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Gender Identification of Domesticated Chicken Using a PCR-based Method

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ABSTRACT

The monomorphism of newly hatched chicks poses a problem for farmers who need to separate them. Currently, the most widely used technique is the cloacal sexing method which has a low accuracy rate. Thus, a PCR based gender determination protocol was established to obtain a higher accuracy rate than conventional sexing methods. Feathers of a day up to a week old chicks were collected for the molecular analysis using the PCR targeting the *CHD-W* and *CHD-Z* genes in the chicken's chromosome Z and chromosome W, respectively. The results of this study showed that the PCR based gender determination protocol was a sensitive and accurate method for determining the gender of monomorphic chicks as compared to the conventional sexing method.

Keywords: Monomorphism, PCR based gender determination protocol, *CHD-W* and *CHD-Z*

INTRODUCTION

Chicken sexing has been an integral part in the breeder, broiler, and layer industries since 1935. An early determination of chick's gender will enable farmers to reap more profits based on the difference in growth rates exhibited by the gender of chickens and the female's role in producing eggs in the layer industry. This, in turn, will reduce unnecessary cost in raising unwanted chicken such as food, water, and vaccination of chicken. The first sexing technique was vent sexing which was introduced by Professor Masui and Mashimoto (Cerit and Avanus, 2006) in 1935. Vent sexing is a sexing method which is based on the appearance of the chick's sexual organs. Unfortunately, this widely practiced method is not very accurate due to certain factors such as the morphological variation of the sex organs in different breeds of chicken and the personnel's experience in handling the sexing exercise. The accuracy of vent sexing has been determined at 98% (Phelps, 2001). Unfortunately, this 2% margin of error may cost big monetary loss considering the fact of the current scale of the poultry industry.

Chromosomally, the gender of chicken is determined by the sex chromosome ZW with males having ZZ (Saitoh *et al*., 1991; Saitoh *et al*., 1993). On the other hand, females are heterogametic WZ (Griffith and Tiwari, 1996; Griffith and Korn, 1997; Griffith *et al*., 1998; Ellegren, 2001). In 1996, Griffith developed universal primers for PCR based application of sex determination on non-ratite birds. These primers targeted the Chromodomain Helicase DNA binding protein (*CHD*) gene. The protein itself is an ATP-dependent chromation remodelling factor that controls how DNA is being packed. Cerit and Avanus (2006) state that the *CHD* gene chromosomes Z and W are avian specific sex linked gene. Subsequently, Griffith *et al*. (1996) designed a pair of primers (P8 and

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P2) that are able to amplify both *CHD-W* and *CHD-Z* simultaneously with different product sizes for both sex chromosome linked genes in non-ratite avian. This results in females having double bands and males with a single band of PCR products after agarose gel electrophoresis due to the amplification of both targeted genes. Thus, an attempt was done in this study to test the application of the existing universal primers, described by Griffith *et al*. (1998), in determining the gender of a day up to a week old chicks, as well as to compare the accuracy rate of this PCR-based sexing method with the conventional sexing method. Subsequently, two sets of primers were also designed to confirm the results obtained from the primers described by Griffith *et al*. (1998) and Richard *et al*. (2009).

MATERIALS AND METHODS

Collection of DNA Sources and DNA Extraction

Primary feathers were collected from 30 chicks and each chick was tagged for verification exercise after four weeks beginning from the date of samples collection. DNA was extracted from these 30 samples using the Wizard® Genomic DNA Purification Kit (Cat. No. A1125, Promega, Madison, USA) and the resultant DNA extract was qualitatively checked and quantified at 260nm/280nm using a spectrophotometer. This was followed by standardisation of all DNA extractions into concentration of 0.1µg/µl DNA each. All the samples were also electrophoresed in a 1% agarose gel to determine the intensity of the bands for the DNA extracts.

PCR

The PCR was done following the protocol by Mulis (1987) and Sambrook *et al*. (1989). Meanwhile, the final reaction mixture of 25µ1 was prepared as follows: 1x PCR buffer; 0.25mM dNTP; 1U/25µl *Taq* DNA polymerase; 1pmol/µl of primers; 0.2 µg/25µl DNA template and topped up with ddH_20. The PCR primers used in this study were P8 (5’- CTY CCR AGR ATG AGA AAC TG -3’) and P2 (5’- TCT GCA TCA CTA AAT CCT TT -3’), adapted and modified from Griffith *et al*. (1998); 2 sets of primers were designed from the National Centre for Biotechnology Information (NCBI) Genbank sequences *viz*. JVK1 (5’- CTC AGG AGA TGG ATA TAG-3’) and JVK2 (5’-GTT AGC TAC CTT GAA CTG -3’) from the accession number AF00659; ZR (5’- CAG AGG TCT CCT TAT GGT TC -3’) and ZF (5’- GTC CTT GAG AGT TCT CTA CC -3’) were from the accession number AF006660. The product sizes for the primer pair P2 and P8 are 345 bps for the *CHD-W* gene and 362 bps for the *CHD-Z* gene, respectively. The primer pair JVK1 and JVK2 yielded a PCR product with a size of 92 bps from the *CHD-W* gene, whereas the primer pair of ZF and ZR yielded a PCR product with the size of 527 bps from the *CHD-Z* gene, respectively. *Figs. 1* and *2* show the amplicon sites of the primers used in this study.

The optimisation of the PCR conditions was done using both the adult male and female chicken archived DNA extracts which were available at the Molecular Biology Laboratory, Biotechnology Department in UCSI University. The PCR was performed in a Mastercycler Gradient thermocycler (Eppendorf, Germany), with the following temperature profile: initial denaturation at 95°C for 5 mins, followed by 30 repeating cycles of denaturation at 95°C for 1 min 30 sec, annealing at 41.5^{o}C for 1 min 30 sec, extension at 72°C for 1 min 30 sec, and a final extension of 72°C for 3 mins. The PCR products were then separated in 3% agarose gel electrophoresis stained with ethidium bromide at 90 V for an hour, and they were then visualised under UV illumination and photographed.

RESULTS AND DISCUSSION

Apart from extracting DNA from feathers, other non-invasive approaches such as DNA extraction from egg shell membranes (Yun and Xiao, 2008) and DNA from faeces (Wasser *et al*., 1997) can be good sources of DNA (Taberlet *et al*., 2006) for the molecular analysis of chicken sex determination. The spectrophotometric ratios of 260nm/280nm in this study were found to range

Gallus gallus chromosome W chromodomain helicase DNA binding protein 1 (_CHD-W_) mRNA.

1741 gctagagctc ataggattgg accaaagaaa caggttaata tttatcggct agtcaccaaa

1801 ggatcagtag aagaagatat tcttgaaaga gccaagaaaa agatggtgtt agatcattta

1861 gtgattcaga gaatggacac cacagggaaa actgtactac atacaggctc tactccttca

1921 agctcaacac cttttaataa ggaagagtta tcagcaattt tgaagtttgg tgctgaggaa

1981 ctttttaaag aacctgaagg ggaggaagag gagcctcagg agatggatat agatgaaatc

2041 ctgaagaggg ctgaaactcg agaaaatgag tcaggcctat taactgtagg agatgagtta

2101 ctttcacagt tcaaggtagc taacttttcc aatatggatg aagatgacat tgaattggaa

2161 ccagaacaaa atctaagaaa ctgggaagaa atcattccag aagttcagtg gcgacgaata

2221 gaagaggagg aaagacaaaa agaacttgaa gaaatatata tgcttccaag aatgagaaac

2281 tgtgcaaaac agatcagctt taatggaaat gaagggagat gcagtaggag cagaagatat

2341 tctggatctg atagtgattc catctcagaa agaaaacgac caaaaaaacg tggacgacca

2401 cgaactattc cccgtgaaaa cattaaagga tttagtgatg cagagattag acgatttatc

2461 aagagttaca agaaatttgg tggcccactt gaaaggttag atgctatagc tagagatgct

2521 gagctagttg ataaatctga aacagacctt agacgtctgg gagaacttgt acataatgga

2581 tgcattaagg ctttaaatga taatgacttt ggtcaaggaa gaacaggtgg tagatttggg

2641 aaagttaaag gcccaacatt ccgaatagca ggagtgcagg tgaatgcaaa gctagtcatt

2701 tctcacgaag aagagttggc accattgcat aaatcgattc cttcagatcc agaagaaagg

2761 aaaagatatg tcatcccata ccacaccaaa gcagctcatt ttgatataga ttggggtaaa

2821 gaagatgatt ccaatctgtt aataggcatc tatgaatatg gttatggcag ctgggaaatg

Fig. 1: Nucleotides in grey show primers JVK1 and JVK2 aligned to the CHD-W in G. gallus genome (Accession number AF181826), while nucleotide (in yellow) indicates the locations of primers P8 and P2 align in CHD-W. The estimated size for the product of primers JVK1 and JVK2 was 92 bps

Gallus gallus **chromo-helicase-DNA-binding on the Z chromosome protein (*****CHD-Z*****) mRNA.**

3241 cattgactgt aggggatgag ttgctttcac agttcaaggt ggcgaacttt tccaatatgg

3301 atgaagatga tattgagttg gaaccagaaa gaaattcaag aaattgggaa gaaatcatcc

3361 cagaatccca acggagaagg atagaggagg aggaaagaca aaaagaactt gaagaaatat

3421 acatgctccc gaggatgaga aactgtgcaa aacagatcag ctttaatggg agtgaaggaa

3481 gacgcagtag gagcagaaga tattctggat ctgatagtga ctccatcaca gaaagaaaac

3541 ggccaaaaaa gcgtggaaga cctcgaacca ttcctcgaga aaatattaaa ggatttagtg

3601 atgcagagat caggcggttt atcaagagtt acaagaaatt tggtggccct ctggaaaggt

3661 tagatgctgt agctagagat gctgaactgg ttgataaatc tgagacagac cttagacgtt

3721 tgggtgaact tgtacataat ggatgcatta aggctttaaa ggacaattca tctggacaag

3781 aaagagcagg aggtagactt gggaaagtta aaggcccaac gtttcgaatc tcaggagtgc

5461 aatcttcgag agattataga taccactcag actggcaaat ggaccacaga gcttctggta

5521 gtggcccgag gtcaccacta gatcagaggt ctccttatgg ttcaagatct cccctaggac

5581 acagatctcc atttgaacac tcatcagatc acaaaagtac acctgaacat acatggagta

5641 gccggaagac ataacaaaga ctgacatttt ctggaccttc tttttagcca tatacagtaa

5701 actaacacag taattgcctt acatgacttg aaagatatgg actggatatt ctatcagtag

5761 cagtattgtt acttctttcc aggatgcaag gtctattatc ccaacagaag aaaaatattt

5821 ttgtatttaa agtttatgct gcactgtgct gcaaatgttg tggcactttt tttttaagaa

5881 atggaagatg tttactttta cagggacctc aacactgccc ctttcagact ggatcttact

5941 ataaaactct tcatgtcaaa gtggttctag gctgaacaca gattaaatta tgtttgtaaa

6001 tgaacactta aacactgacc tgtgcttatg tttcaggaaa gaatggggga tttattttgt

6061 tttatttctt ggtagagaac tctcaaggac tttgttcact ttccaaagct acttgtttac

Fig. 2: Nucleotides in grey show primers ZF and ZR align to the CHD-Z in G. gallus genome (Accession number AF004397). On the other hand, nucleotides (in yellow) show the location of the primer pair P8 and P2 aligned to the CHD-Z gene. The estimated size for the product of primer pair ZF and ZR was 527 bps

from 1.7 to 2.3, with the values falling below 1.8 due to protein contamination in the extracted DNA sample (Joseph, 2007). Meanwhile, the values more than 2.0 might indicate the presence of RNA contamination or the presence of high GC content substances in the extracted DNA (Sambrook and Russell, 2001). The authors were able to extract high yield and high purity DNA using primary feathers in this study. Subsequently, if the bands of the DNA extract were too intense, further dilution was then carried out to ensure that all the samples had the DNA concentration of 0.1µg/µl and that their intensities were comparable to one another.

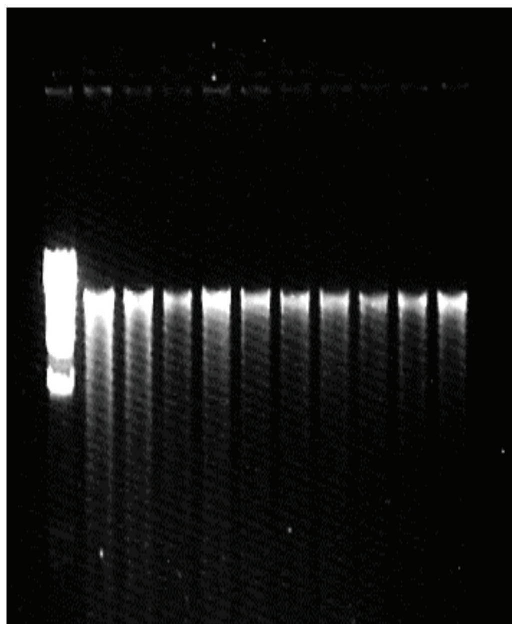

Fig. 3: The results of DNA extraction performed on 10 primary feather samples

Then, the positive controls underwent PCR optimisation (Vernon *et al*., 2001) and the results are shown in *Fig. 4*. The female sample produced two bands at the estimated size of 345 bps and 362 bps, respectively, whereas the adult male chicken produced a single band of 345 bps size, which is congruent with the finding by Griffith *et al*. (1998).

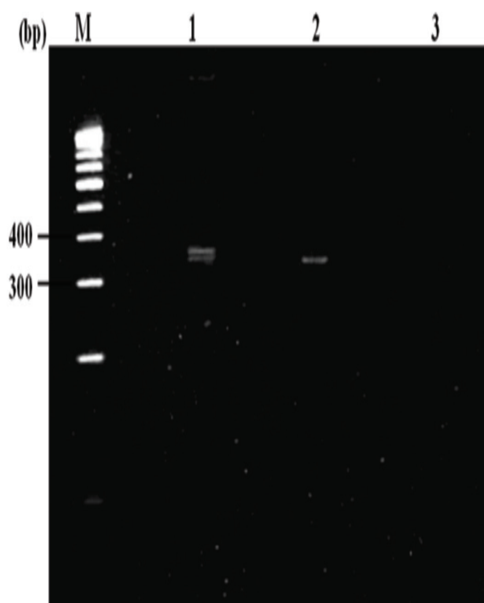

Fig. 4: A female sample in lane 1 was with double bands due to the CHD-Z (345 bp) and CHD-W (362 bp), while a single banded male sample CHD-Z (345 bps) in lane 2; M is the 100 bps molecular marker and lane 3 is the negative control

The results gathered for the PCR amplification of both the male and female samples, using the JVK1/JVK2 primer set which targeted the CHD-W gene, are shown in *Fig. 5*. The female samples produced a single band at 92 bps, and this is congruent with the predicted amplicon size designed using the NCBI Genbank accession no. AF181826. On the other hand, the male samples did not produce any band as they do not have chromosome W in their karyotype. *Fig. 6* shows the PCR amplification of both the male and female samples using the ZF/ZR primer set. Both the samples produced bands as the primers targeted the CHD-Z genes in both the male and the female chicken genome. The size of the products, on the contrary, was about 527 bps, which is the same as the predicted amplicon size designed using the NCBI Genbank accession no. AF004397.

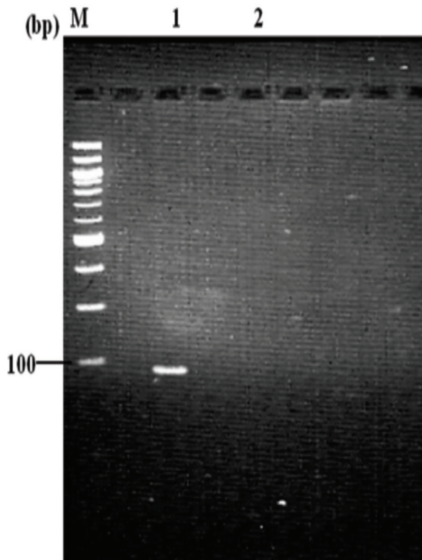

Fig. 5: A positive sample was detected in lane 1 where CHD-W (92 bps), while lane 2 shows the absence of CHD-W, and M is the 100 bps molecular marker

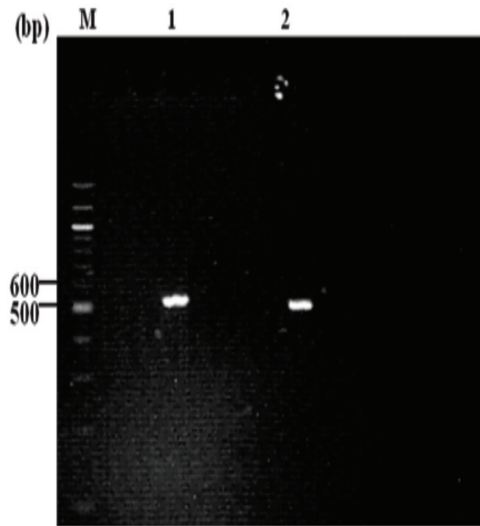

Fig. 6: Positive results of CHD-Z for both the samples using the ZF/ZR primer set due to presence of CHD-Z in both the male and female chicken genomes

Both the positive control samples showed positive results as CHD-Z (527bp) was present in both the male and female genomes (*Fig. 6*). *Fig. 7* reveals the PCR results of 10 out of the 30 samples of chicks using the P8/P2 primer set. In this study, the molecular analysis results were verified by observing the chickens that were sampled earlier. As a result, out of 30 the chicks, there were 16 male chicks and 14 female chicks. The PCR-based results are congruent with the results collected from the farm site after four weeks of captivity. Therefore, this method has been proven to be reliable and is 100% accurate as the gender for all the 30 samples has been correctly identified using the established PCR-based protocol. Thus, it is a better sexing method compared to vent sexing which only has 98% accuracy rate. Apart from that, using feathers as a source of DNA is a good approach as it is a non-invasive method compared to other sources, such as blood, biopsy, and liver samples.

As the PCR-based sexing method is an accurate method, it should be proposed to be used by farmers from various industries to replace the conventional vent sexing method which has an accuracy rate of 98% only. This will increase productivity and reduce cost associated with loss due to raising chicks not desired in the poultry industry. Despite all the advantages and benefits of using this method, the cost incurred is higher than the vent sexing method due to the equipment, reagents and laboratory setup, which will become more profitable in a long run, especially for the large-scale poultry farms. In addition, the PCR screening is also capable to sex 10,000 chicks within 6 days or less, without any error. Thus, this may also serve as a potential new job opportunity for biotechnology graduates as trained personnel in the field of molecular biology are required to use the proposed method in conducting tests.

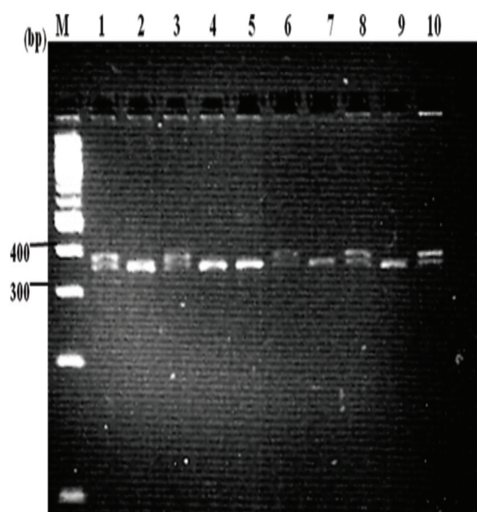

Fig. 7: 10 samples of chicks and M is the 100bp molecular marker. Samples 2, 4, 5, 7, and 9 were males while the rest were females

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