



Chicken Sex Determination using SWIM Gene Revealed its Location in Both Autosomes and Sex Chromosome

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ABSTRACT

Background: In chickens, female has heterogametic sex chromosomes (ZW) whereas it is homogametic (ZZ) in males. In chicken, the sex can be determined by genes specific to the W chromosome.

Methods: Ten fertile eggs at 18.5th day of incubation were selected for liver tissue sampling and further DNA isolation. The SWIM gene located on chromosome W was amplified using gene specific primers.

Result: The PCR results revealed a 212 bp band that is specific to female birds and additionally a non-specific band appeared both in male and female birds at 900 bp. DNA sequencing, alignment and BLAST analysis of the bands specific to females and non-specific bands of males and females has revealed the sequence similarity of the non-specific bands with autosomes on chromosome 9 and W chromosome.

Key words: Chicken, Liver, PCR, Sex determination, SWIM.

INTRODUCTION

In contrast to mammals, birds have heterogametic females with two distinct sex chromosomes (ZW) and homogametic males with one sex chromosome (ZZ). The W chromosome is transferred through the ovum of female birds to determine the sex of the progeny (Stevens, 1997; Bachtrog *et al.*, 2011). Males have two Z sex chromosomes in this arrangement while females have Z and W sex chromosomes. The W chromosome only contains a small number of genes, but the Z chromosome is bigger and carries many genes (Graves, 2014). During the embryonic stage, the chick's sex can be determined using gene sequences specific to the W chromosome. Gender in chickens and other bird species is determined manually via cloacal or vent sexing, as well as feather sexing based on plumage colour. Although manual gender determination approaches are less expensive, they require highly skilled workers and are more susceptible to errors (Seemann, 2003). However, sexing of the chick at embryonic stage can also be performed using polymerase chain reaction (PCR) with genes located on both Z and W chromosomes. The PCR of chromo helicase DNA binding 1 (CHD-1) which is present in both Z (CHD-1Z) and W chromosome (CHD1-W) are used for sexing in non-ratite birds which had produced two bands in females and one band in males on agarose gel electrophoresis (Fridolfsson and Ellegren, 1999). Similarly, PCR based assay on XhoI repeats on the W chromosome was detected using gene specific primers where a band of size 415 bp appeared in females and no band in males (Clinton *et al.*, 2001). While the amplification of ECoRI repeats on W chromosome will help in sexing of the carinatae species (Itoh *et al.*, 2001). The zinc finger SWIM domain containing protein 6 like (SWIM), is located on W-chromosome; therefore serves as specific marker for

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female. However, comprehensive investigation of the W chromosome has yet to be undertaken in birds due to the presence of smaller and highly hetero-chromatic genomic regions in the W chromosome. The W chromosome has recently been shown to have several roles in crucial sex-specific evolutionary processes and sex determination (Moghadam *et al.*, 2012). The PCR amplification of SWIM gene on W chromosome is the simple, robust method for sex determination in chicken. In an attempt to determine the sex of the chicken embryos using the primers reported by He *et al.* (2019), there appeared non-specific bands in male and female samples and specific bands in the females. Previous research has demonstrated the significance or specificity of SWIM primers in detecting the sex of birds. The present investigation was undertaken to study the presence of non-specific bands in male and female chicken and to establish an even more specific primer for sex determination.

MATERIALS AND METHODS

Experimental birds

The present study was carried out in Kadaknath chicken that is being bred and maintained at ICAR-Directorate of Poultry Research in Rajendranagar, Hyderabad. To conduct the experiment, female Kadaknath chicken were artificially inseminated with Kadaknath semen. Subsequently, the laid eggs were carefully collected and placed in an incubator, maintaining a constant temperature of 99.5°F and a relative humidity ranging between 55-60%. On the 18th day of incubation, the eggs were subjected to candling. The infertile eggs were not processed further and only fertile eggs were selected for the experiment.

Collection of sample and Isolation of Genomic DNA

The fertile eggs of Kadaknath birds were chosen at random on the 18.5 day of incubation. A total of 10 liver tissue samples were then collected under sterile conditions, rinsed with saline to remove blood and placed in 1.5 ml centrifuge tubes containing 1X PBS and stored at -80°C for subsequent processing. Using a mortar and pestle, the liver tissue from the individual samples were homogenised. One millilitre of 1X PBS was then added and the mixture was centrifuged at 2000 rpm for 10 minutes and supernatant was decanted. In order to further break down the cells in the pellet, 1 ml of cell lysis solution was added, incubated for 20 minutes and centrifuged at 2000 RPM for 10 minutes. Later, genomic DNA was recovered with a few minor alterations utilising the phenol-chloroform extraction method (Sambrook *et al.*, 1989). The extracted DNA was then stored at 4°C till use. Further, purity of DNA was evaluated by measuring the optical density (OD) at wave lengths 260 nm and 280 nm in a UV-spectrophotometer.

Genomic DNA quality was checked to ensure intact DNA without any shearing using horizontal agarose gel electrophoresis.

Polymerase chain reaction (PCR) and sequencing

The composition of PCR reaction mix for amplification of SWIM primers were 10X Taqbuffer, DNTPs (0.4 Mm), Primer-Forward (4 Picomoles), Primer-Reverse (4 Picomoles), Taq Polymerase (1 unit/μl), Autoclaved Mille Q water with a final volume as 11.5 ul and 1 ul DNA as template. The PCR reaction conditions for SWIM primers includes, initial denaturation at 94°C for 5 min, cyclic denaturation at 94°C, Primer annealing at 55°C, extension at 72°C repeating over 36 cycles and Final extension at 72°C and hold at 4°C. The amplification of the SWIM gene with specific primers (Table 1) was carried out in 0.2 ml capacity PCR tubes, using a thermal cycler (Himedia). The amplified product was checked in gel electrophoresis using UV transilluminator. The specific and non-specific PCR products were purified using GeneJET gel extraction kit (ThermoFisher) following user manual. The purified products were custom sequenced for Sanger sequencing. The obtained DNA sequences were subjected to multiple sequence alignment using CLUSTALW and BLAST (Samal *et al.*, 2021) analysis using NCBI BLAST tool.

RESULTS AND DISCUSSION

In the breeding industry, the most reliable way of establishing the gender of bird species is PCR analysis. The number of studies on sex-linked markers and PCR-based approaches for detecting gender in bird species is continuously increasing. The bulk of PCR-based methods for detecting the birds sex concentrate on sex chromosome

Table 1: Details of primer used for embryonic chicken sexing.

Targeted gene	Primer sequence	Product length	Authors
SWIM	F: 5'GAGATCACGAACCTCAACCAG 3' R: 5'CCAGACCTAATACGGTTTTCAG 3'	212 bp	(He <i>et al.</i> , 2019)

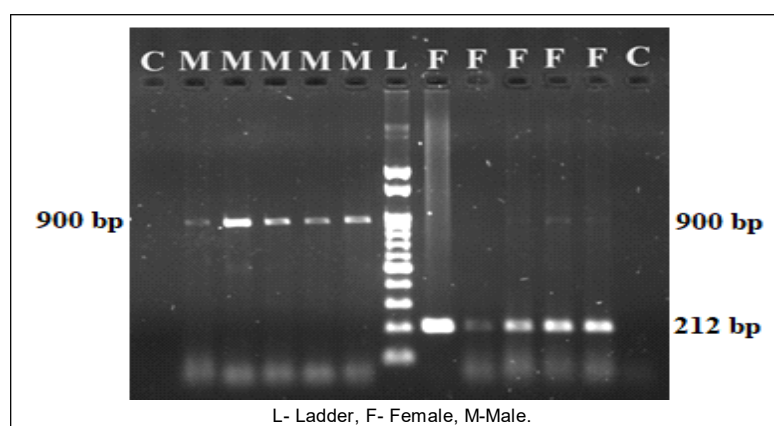


Fig 1: Agarose gel electrophoresis showing PCR amplified product of SWIM (212 bp) and Non-specific bands (900 bp) in male and female samples.

C3MR	TGAGATCACGAACTCAACCAGGGCATGGTCACTGCAGCCAGGCTGCCTCCAACCAACGC	60
FUC5R	TGAGATCACGAACTCAACCAGGGCATGGTCACTGCAGCCAGGCTGCCTCCAACCAACAC	60
FUC2R	TGAGATCACGAACTCAACCAGGGCATGGTCACTGCAGCCAGGCTGCCTCCAACCAACAC	60
FLC5R	TGAGATCACGAACTCAACCAGGGCATGGTCACTGCAGCCAGGCTGCCTCCAACCAACAC	60
FLC1R	TGAGATCACGAACTCAACCAGGGCATGGTCACTGCAGCCAGGCTGCCTCCAACCAACAC	60
FLC2R	TGAGATCACGAACTCAACCAGGGCATGGTCACTGCAGCCAGGCTGCCTCCAACCAACAC	60
FLC4R	TGAGATCACGAACTCAACCAGGGCATGGTCACTGCAGCCAGGCTGCCTCCAACCAACAC	60
FUC1R	TGAGATCACGAACTCAACCAGGGCATGGTCACTGCAGCCAGGCTGCCTCCAACCAACAC	60

C3MR	CTTTAATGATCTCTCCGATTGGTGAGCAGCAGGTCCAGCACCTTTTTTCTCTGGT	120
FUC5R	CTTTAATGATCTCTCCGATTGGTGAGCAGCAGGTCCAGCACCTTTTCTCTGGT	119
FUC2R	CTTTAATGATCTCTCCGATTGGTGAGCAGCAGGTCCAGCACCTTTTCTCTGGT	118
FLC5R	CTTTAATGATCTCTCCGATTGGTGAGCAGCAGGTCCAGCACCTTTTCTCTGGT	118
FLC1R	CTTTAATGATCTCTCCGATTGGTGAGCAGCAGGTCCAGCACCTTTTCTCTGGT	118
FLC2R	CTTTAATGATCTCTCCGATTGGTGAGCAGCAGGTCCAGCACCTTTTCTCTGGT	118
FLC4R	CTTTAATGATCTCTCCGATTGGTGAGCAGCAGGTCCAGCACCTTTTCTCTGGT	118
FUC1R	CTTTAATGATCTCTCCGATTGGTGAGCAGCAGGTCCAGCACCTTTTCTCTGGT	118

C3MR	CGGTCTGTCCAATAACAGAAACCGAGAAAGTTA	152
FUC5R	CGGTCTGTCCAATAACAGAAACCGAGAAAGTTAT	151
FUC2R	CGGTCTGTCCAATAACAGAAACCGAGAAAGTTA	150
FLC5R	CGGTCTGTCCAATAACAGAAACCGAGAAAGTTA	150
FLC1R	CGGTCTGTCCAATAACAGAAACCGAGAAAGTTA	150
FLC2R	CGGTCTGTCCAATAACAGAAACCGAGAAAGTTA	150
FLC4R	CGGTCTGTCCAATAACAGAAACCGAGAAAGTTA	150
FUC1R	CGGTCTGTCCAATAACAGAAACCGAGAAAGTTA	150

C3M: Sample 3 male; FUC: Female upper band sample number C1, C2, C5; FLC: Female lower band sample number C1, C2, C4, C5; R: Reverse sequence.

Fig 2: CLUSTALW alignment of SWIM gene sequences in male and female samples in chicken.

Descriptions	Graphic Summary	Alignments	Taxonomy					
Sequences producing significant alignments								
Download Select columns Show 100								
select all 100 sequences selected								
GenBank Graphics Distance tree of results MSA Viewer								
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc. Len	Accession
Galus gallus breed Huxu chromosome W	Galus gallus	46.1	2217	100%	1e-04	100.00%	14151827	CP100593.1
Galus gallus BAC clone CH261-54P4 from chromosome w complete sequence	Galus gallus	46.1	167	100%	1e-04	100.00%	241961	AC239503.4
PREDICTED: Galus gallus BTR domain containing 7 (BTB07) transcript variant X2 mRNA	Galus gallus	30.2	30.2	65%	7.9	100.00%	7764	XM_040673026.2
PREDICTED: Galus gallus BTR domain containing 7 (BTB07) transcript variant X1 mRNA	Galus gallus	30.2	30.2	65%	7.9	100.00%	7898	XM_046942437.1
PREDICTED: Galus gallus uncharacterized LOC121118165 (LOC121110165) transcript variant X1 ncRNA	Galus gallus	30.2	30.2	82%	7.9	94.74%	11981	XR_005847754.1
PREDICTED: Galus gallus uncharacterized LOC121118165 (LOC121110165) transcript variant X2 ncRNA	Galus gallus	30.2	30.2	82%	7.9	94.74%	12059	XR_005858151.1
PREDICTED: Galus gallus uncharacterized LOC121118165 (LOC121110165) transcript variant X4 ncRNA	Galus gallus	30.2	30.2	82%	7.9	94.74%	12140	XR_005858150.1
PREDICTED: Galus gallus uncharacterized LOC121118165 (LOC121110165) transcript variant X1 ncRNA	Galus gallus	30.2	30.2	82%	7.9	94.74%	11972	XR_005858149.1
PREDICTED: Galus gallus uncharacterized LOC121118165 (LOC121110165) transcript variant X3 ncRNA	Galus gallus	30.2	30.2	82%	7.9	94.74%	12053	XR_005858148.1
Galus gallus chromosome UNK clone CH261-17M19 complete sequence	Galus gallus	30.2	30.2	82%	7.9	94.74%	1183975	VAC145959.3
Galus gallus breed Huxu chromosome 9	Galus gallus	30.2	301	95%	7.9	94.74%	23996833	CP100583.1

Fig 3: Result of BLAST analysis of specific and non specific nucleotide sequences in male and female samples in chicken.

genes. PCR-based sex determination methods have steadily improved in terms of accuracy, speed and high-throughput applicability (Dhanasekaran *et al.*, 2016). The extracted DNA from different liver samples has shown purity level ranging from 1.8 to 1.9 in spectrophotometer. The PCR products were further resolved in 1% gel electrophoresis that revealed a 212 bp which is specific to female birds and a non specific band in both male and female birds at 900 bp (Fig 1). The CLUSTAL W (Fig 2) and BLAST analysis of the sequences belonging to the 900 bp PCR product revealed its homology in female and male embryos (Fig 3) and 212 bp is unique to females. The non-specific bands in females (FLC 1,2,4 and 5) has same base alignment with upper bands of both sexes. BLAST analysis confirmed the sequences of the upper extra band appearing in both females and males due to the sequence similarity with autosomes and W chromosome. However,

SWIM primers can be used to differentiate male and females sexes through PCR.

CONCLUSION

In this study, the primers that was used for amplification of SWIM gene for sex determination in Chicken, that was previously reported to amplify the SWIM gene on chromosome W was also found to amplify a region in autosomes on chromosome 9.

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Conflict of interest

The authors declare that there is no conflict of interest.

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