A Non-Invasive and Inexpensive PCR-Based Procedure for Rapid Sex Diagnosis of Chinese Gamecock Chicks and Embryos

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Abstract: We sought to design a simple and rapid diagnostic kit for sex identification in chicks and embryos of Chinese gamecocks, using DNA extracted from chicks and embryos through non-invasive methods. This diagnostic process is based mainly on the amplification of intron length polymorphisms in chromo-helicase DNA-binding (CHD) genes in both Z and W chromosomes by Polymerase Chain Reaction (PCR). This approach offers some advantage over the old diagnostic method developed by Fridolfsson and Ellegren, in which one of the primers is mismatched to the chicken CHD-Z gene and it can additionally be used for the rapid sexing of chicks and embryos. In this study, we demonstrate that the improved method of sex identification is more stable than Fridolfsson's and uses simple, non-invasive DNA extraction, to provide a rapid and inexpensive procedure for sexing chicks. We made successful application of this procedure for sexing chicken in two diagnostic cases. We further tested another pair of primers targeting chicken chromo-helicase-DNA binding (CHD) genes as well. And lastly, we used this technology to develop a PCR-based diagnostic kit with an internal control for the sex identification of gamecock chicks and embryos.

Key words: Gamecock, sex diagnosis, non-invasive, chromo-helicase-DNA binding gene, PCR, chick, embryo

INTRODUCTION

In China, gamecocks (also known as Douji) are a traditional chicken breed reared solely for cockfighting. The history of cockfighting can be traced back to 2500 years ago (West and Zhou, 1989; Xie, 1992; Zhu, 1999). Cockfighting and gamecocks remain popular in China to this day. Male gamecocks generally need to be identified and segregated early for special handling and training. However, the early sex identification of chicks can be present problems as, with the exception of highly commercialized chicken breeds, male and female hatchling or fledgling birds are often morphologically indistinguishable. It is difficult to identify gamecocks by sex-linked morphological traits when they are required to be segregated at no >7 or 8 weeks of age. Methods of sexing should be accurate and non-invasive. Traditional identification methods used in monomorphic birds, such as vent sexing, laparoscopy, steroid sexing and karyotyping, are time-consuming and involve some amount of risk to birds (Cerit and Avanus, 2007). The rapid feathering measures (automatic sexing by feather colour) used in commercialized chicken demand a

particular breed of pure fowl. It is unrealistic, however to use this approach in genetic and breeding studies involving native chicken breeds, such as gamecocks. More rapid and flexible approaches are therefore desirable.

Molecular sexing based on the Polymerase Chain Reaction (PCR) is one attractive option, as PCR is simple to perform, rapid and requires only a minute quantity of DNA, which can be obtained from a single feather or a drop of blood. The DNA can then be used to identify a sex-specific marker. Consequently, with the development of molecular sex identification techniques (Griffiths et al., 1996; Griffiths and Orr, 1999; Kahn et al., 1998; Fridolfsson and Ellegren, 1999), the sex of previously indistinguishable individuals may be assigned unequivocally. Itoh et al. (1997) used a W chromosome specific primer pair to differentiate between males and females using PCR. Griffithsm et al. (1998) suggested a single set of PCR primers to amplify homologous sections of 2 conserved CHD (Chromo-Helicase-DNA binding) genes, located on the sex chromosomes of all birds. The primers amplify homologous sections of the 2 CHD alleles, incorporating introns that usually vary in size. The CHD

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alleles are flanked by highly conserved nucleotide sequences. Alleles can therefore be distinguished by their molecular length using electrophoresis separation. After Griffithsm *et al.* (1998) and Fridolfsson and Ellegren (1999) designed another pair of PCR primers (2550F/2718R), which made the PCR-based procedure simpler and more rapid, by amplifying an intron from the CHD-Z and CHD-W alleles, followed by agarose gel electrophoresis. The Fridolfsson's method had been applied in the sexing of many bird species (Fridolfsson and Ellegren, 1999). However, this technique fails when the PCR system is unstable such that the amplified products cannot be detected on agarose gels (Pagliani *et al.*, 1999) or when length difference between the CHD-Z and CHD-W alleles is too small. Some bird species have no or only very small variations in intron length under normal conditions and several modifications of Griffiths' and Fridolfsson's methods that seek to address this shortcoming have been reported (Ito *et al.*, 2003; Sacchi *et al.*, 2004; An *et al.*, 2007; Cerit and Avanus, 2007; Reddy *et al.*, 2007).

In this study, we demonstrate that Fridolfsson's method is unstable for sexing chicken, as one of the primers (2550F) is mismatched to the CHD-Z sequences. In order to resolve this problem, we modified the existing technique using a new chicken CHD-Z specific primer (2376F) to assign sex in adult chickens and chicks. The improved PCR-based diagnostic method is simple, rapid, cheap and non-invasive. We also used a pair of PCR primers (1132F/1277R) developed recently by Chen (2009) base on CHD-W genes to test the efficiency of the new primer pair (2376F/2524R). Finally, we report two cases of application of the new PCR-based sexing technique to chicks and embryos.

MATERIALS AND METHODS

DNA preparation: For feather samples, genomic DNA was extracted by two optional methods.

Method 1 was adopted from Zhao and Li (2003) with necessary modifications. Two to three breast feather tips (calami) were collected. Each feather of 0.5 cm long cut from the tip was washed by 75% ethanol and distilled water, fully sheared with scissors. The material was mixed with 1 ×PCR buffer (50 mmol L^{-1} KCl, 10 mmol L^{-1} Tris-HCl and 0.01% glutin) with MgCl$_2$ added to 2 m mol L^{-1} and Proteinase K to 20 mg L^{-1}. The mixture was put on a thermal cycler with the following programme: 65°C for 30 min, 95°C for 15 min and 4°C for 10 min. The heated mixture was stored in a refrigerator at -20°C for subsequent PCR amplification.

Method 2 was modified from Higuchi *et al.* (1988). In brief, a single 0.5 cm feather tip was mixed first with 10 µL 1 mol L^{-1} DTT and then with 400 µL 1×TNE (10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA, 100 mmol L^{-1} NaCl, pH 8.0). After addition of 40 µL SDS (10%) and 10 µL Proteinase K (10 mg L^{-1}), the mixture was incubated at 56°C at least for 10 h. DNA was extracted using the phenol chloroform method. DNA was precipitated by isopropyl alcohol and washed by 75% ethanol and then dissolved in TE (Tris-EDTA, pH 8.0) buffer at a final concentration of 100 ng µL^{-1}. About 1 µL genomic DNA was used for the PCR reaction.

For embryo samples, genomic DNA was extracted using the alkaline method. Embryos of Chinese Luxi Gamecock (indigenous) and White Rock (commercial) chickens were used in the present study. Fertile eggs were collected and incubated at 38±0.5°C and 50-60% humidity (before the 8th day of egg candling) in the Chinese Agricultural University experimental incubator. Eggs were taken from the incubator between 7 and 8 days after the setting of the embryos and their associated membranes and were kept in sterile Petri dishes. Subsequently, tissue samples of embryos were collected aseptically and carefully in sterile micro-centrifuge tubes. About 25 mg of soft tissue of embryo was suspended in 600 µL of cell lysis buffer with 10 µL Proteinase K (10 mg L^{-1}), vortexed and incubated at 37°C overnight. DNA was extracted using the phenol chloroform method described above. Finally, 2-5 µL of the solution was used as template DNA for PCR amplification.

***In silico* PCR amplification and sequence alignment:** In silico PCR reactions were executed for the examination and pre-analysis of CHD gene primer designing. This job was completed using the chicken genome sequences at the UCSC genome browser homepage (http://genome.ucsc.edu/) with default settings. Reports and the *in silico* amplified chicken CHD-Z and CHD-W gene sequences were downloaded for reference. In addition, three avian species' CHD-Z and CHD-W gene sequences were also downloaded from the National Center for Biotechnology Information (http://www.ncbi. nlm.nih.gov/). These sequences are AB080660.1 (*Phalacrocorax capillatus* CHD-Z, abbreviated as PcCHD1Z), AB080661.1 (*Phalacrocorax capillatus*, CHD-W, abbreviated as PcCHD1W), AY464013.1 (*Platalea minor*, CHD-Z, abbreviated as PmCHD1Z), AY464014.1 (*Platalea minor*, CHD-W, abbreviated as PmCHD1W), AY517719.1 (*Columba livia*, CHD-Z, abbreviated as ClCHD1Z) and AY517718.1 (*Columba livia*, CHD-W, abbreviated as ClCHD1W). All of these CHD-Z and CHD-W alleles were aligned using the ClustalW online software (http://www.ebi.ac.uk/ clustalw/), displayed and modified by the BioEdit package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Table 1: Primers used in this study

Primers	Primer sequence	References
2550F	GTTACTGATTCGTCTACGAGA	Fridolfsson and Ellegren (1999)
2718R	ATTGAAATGATCCAGTGCTTG	
2376F	GCTACTGATTCGTCTGCGAGA	This study
2524R	ATTGAAATGATCCAGTGCTTG	
1132F	AGGATGGAAATGAGTGCATTG	Chen (2009)
1277R	CATCTGGTGGTTTAATGAGGT	

PCR amplification: PCR amplifications were carried out in a total volume of 25 μL. The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM of dNTP each, 0.01 mg Bovine Serum Albumin (BSA), 50 ng of each primer (P2 and P8) 12, 0.05 units of Taq polymerase and about 50 ng of genomic DNA. The graded PCR reaction programmes were designed with an initial denaturing step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 55±8°C in Experiment 1 or 50±6°C and 55±6°C in Experiment 2 for 30 sec and 72°C for 30 sec, with a final hold at 4°C for 10 min to complete the programme. The PCR products were then separated by electrophoresis on 2% agarose gel for 30 min at 120 V and stained with ethidium bromide for visualization under UV light.

Experiment 1: Comparing the effects of the modified and the original primers: PCR amplification productions of the new primer pair (2376F/2524R) and the original pair (2550F/2718R) were compared. Differences were analyzed in detail.

Experiment 2: Comparing the effects of two new pairs of primers: Other than the new primer pair (2376F/2524R), Chen (2009) designed a pair of primers (1132F/1277R, Table 1) based on another intron site of CHD-W and found it matched CHD-Z as well, but with shorter PCR products.

Primers used in these two experiments are shown in Table 1 and the graded PCR reaction programmes were set as described.

RESULTS AND DISCUSSION

Comparison of the effect of different PCR primers
Experiment 1: Comparing the effects of the improved and old primers: The results of PCR amplification using the newly modified primers and Fridolfsson's original primers are shown in Fig. 1. The graded annealing temperatures (Tm) were set at 55±8°C. These two pairs of primers were denatured and then annealed at graded temperatures around their optimized annealing temperatures as predicted by primer designer software. DNA was extracted from feather samples using the two methods described. The graded annealing temperatures were 47.2, 48.1, 49.5, 51.3, 53.4, 55.6, 57.8, 59.8, 61.6 and 62.8°C (from left to right), respectively.

A band of approximately 650 bp parallel amplified fragments was visible in the male and female samples with no obvious size variation. An additional band of approximately 450 bp was also visible in the female samples. Gel electrophoresis revealed one band in male and two bands in female birds (Fig. 1).

As demonstrated in Fig. 1, the ratios of positive or fully completed PCR products of CHD genes amplified with the primers (2376F/2524R; bottom) in male and female chicks were 10/10 and 7/10, while those amplified with the primers (2550F/2718R; bottom) were 6/10 and 5/10, respectively. It is clear that the new pair of PCR primers was more efficient than the old or original one.

Experiment 2: Comparing the effects of two new pairs of primers: The results of PCR amplification based on the modified pair of primers and that of Chen (2009) are shown in Fig. 2. The graded annealing temperatures (Tm) were set at 50±6°C in Fig. 2a, b and 55±6°C in Fig. 2c. All primer pairs were amplified at graded temperatures around their optimized annealing temperatures as predicted by primer designer software. In Fig. 2a, b the annealing temperatures are the same, i.e., 44.1, 44.8, 45.9, 47.2, 48.8, 50.4, 52.1, 53.6, 54.9 and 55.9°C (from left to right), respectively. In Fig. 2c, the annealing temperatures are 55±6°C, i.e., 49.2, 49.8, 50.9, 52.3, 53.8, 55.5, 57.1, 58.7, 60.0 and 60.9°C (from left to right), respectively.

Two bands of approximately 500 and 150 bp were parallel amplified fragments in the male and female samples. An additional band of approximately 300 bp was also visible in the female samples. Thus, gel electrophoresis reveals two bands in males and three bands in females using Chen's primers (Fig. 2). As showed in Fig. 2a and b, the proportions of CHD gene's PCR products amplified with these two primers in male and female chicks were all 10/10 and 10/10 (Tm = 50±6°C). However, the same numbers of bands are amplified in male and female chicks with Chen's primers, which make it difficult to identify male and female chicks. At higher annealing temperatures, however, this is expected to change. Further PCR amplifications with higher graded annealing temperatures (Tm = 55±8°C) did lessen the non-specificity (Fig. 2c). Thus, we suggest the use of a higher annealing temperature in PCR amplifications when using Chen's primers. The comparison showed that the new pair of primers was clearer than that of Chen's, especially in male chicks.
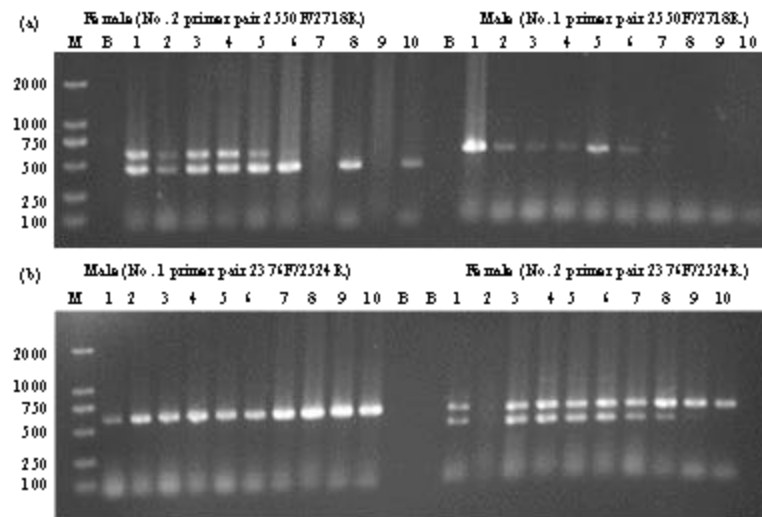
Fig. 1: PCR products of CHD alleles in single male and female chicks amplified with the old primers 2550F/2718R (a) and the new primers 2376F/2524R (b) on a 2% agarose gel with a 2000-bp ladder (DL2000, noted as M). Marker B shows blank lanes working as a negative control. The annealing temperatures (Tm) were designed as ten graded temperatures proximal to the corresponding primer pairs' optimized annealing temperatures predicted by primer designer software, 6.5 µL of PCR product was added per lane. The female had 2 bands of approximately 650 and 450 bp, while the male showed 1 band of approximate 650 bp. This programme was carried out using samples from many male and female chicks and demonstrated that the new primer pair was more efficient than the old one, especially in male individuals

In silico PCR amplification, sequence alignment and primer analysis: *In silico* PCR products were copied and analyzed for use in CHD gene primer designing. Lengths of PCR products amplified with primers (2376F/2524R) were 988 bp (for the CHD-Z allele) and 447 bp (for the CHD-W allele) across the chicken Z and W chromosomes. Since the PCR amplification was designed to make clear the intronic length polymorphism in CHD alleles, we tried to obtain the full allele sequences. The predicted fragment for the CHD-W allele is in accord with actual PCR amplifications in many birds, while that for the CHD-Z allele is much longer than previously reported PCR products. However, the completed genomic sequences of the chicken CHD-Z allele and Z chromosome sequencing remain unknown. The in silico PCR production of CHD-Z allele (Fig. 3) was full of many vague nucleic acids N.

Sequence alignment was made using the sequences of *in silico* PCR products of the chicken CHD-W allele and CHD-Z and CHD-W alleles of three avian species. The names for each sequence were abbreviated following the binomial nomenclature for each species. The complete sequence of the chicken CHD-Z allele is presently unavailable and its *in silico* PCR product was not used. The differences between the CHD-W and CHD-Z allele fragments were analyzed as shown in Fig. 4a. The borders

between exons and introns are indicated and the matching sites in CHD alleles for primers (2376F/2524R) are underlined. In all avian species, introns in CHD-Z alleles are much longer than those in CHD-W alleles, probably due to differences in rates of intronic evolution between the CHD-Z and CHD-W alleles (Fig. 4a).

For these avian CHD alleles, the matching sites for primers (2376F/2524R) were substantially conserved (Fig. 4a). Figure 4b, on the contrary, shows the matching sites between primer 2550F and the CHD-Z allele as determined by BLAST search. Evidently, two crucial non-matching sites in primer 2550F mutated from the corresponding sites in CHD-Z allele. These two point mutations were C→T in the 5' end and 6→A in the 3' end between the optimized or perfect primer and primer 2550F. Sites in the 3' end of PCR primers are lethal. The G to A mutation was particularly crucial, as it lies at the 3' end of the PCR primer 2550F, in which problems might occur due to high annealing temperatures resulting in unstable combinations between the primer and target sequence.

Applications in chicken sex diagnosis: After establishing our PCR-based sex diagnosis procedure for Chinese gamecocks, we made two practical applications. One was for the sex diagnosis of gamecock chicks that were previously vent-sexed as male (case 1). The accuracy of
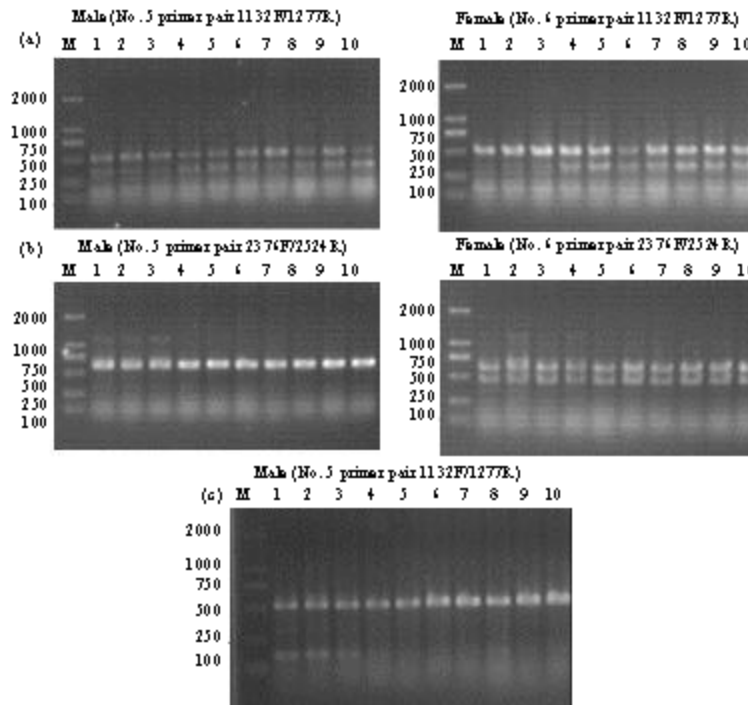
Fig. 2: (a) PCR products of CHD alleles in single male and female chicks amplified with the primers 2376F/2524R and (b) the primers 1132F/1277R (left) on 2% agarose gel with a 2000-bp ladder (DL2000). (c) PCR products of CHD alleles in male and female amplified by the primers (1132F/1277R) on 2% agarose gel with a 2000-bp ladder (DL2000, noted as M), 6.5 μL of PCR product was added per lane. Male chicks should show 2 bands (about 500 and 150 bp) with no nonspecific bands, whereas female chicks should show 3 bands (about 500, 300 and 150 bp). The PCR product of CHD gene in male chicks showed 3 bands, including a nonspecific band. Further PCR amplifications eliminated the additional band, as shown in Fig. 2c. The annealing temperatures (Tm) were designed as ten graded temperatures around corresponding primer pairs' optimized annealing temperatures predicted by primer designer software

artificial vent sexing was then estimated in two chicken breeds (Luxi gamecock and White Rock). These gamecock chicks were bred and raised solely for cockfighting. In this experiment, White Rock chicken served as control. DNA was extracted from feather samples using methods 1 and 2. The results of sex diagnosis are shown in Fig. 1, 2 and Table 2. Table 2 shows the artificial sexing accuracies are 90.22 and 93.48% in Chinese gamecock and White Rock chicks respectively, assuming that the molecular sexing method was absolutely reliable. This also reconfirms that the gamecock chicks are difficult to sex.

We also tested the procedure in the sex diagnosis of Luxi gamecock and White Rock embryos (case 2). PCR products are partly shown in Fig. 5. The embryos used in these tests were donated from other experiment designs, such as microarray research on developmental problems and transgenic studies. In total, 80 fertile eggs were collected and diagnosed. DNA extraction was also done

Table 2: Gamecocks tested in sex diagnosis

Chicken breeds	Sex	Case 1	Total	Case 2	Total	Artificial accuracy in case 1 (true : total) (%)
Gamecock	Male	83	92	9	30	90.22
	Female	8	-	21		-
White rock	Male	86	92	15	30	93.48
	Female	6	-	15	-	-

according to the previously described methods. A sex diagnostic kit for gamecock and other chickens was then designed and prepared accordingly.

Sex identification in different species is one of the key procedures for animal breeding, genetic and evolutionary researches. However, male and female hatchling or fledgling birds are often morphologically indistinguishable. About 50% of the world's bird species do not show sexual dimorphism (Griffithsm *et al.*, 1998). The sexes of chicks among most native chicken populations also cannot be distinguished visually due to the morphological similaries between male and female hatchling birds. The accurate sexing of chicks of many

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>Chicken CHD-Z gene In-Silico PCR product in chromosomeZ

gctgcaatgt gtgttagtgt tgagatggac tgattttgta atacatattt   50184551
ctttaacagC ATTTGATACG TAGCAGCGGG AAACTAATCC TTCTTGACAA   50184601
GCTACTGATT CGTCTGCGAG AACGTGGCAA CAGAGTTCTG ATTTTCTCTC   50184651
AGATGGTGAG GATGCTGGAC ATCCTAGCAG AATATCTGAA GTATCGCCAG   50184701
TTTCCCTTCC AGgtaacaat nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50184751
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50184801
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50184851
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50184901
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50184951
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185001
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185051
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185101
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185151
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185201
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185251
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185301
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185351
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185401
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185451
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn   50185501
nnnnnnnnnn nnnnnnnnnn natctctta gAGACTTGAT GGATCAATAA   50185551
AAGGGGAATT GAGGAAGCAA GCACTGGATC ATTTCAATGC AGAAGGATCA   50185601
GAGgtatggt aattattttt tcctaacttg ctttttttcc cattaaacat   50185651
tgtataaaaa tgaagtttat ggcattgaat cttataca

>Chicken CHD-W gene In-Silico PCR product in chromosomeW

agtgtatgtt agtgttgagg taaacttatt ttgcaataca caatttctta   430295
tctttgtagC ATTTAATACG TAGCAGTGGG AAACTAATCC TCCTTGACAA   430345
GTTACTGATT CGTCTACGAG AACGTGGCAA CAGAGTACTG ATTTTCTCTC   430395
AGATGGTGAG GATGCTAGAC ATCCTAGCAG AATACTTGAA GTATCGTCAG   430445
TTTCCTTTTC AGgtaagaat tttgatggta gtagccaaga agccttgatc   430495
tttgccactt tatcttaagt aaaagtgtcc tttctgtaga aaagacttct   430545
aaaagtttaa ttttatgtat agaaaaagac tggcaattac tatggtgtga   430595
ggtgttgcat tattctcctc ctcctccttc ccccccattc ctccccttgc   430645
cctcagttgt tttggcaatt gagtattcag gttgctctga ttagaatata   430695
gtatgagttc ctttttaact gtaatatttg atctctttag AGACTTGATG   430745
GATCAATAAA AGGAGAACTG AGGAAACAAG CACTGGATCA TTTCAATGCA   430795
GAAGGATCAG AGGtatggca ttagtgggtg gtggtttttt aatctatatg   430845
gcaggaaaac tgaaaacaga aaaagtaagg gatggatact gttgaaa
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Fig. 3: Reports of *in silico* PCR amplification for chicken CHD alleles. Since practical PCR amplifications were designed to make clear the intronic length polymorphism in CHD alleles, the improved primers (2376F/2524R) were used to pre-amplify the expected introns through the CHD-Z and CHD-W alleles. Reports were automatically generated by the UCSC genome browser

endangered species, such as Chinese gamecocks and other rare breeds of chicken, by non-invasive methods is essential not just for the purpose of captive breeding but in molecular ecological studies as well (Itoh *et al.*, 2001). Meanwhile, the quick and accurate sexing of embryos at an early stage of development is also crucial in sex reversal, sex differentiation researches and the generation of germline chimeras for transgenic chicken studies. However, reliable sexing of embryos by morphological differences before day 8 of incubation is impossible (Clinton *et al.*, 2001). This has driven the development of novel diagnostic approaches, including molecular methods of sex identification or diagnosis of embryos, such as southern blot hybridization of W chromosome repetitive sequences (Uryu *et al.*, 1989), fluorescent *in situ* hybridization (Klein and Ellendorff, 2000) and more recently, PCR-based protocols using W chromosome-specific DNA sequences (Petite and Kegelmeyer, 1995; Griffiths *et al.*, 1996; Griffithsm *et al.*, 1998; Griffiths and Orr, 1999; Kahn *et al.*, 1998; Fridolfsson and Ellegren, 1999; Clinton *et al.*, 2001). Hence, more accurate and definitive sexing techniques are desirable.

The CHD gene was the first protein-coding gene found in the avian sexual system. The female bird is heterogametic (ZW), meaning that the W chromosome must be the source of sex-linked markers. The first W chromosome gene discovered was CHD-W. This gene is highly conserved and it has been shown that a single pair of PCR primers (P2 and P8, Griffithsm *et al.*, 1998) can be used to sex most avian species. These primers anneal to

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                                                                                                            exon│intron
Length (bp)  5'—....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ..|..|..|
                    5         15         25         35         45         55         65         75         85         95        105      │115
Gg_chrW      GTTACTGATT CGTCTACGAG AACGTGGCAA CAGAGTACTG ATTTTCTCTC AGATGGTGAG GATGCTAGAC ATCCTAGCAG AATACTTGAA GTATCGTCAG TTTCCTTTTC AGGTAAGAAT
C1CHD1W      GTTACTGATT CGTCTACGAG AACGTGGCAA CAGAGTACTG ATTTTCTCTC AGATGGTTAG GATGCTAGAC ATATTAGCAG AGTATTTGAA GTATCGTCAA TTTCCCTTTC AGGTGAGAAT
PmCHD1W      ----CTGATT CGTCTACGAG AACGTGGCAA CAGAGTACTG ATTTTCTCTC AGATGGTGAG GATGCTAGAC ATCCTAGCAG AGTATTTGAA GTATCGTCAG TTTCCCTTTC AGGTAAGAAT
PcCHD1W      GTTACTGATT CGTCTACGAG AACGTGGCAA CAGAGTACTG ATTTTCTCTC AGATGGTGAG GATGCTAGAC ATCCTAGCAG AGTATTTGAA ATATCGTCAG TTTCCCTTTC AGGTAAGAAT
PcCHD1Z      GTTACTGATT CGTCTACGAG AACGTGGCAA CAGAGTTTTG ATTTTCTCAC AGATGGTGAG GATGCTGGAC ATCCTAGCAG AATATCTCAA GTACCGTCAG TTTCCCTTTC AGGTAAGAAT
PmCHD1Z      GTTACTGATT CGTCTACGAG AACGTGGCAA CAGAGTTCTG ATTTTCTCAC AGATGGTGAG GATGCTGGAC ATCCTAGCAG AATATCTGAA GTATCGTCAG TTTCCCTTTC AGGTAAGAAT
C1CHD1Z      GTTACTGATT CGTCTACGAG AACGTGGCAA CAGAGTACTC ATTTTCTCAC AGATGGTGAG GATGCTGGAC ATCCTAGCAG AATATCTGAA GTATCGTCAG TTTCCCTTTC AGGTAAGAGT
Consensus    ****** ********** ********** ****** * ******** * ****** ** ***** *** ** ***** * ** ** ***** ***** **** **** *** *

                    125        135        145        155        165        175        185        195        205        215        225        235
Gg_chrW      TTTGATGGTA GTAGCCAAGA AGCCTTGATC TT------TG CCA------- ---CTTTATC TTAAGTAAAA GTGTCCTTTC TGTAGAA--- ---AAGACTT CTAAAAGTTT AATTTTTATGT
C1CHD1W      TTTTCTGGTA GTAGCCAAGA AGCCTTGATC TT------TA CCA------- ----CTTTT TGTAGA---- ------TTT ATGAAAGTTT AATTTTACAT
PmCHD1W      TTTGCTGGTA GTAGCCAAGA AGCCTTGATC TT------TA CCG------- ---CTTTATC TCAAG-AAAA GTGTCCTTTT TGTAGAA--- ---A-GATTT ATGAAAATTT AATTTTTATGT
PcCHD1W      TTTGCTGGTA GTAGCCAAGA AGCCTTGATC TT------TA CCA------- ---CTTTATC TTAAG-AAGT GTGTCCTTTT TGTAGAA--- ---A-GATTT ATGAAAGTTT AATTTCACGT
PcCHD1Z      CTTGGAGGTA GTAGCAAAGA AGTTTTGATC CT--GAATGTA AGAAAAATCT TTTCTTTACT CTGAGAGTGA CAGAGCACTG CAAAAAGCTG TCCAGAGGTT ATGGAATCTC CATCCTCTGT
PmCHD1Z      CTTGGTGGTA GTAGCCAAGA AGTTTTGATC TTTGGAATA AGAAAAATCT TTTCTTTACT CTGAGGGTGA CAGAGCACTG GAACAAGTTG TTCAGAGGTT ATTGCATCTC CATTCTCTGT
C1CHD1Z      TGTGGTGGTA GTAGCCAAGA AGCTTTGACC TT-GAATATA AGAAAAATCT TTTCTTTACT CTGAGGCTGA CGGAGCACTG -AACAAGTTG CCCAGAGGTC ATGGAATCTC CATCCTCTTT
Consensus    *  **** ***** **** ** ** ** * *    *  ***** *   *   *   *  * *  **   *

                    245        255        265        275        285        295        305        315        325        335        345        355
Gg_chrW      ATAGAAAAAG ACTGGCAATT ACT--ATGGT GTGAGGTGTT GCATTATTCT CCTCCT---- ---------- -----CCTCC TTCCCCCCCA TT---CCTCC CCTTGCCCTC AGTTGTTTTG
C1CHD1W      ACAGGAAAAG ACTGGCAATT ACTATATGCT GAATAGTATT TTGAAGTTAA ACTGATGAAT TAGAAAGATG AAGT-GTTAC ATTACTTTTA TT---CCACC CCACCCCCTC AGTTGTTTTG
PmCHD1W      ACAGGAAAAG ACTGGCAATT ACTATATGCT GAATAGTATT TTGAAATGAA ACTGATGAAT TAGAAAGATG AAGT-GTTAC ATTACTCTTA TTTCCCCCCC CCCCCCCCCC CATTGTTTTG
PcCHD1W      ATAGGAAGAG ACTGGCAATT ACTAAATGCT AAATAGTATT TTGAAATGAA ACTGATGAAT TAGAAAGATG AAGT-GTTAC ATTCCTCTTA TT-------- -CCACCCCCC AATTGTTTTG
PcCHD1Z      GACATTCAAA AGCCACCTGG ACAAGACCTT GGGTAACCTG CTTTATCTGT CCCTGCC--T GAGTAGGGGA GTTAGACAAG GTGATCTCCA GAG--GTCCC TTCCAACCTC AACTGTGTTG
PmCHD1Z      GACATTCAAA AGCCACCTGG ACATGACCTT GGACAACCTG CTTTAGCCGC CCCTGCC--T GAGCAGGGGA GTTAGTCAAG ATGACCTCCA GAG--GTCCC TTCCAACTTC AACTGTTTTC
C1CHD1Z      GACATTCAAA AGCCACCTGG ACATAACCGT GTGAAACCTG CTTTAACTGT CCCCACC--T GAGTAGGGGA CTTAGACAAG ATGACCTCCA GCA--GTCCT TTCCAAATTC GACTGTTTTG
Consensus    * *   *    *  * ** *   *       *                *      *  *     *    *   *  *  ** *** **

                                                                                                                    intron│exon
                    365        375        385        395        405        415        425        435        445      │455        465        475
Gg_chrW      GCAATTGAGT ATTCAGGTTG CTCTGATTAG AATATAGTAT GAGTTCCTTT TTAACTGTAA TATTTGATCT ---------- ---------- ---CTTTAGA GACTTGATGG ATCAATAAAA GGAGAACTGA
C1CHD1W      GCAATTGAGA ATTAAAGTTG CTCTGATTAG AATATAGAAG GAATTCCTTT TTAACTGTAT TATTCAATCT ---------- ---------- ---CTTTAGA GACTTGATGG ATCAATAAAA GGGGAATTGA
PmCHD1W      GCAATTGAGA ATTCAAGTTG CTCCAATTAG AATATAGTAG GAGTTCCTTT TTAACTGTAT TATTCAATTT ---------- ---------- ---CTTTAGA GACTTGATGG ATCAATAAAA GGGGAATTGA
PcCHD1W      GCAATTGAGA ATTCAAGTTG CTCTGATTAG AATATAGTAG GAGTTCCTTT TTAACTGTAT TGTTCAATCT ---------- ---------- ---CTTTAGA GACTTGATGG ATCAATAAAA GGGGAATTGA
PcCHD1Z      TCATCATGTG ATCTTTACCA CTTTGCTTAA GAAAAGTCTT AAGAA---GT GTGTTTTTTC TAGAAAGGCT GGCAATCGCT ATATGCTAAA TAGTATTTTG AAATTTAACA AATGAATTAA
PmCHD1Z      TGATT----- -TCTTTACCA CTTTGCTTAA GAAAAGGTAT AAGAAAAAGC GTTCTTTTTC TAGAAAGAGT GGCAATCGCT ATATCCTATG TAGTATTTTG AAATTAAACT GGTGAATTAA
C1CHD1Z      CAATTATATG ATCTTTACCA CTTTGCTTAA GAAAAGATAA AAGAA--AAT GTGGTTTT-- ---------- ---------- --GTACTAAG TAGTATTTTG AAAGGAAATA GATTATTTTA
Consensus    *          *          **  ***  * *        *          * *  *                            **     * *  * * * **       ** ***  * *

                    485        495        505        515        525        535        545        555        565        575        585        595
Gg_chrW      GGAAA----- CAAGCACTGG ATCATTTCAA T--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C1CHD1W      GGAAA----- CAAGCACTGG ATCATTTCAA T--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
PmCHD1W      GGAAA----- CAAGCACTGG ATCATTTCAA T--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
PcCHD1W      GGAAA----- CAAGCACTGG ATCATTTCAA T--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
PcCHD1Z      AAAAT----- GAAGTGTTGC ATTACTTTTT TTCCCTTCAC ATAACAGTTT TGGCAGTTGA GAATTCAAGT AGCTCTGA-- CTTTGAATAT AGTATA---- ---------- ----AGAATT
PmCHD1Z      AAAATTACAT GAAGTGATGC ATTACTTTTT TTTCCTTCAC ATAACAGTTT TAGCAGGTGA GAATTCAGGC TTCTCTGA-- TTTTGAATGT AGTATA---- ---------- ----AGCATT
C1CHD1Z      AAAATTATGT GCAGTGTTGC GTTACTTTTT TCACCTTCAC ATAACAGTTT TAGCAGTTGA GAATTCAAGG TGCTCTGAGA TTTTGTATAG AGAATATTAG AATATTTTGA ATATAAAATT
Consensus    **         **  **   *  *  * **   *

                    605        615        625        635        645        655        665        675        685    —3'
Gg_chrW      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
C1CHD1W      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
PmCHD1W      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
PcCHD1W      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------
PcCHD1Z      ACTTTTTAAC TGTAGTATTC AATCTTTTTA GAGACTTGAT GGATCAATAA AAGGGGAATT GAGGAAACAA GCACTGGATC ATTTCAAT
PmCHD1Z      ACTTTTTAAC TGTAGTATTC AATCTCTTTA GAGACTTGAT GGATCAATAA AAGGGGAATT GAGGAAACAA GCACTGGATC ATTTCAAT
C1CHD1Z      GCTTTTTAAC TATAGTATTC AATCTCTTTA GAGACTTGAT GGATCAATAA AAGGGGAACT AAGGAAACAA GCACTGGATC ATTTCAAT
```

B   **Binding sites of primer 2550F to CHD-Z gene**

CHD-Z gene

↑

3'- CGATGACTAAGCAGACGCTCTTGCACCGTTG-5'

| |||||||||||||| |||||

5'-GTTACTGATTCGTCTACGAGA -3'

↓

Primer 2550F

Fig. 4: Sequence Alignment and the differences between CHD-W and CHD-Z allele fragments. (a) Sequence alignments of the partial exons and introns of CHD-Z allele and partial exons of CHD-W allele. The names for each sequence are abbreviated according to the binomial nomenclature. Two arrowheads and partial boxes indicate the boundaries of the intron sequence. The short underlined sequences at the two ends are primers proposed for use in PCR. The sequence of the chicken CHD-W was determined from the product of in silico PCR. The complete sequence of chicken CHD-Z is presently unavailable. Its *in silico* PCR product included too many gaps or unknown nucleic acid bases and was out of the alignment. (b) Identical viewing of primer 2550F matched to CHD-Z by BLAST (NCBI). Two non-matching sites (T and A) were found

conserved exon regions and amplify across an intron that varies in size between CHD-W and CHD-Z (Fig. 4).

Because there are some nucleotide differences between CHD-W and CHD-Z fragments, gel electrophoresis
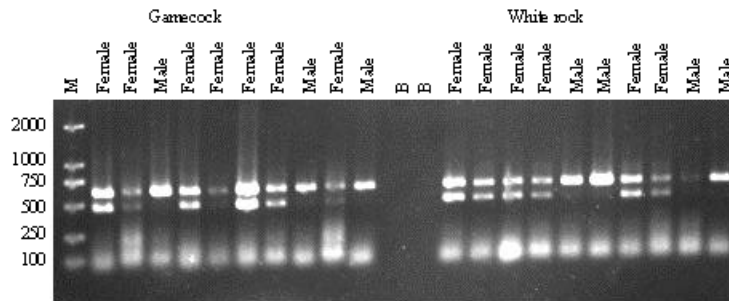
Fig. 5: PCR products of CHD alleles in diagnosing chicken embryos. PCR products of CHD alleles in male and female amplified by primers (2376F/2524R) were showed on 2% agarose gel with a 2000 bp ladder (DL2000), 7.5 µL of PCR product was added per lane. Marker M notes the ladder DL2000 while marker B denotes the blank lanes serving as negative controls

reveals one band in males and two bands in females in most of the bird species (Fridolfsson and Ellegren, 1999). This molecular method is simple and definitive for almost all birds and is therefore widely used. However, if PCR products are not sufficiently amplified or when length differences between CHD-Z and CHD-W alleles cannot be visualized using agarose, these techniques do not work.

Fridolfsson's method is attractive but not universally applicable in sexing chicken. In this study, we showed that Fridolfsson's method is unstable for the sexing of chicken due to a mismatch between one of the primers and the CHD-Z gene. We improved and applied this molecular sexing technique using PCR in adult chicken and chicks. A PCR-based diagnostic kit with an internal control was prepared for sex identification of gamecock chicks. We also compared and tested our primer pair with another pair of primers (1132F/1277R) developed recently by Chen (2009). The Chen's primer pair was designed based on the CHD-W allele, similar to the primer pairs P2 and P8 described by Griffithsm *et al.* (1998). It amplifies both CHD-W and CHD-Z allele fragments, while P2 and P8 only amplify part of the CHD-W fragment. We demonstrated that Chen's primers are valid but non-specific for female chicken and suggested higher annealing temperatures should be during PCR.

CONCLUSION

In the study, we demonstrate that the improved method of sex identification is more stable and efficient than the old one for sexing chicken. We made successful application of this procedure for sexing chicken in two diagnostic cases. The improved PCR method, combined with the non-invasive nature of the sampling approach and proper DNA extracting methods, provides a simple, rapid, inexpensive and non-invasive procedure for the sexing of gamecock chicks and the PCR reaction system is more stable than previously proposed approaches. We suggest that this PCR-based technique can be incorporated into the sex diagnosis or identification of chicks and embryos. This procedure may be particularly useful for species in which sexes should be discriminated in early chicks or for critical samples in which blood or tissue is not available. The ease of use, moderate costs, non-invasive nature, accuracy and repreproducibility of this approach makes the procedure and thus its kit, more attractive than other sex diagnosis techniques for use in chicken research.

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