; Wang

et al., 2011), and this makes the procedure time consuming and expensive. The melting curve-based method, that is, melting curve analysis, does not involve post-PCR manipulations but is less sensitive to the variations present in DNA molecules so that the assay may have to make use of more primers common for ZW and specific for W (Chang et al., 2008; Huang et al., 2011; Chen et al., 2012). This makes the melting curve profiles more complicated for identifying the sex of birds. Furthermore, there is a possibility of a false-negative amplification of female birds by the W-specific primer which may be identified as male, thus causing an error in allocating genotype. The method developed using mRNA instead of genomic DNA requires the use of chicken tissues making it an invasive technique which is not practical. It should also be noted that although a successful HRM sexing method using the P2/P8 primers was reported in other bird species (Morinha et al., 2011, 2013), the use of the degenerate primer P8 can possibly reduce the accuracy of this analysis as the primer may produce heterologous PCR products even with a homozygous genotype (Dawson et al., 2001). Chapman (2012) developed a method to sex the Caribbean flamingo based on short amplicon HRM analysis which targets single-nucleotide polymorphisms instead of intron-size differences between CHD-Z and CHD-W genes. However, because HRMA relies on single-nucleotide polymorphisms to distinguish homozygotic males from females, primers corresponding to the CHD-Z allele must be a perfect match. Scoring may be misleading in instances where mismatched nucleotides of primers cause a null allele for CHD-W, resulting in misclassification of heterozygotic females as homozygotic males (Carlson et al., 2006). In addition, HRM analysis was used to sex ratite birds with the use of 4 different markers which resulted in the distinct separation of male and female birds (Morinha et al., 2015). This method is made applicable specifically for sexing ratite bird species as the divergence of the sex chromosomes of ratite birds have hindered the utilization of the sex-linked markers developed for non-ratite species.

The accuracy of HRM analysis to detect small DNA variations down to single nucleotide differences has been considered to rely on a variety of factors including the primer sequences and resulting amplicons, DNA purity, Taq polymerase quality, and salt concentrations in the reactions (Nolan et al., 2013). It has been described that DNA quality and quantity could impact HRM melt profiles, in particular, the carryover of salt from the DNA isolation procedure into the HRM reaction causing low sensitivity, poor reproducibility, and/or incorrect genotype calls (Twist et al. 2013). It has long been shown that high-quality DNA can only be secured with lengthy extraction and purification procedures from tissue or blood with a high DNA content by using in-house protocols or commercial kits. This is normally time-consuming and expensive. To sex broiler chickens to be used in a relatively large scale experiment such as nutritional trials, a method is needed that is both quick

and cost effective such as the one in the current study. With efforts to optimize the DNA extraction protocols and HRM analysis, we have been able to extract crude DNA in a fast and efficient manner for the accurate analysis of the variations present in *CHD1Z* and *CHD1W* genes. Our protocol uses crude DNA that is extracted by the incubation of the feather tip content in a buffer for 15 min while still overcoming the possible interference from the extraction being carried over to the HRM analysis. This may be because of the use of proteinase K to catalyze proteins such as hemoglobin in blood and other compounds such as lactoferrin, IgG, and myoglobin known to inhibit polymerase activity (Al-Soud and Rådström, 2001). For the primer pairs used in the current study, primer pair HD1U-NEHRM3F/R showed better separation between the normalized HRM curves and distinct peaks of derivative melt curves of male and female birds. Therefore, we recommend to use the primer pair HD1UNEHRM3F/R for HRM DNA sexing of chickens. The other 2 pairs of primers can be used if confirmation is needed because of poor DNA quality for individual birds or possible PCR reagent inconsistency between different laboratories. In addition, the protocol shortened the time required for PCR amplification and HRM analysis to a total of approximately 70 min which allows for a fast turnaround time of the assay as well.

Overall, the technique described in this study offers researchers and possibly producers the ability to sex day-old broiler chicks in a noninvasive, efficient, cost-effective, and highly sensitive way, allowing for high throughput genotyping. The method developed in this study is much shorter than other molecular methods used to determine sex in chickens. This significantly contributes to the industry particularly in research where the uniform distribution of sex may help to reduce the variation in results. However, it is important to note that many factors such as DNA concentration and quality (in spite of crude DNA), proper labeling of birds and samples, and real-time PCR machine performance may influence the accuracy of this method. Therefore, the protocol should be followed precisely to produce accurate results, and any analysis done should be carried out with the use of control samples in which the sex is known to avoid errors in sex assignment. This method can also be considered for use in other avian species as they may have homologous DNA sequences that can be amplified with the use of the same primers designed in this study. Future work can definitely be explored to determine if this protocol can be applied to determine the sex of other avian species.

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DISCLOSURES

The authors declare that they have no conflicts of financial interest.

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