Indian Journal of Animal Sciences **94** (7): 620–625, July 2024/Article
https://doi.org/10.56093/ijans.v94i7.149461

Differential expression and genetic polymorphism of *DMRT1* and *FOXL2* genes in the gonads of *Kadaknath* chicken

SHIVANI BACHAMOLLA[1], JAYAKUMAR SIVALINGAM[2], VELPULA CHINNIPREETAM[1],
DAIDA KRISHNA[1], SHANMUGAM MURUGESAN[2], B RAJITH REDDY[2],
TARUN KUMAR BHATTACHARYA[3] and RAJKUMAR ULLENGALA[2]✉

ICAR - Directorate of Poultry Research, Hyderabad, Telangana 500 030 India

Received: 20 March 2024; Accepted: 20 May 2024

ABSTRACT

In chicken, the left ovary is functional and the right ovary becomes rudimentary in the later stage of the embryonic development. The expression and genetic polymorphism of the *DMRT1* and *FOXL2* genes are assessed in order to understand the distinct developing process of gonads in male and female embryonic gonads. In the current study, 19th day *Kadaknath* chicken gonadal tissue samples were collected from the embryos and RT-PCR analysis and DNA sequencing was done. According to the expression studies, the left ovaries relative expression of *FOXL2* was 2.14 and 12.65 factor greater than that of the right ovaries and testes, while the right testis relative expression of *DMRT1* was 3.78 and 3.83 fold higher than that of the left and right ovaries. DNA sequence analysis in the left ovary identified a nonsynonymous alteration (c.289C>G) involving histidine to aspartic acid at the 289th position in the *FOXL2* coding region. The 51st position of exon 6 of *DMRT1* has a G→A transition SNP that is not part of the coding sequence (CDS). Males showed higher levels of the *DMRT1* gene than females, while females expressed higher levels of *FOXL2*. It was found that the *DMRT1* and *FOXL2* genes are polymorphic. The genetic variability in the gonads and the differences in *DMRT1* and *FOXL2* gene expression allow learning more about the critical mechanisms underlying chicken gonadal development.

Keywords: Chicken, *DMRT1*, *FOXL2*, Genetic polymorphism, Ovary, Relative gene expression

In birds, sex is determined by Z and W sex chromosomes where the male is homogametic (ZZ) and the female is heterogametic (ZW). The process of sex determination in birds is complex and currently not clear. However, researchers have postulated different theories which might be due to a dominant ovary-determining gene on the W chromosome or it could be due to Z-chromosome dosage (Ellegren 2000). From the entire 21-day embryonic stage during day 6th, chicken initial bipotential gonads begin morphological differentiation into ovaries or testes. Condensing cells in the medulla indicate sertoli cell differentiation in male embryos. There is a reduction in the germinal epithelium, a concentration of germ cells within the seminiferous cords, and the formation of bilateral testes.

In female embryos, only the left gonad develops as an ovary, characterized by medulla fragmentation and hypertrophy of the germinal epithelium (Carlon and Stahl 1985). According to several studies, key genes which are upregulated in male are double sex and the Mab-3 related transcription factor 1 (*DMRT1*), SRY-Box transcription factor 9 (*SOX9*), anti mullerian hormone gene (*AMH*), Steroidogenic factor-1 gene *(SF1*) and female genes that are up-regulated include aromatase, Forkhead/winged-helix transcription factor gene 2 (*FOXL2*), female-specific expression transcription factor (*FET1*) and wingless-related MMTV integration site 4 (*WNT4*).

In the present study, the differential expression and genetic polymorphism of *DMRT1* and *FOXL2* genes were studied in *Kadaknath* chicken to explore the usefulness of genes in sex determination. *DMRT1*, a chicken Z-linked gene, has recently gained a lot of attention as a potential testis-determining factor belongs to a family which is characterized for holding a highly-conservative zinc-finger DNA-binding motif (DM domain) rich in cysteines. The name was derived from structurally and functionally related genes found in invertebrates – double sex in Drosophila and Mab-3 in *Caenorhabditis elegans*, which encode the DM domain-containing transcription factors and are involved in the sex determination and differentiation process (Hodgkin 2002). The cDMRT1 (chicken *DMRT1*) is solely expressed in the embryonic urogenital system and expression is higher in male (ZZ) gonads than in female (ZW) gonads

Present address: [1]College of Veterinary Science, PVNR Telangana Veterinary University, Hyderabad, Telangana. [2]ICAR–Directorate of Poultry Research, Hyderabad, Telangana. [3]NRC on Equines, Hisar, Haryana. ✉Corresponding author email: ullengalaurk@gmail.com

during the period of gonadal sex differentiation (Smith *et al*. 1999). *DMRT1* gene plays an important role in sex determination, differentiation and maintenance of organ functions in a diversity of species, including fish, mammals, reptiles, birds and amphibians (Picard *et al.* 2015).

FOXL2 is a putative winged helix/forkhead transcription factor that is implicated in the development of gonads, in particular, granulosa cell differentiation, follicle development and maintenance in many vertebrates, including chickens. In chicken, *FOXL2* is a single-exon gene consisting of 2.9 kb located on 9th chromosome. In the chicken embryo, *FOXL2* expression is seen in female but not in male gonads (Govoroun *et al*. 2004; Hudson *et al*. 2005). Mutations in *FOXL2* produce blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) type I in humans, which is an autosomal dominant condition linked to premature ovarian failure (Crisponi *et al*. 2001). In mouse, knockout of *FOXL2* also shows premature ovarian failure (Schmidt *et al*. 2004; Uda *et al*. 2004).

In female chicken, the left ovary is functional and the right ovary becomes rudimentary during the later embryonic stage of the development. Many researchers are working to unravel the underlying mechanism of differential gonadal development in female chicken. Hence, keeping this in view, an attempt was made to study the expression and polymorphism of *DMRT1* and *FOXL2* genes controlling gonadal development in chicken.

MATERIALS AND METHODS

Tissue collection: In the current study, 15 eggs were collected from 15 *Kadaknath* hens which were inseminated with the pooled semen obtained from five males. Further, on the 18th day of incubation, candling of eggs was done and 12 fertile eggs were kept in the incubator at 37.5°C and relative humidity of 55-60%. Eggs were withdrawn from the incubator on the 19th day of incubation and embryos were sacrificed. The male and female gonads were identified visually and right testes and both ovaries were collected aseptically. The collected tissues were immediately submerged in the RNA later solution and stored at –80ºC till further processing. The experiment was performed in accordance with the relevant guidelines and regulations as approved by the Institutional Animal Ethics Committee (IAEC) of ICAR-DPR, Hyderabad.

RNA isolation and cDNA synthesis: Total RNA was isolated from three testes, three left and right ovaries using the "TriZol method" (Chomczynski and Sacchi 2006) and the concentration and quality of RNA were estimated using a nanodrop spectrophotometer. The cDNA was synthesized from 1µg RNA by using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific).

Quantitative Real-Time PCR: The expression of *DMRT1* and *FOXL2* genes in testes and ovaries was estimated by RT-PCR (Himedia) using1ul cDNA, 5ul SYBR Green and 2 ul of primers (forward and reverse) and 2ul of nucleus free water. The relative quantification of *DMRT1* and *FOXL2* with reference to *β actin* was determined by $2^{-\Delta\Delta CT}$. The primers for amplifying chicken *β actin* gene was designed using Primer 3 software and the details of primers used for RT-PCR are given in Table 1.

Sequencing of DMRT1 and FOXL2 genes: The 118 bp and 188 bp fragment of *DMRT1* and *FOXL2* was amplified by thermocycler using10× PCR buffer 1.25 ul,100 µM of dNTP 0.5 ul, 20 ng forward and reverse primers 1ul, Taq DNA Polymerase (5U/µl) 0.15, nuclease free water 8.6 and cDNA (100ng/µl)1.0 and total volume as 12.5 ul. The amplified PCR product was run in 1% agarose gel along with a 100 bp DNA ladder (gene ruler) and visualized under a gel documentation system. The custom DNA sequencing of genes were carried out by an automated dye-terminator cycle sequencing method of ABI 3730 DNA sequencer. Further, DNA sequence analysis was done using BioEdit (Hall *et al.* 2011), MegAlign and DNASTAR (Burland 1999) software tools.

Data analysis: To differentiate the mRNA expression levels of *DMRT1* and *FOXL2* genes in testes and ovaries, an independent sample t-test (SPSS V.12) was performed.

RESULTS AND DISCUSSION

The relative expression of the *DMRT1* and *FOXL2* genes were studied in the right testes and both the ovaries of *Kadaknath* chicken embryos. The *DMRT1* gene expression in right testes (35.14±0.95) was higher compared to left ovaries (33.82±1.33) and right ovaries (33.82±0.56). Higher levels of *FOXL2* gene expression ($P \leq 0.05$) were noticed in the left ovary (38.79±1.09) compared to the right testes (35.85±1.13). It has been showed that *DMRT1* expression was sexually dimorphic on the 19th day of incubation and the expression was higher in testis by 3.83 compared to the left and 3.78 folds than right ovaries. However, the difference was not statistically significant. The statistical analysis is given in Table 2 and 3.

Similar findings were noticed in chicken embryos where the expression was high in male gonads than in females

Table 1. Primer sequences used for amplification of genes

Gene	Primer sequence (5′-3′)	Amplicon size	Annealing temperature	Reference
DMRT1	F: TTCGCGTTGAGTGCCTCGAC R: GAGGACACTGTGAGCCGTTC	118 bp	60°C	Luo *et al.* 2020
FOXL2	F: TGTCCGGGATCTACCAGTAC R: CTCGAACATGTCCTCGCA	188 bp	60°C	Loannidis *et al.* 2021
β actin	F: TCCCTGGAGAAGAGCTATGAA R: CAGGACTCCATACCCAAGAAA	113 bp	60°C	NM_205518.2

Table 2. Relative expression profile of *DMRT1* gene

Tissue	Mean C_T ± SE		ΔC_T ± SE	40-ΔC_T	Fold change ($2^{-\Delta\Delta CT}$)
	DMRT1	*β actin*			
Right ovaries (control)	25.75±0.24	19.59±0.53	6.15±0.56	33.82±0.56	1
Right testes	22.80±0.30	17.94±1.25	4.86±0.95	35.14±0.95	3.78
Left ovaries (control)	25.09±0.39	18.92±1.10	6.17±1.33	33.82±1.33	1
Right testes	22.80±0.30	17.94±1.25	4.86±0.95	35.14±0.95	3.83

Table 3. Relative expression profile of *FOXL2* gene

Tissue	Mean ΔC_T ± SE		ΔC_T ± SE	40-ΔC_T	Fold change ($2^{-\Delta\Delta CT}$)
	FOXL2	*β actin*			
Right ovaries (Control)	21.18±0.72	19.59±0.53	1.58±0.43	38.42±0.43	1
Left ovaries	20.12±0.30	18.91±1.10	1.20±1.09	38.79±1.09	2.14
Right testes (Control)	22.09±0.14	17.94±1.25	4.14±1.13	35.85*±1.13	1
Left ovaries	20.12±0.30	18.91±1.10	1.20±1.09	38.79*±1.09	12.65

Means with '*'as superscripts differ significantly (P≤0.05) (t-test).

during 8.5 and 10.5 days but were not significant (Yang *et al.* 2013). *DMRT1* expression was observed in both male and female chick gonads and expression was more than 2-fold (Addess *et al*. 1997; Yamamoto *et al.* 2003) and 1.6-fold higher (Zhang *et al.* 2010) in control males than in control females. The *DMRT1* was expressed more in males than in female gonadsin chickens (Oreal *et al.* 2002), mice (Smith *et al.* 2003). In turtle and alligator species the level of expression was higher in embryos incubated at the male promoting temperature, implying its role in early testis development (Kettlewell *et al.* 2000). However, *DMRT1* expression in the chicken embryonic ovaries become weaker from day 6.5 onwards and was undetectable on days 10 and 14 (Shan *et al.* 2000). The earlier discussion and present findings clearly suggest that *DMRT1* gene is responsible for the gonadal differentiation with sexual dimorphism.

In the current investigation, the *FOXL2* was sexually dimorphic on the 19th day of incubation, with the left ovaries having 12.65 folds more than the right testes and 2.14 folds higher expression than the right ovaries. The expression was significant (P≤0.05) in left ovaries compared to right testes but not with right ovaries. The chicken *FOXL2* expression was declined subsequently in right gonads and the expression in male gonads was 10 to 250 times lower than in the female gonads (Govoroun *et al.* 2004). In bipotential E4.5 chicken gonads, *FOXL2* expression was sexually dimorphic, with females having higher levels and in E6.0, the female-specific expression became noticeable shortly before sexual differentiation (Ayers *et al.* 2013). The fetal ovaries had significantly higher levels of *FOXL2* expression than fetal testes in mouse, chicken and red-eared slider turtles (Loffler *et al.* 2003), duck and chicken embryos (Koba *et al.* 2008; Raghuveer *et al.* 2011). Likewise, low amounts of *FOXL2* transcript were noticed in goat and mouse testis at fetal and adult stages, (Pailhoux *et al.* 2001; Cocquet *et al.* 2002; Pannetier *et al.* 2003). The relative lower levels of *FOXL2* gene in the right ovary in the present study and the supporting literature might give insights into the degeneration of the right ovary in the chicken.

In present study, sequencing of 118 bp (Fig.1) fragment of exon 6 of *DMRT1* revealed one SNP, a G→A transition (Fig. 2) at the 51st position in three testes samples. Since this SNP is positioned in the 3′ UTR, there is no further involvement in protein formation. The evolutionarily conserved 3′ UTR protein-binding motif has been found to be responsible for *DMRT1* transcript stability and translational efficiency, which is confined to the male gonad (Guo *et al.* 2005; Herpin *et al.* 2009). In the zebra fish 162 SNPs, 28 indels and two simple tandem repeats was discovered in *DMRT1* gene. They recognized two of these as functional candidates with non-synonymous and the central nucleotide of the cis-regulatory motif of the 3′ UTR (Bradley *et al*. (2011).

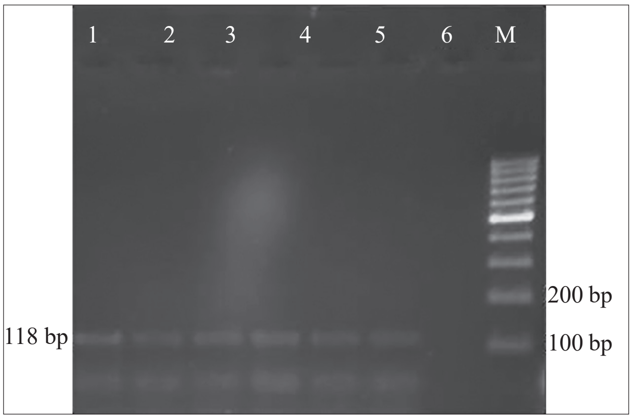
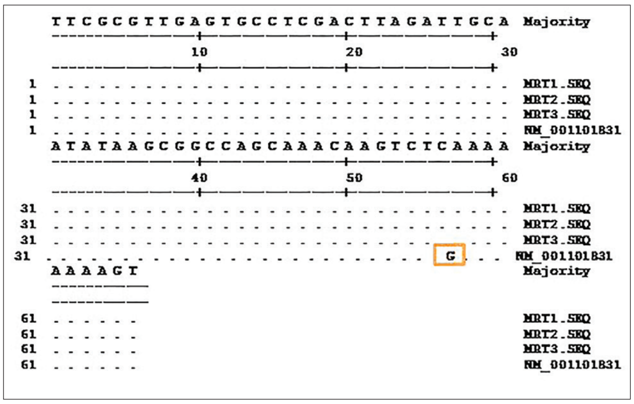


Fig. 1. Agarose gel electrophoresis showing PCR amplified product of *DMRT1* gene. Lane: 1-6: Right testes samples; Lane M: 100 bp Ladder.

However, one indel polymorphism in exon 1 and two silent SNP in exon 2 of *DMRT1* in boar, all of which were outside of the functional DM domain in the coding region and also identified 17 SNPs, 5 indels in the promoter region



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    T T C G C G T T G A G T G C C T C G A C T T A G A T T G C A  Majority
    ---------+---------+---------+
             10        20        30
    ---------+---------+---------+
 1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  MRT1.SEQ
 1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  MRT2.SEQ
 1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  MRT3.SEQ
 1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  NM_001101831
    A T A T A A G C G G C C A G C A A A C A A G T C T C A A A A  Majority
    ---------+---------+---------+
             40        50        60
    ---------+---------+---------+
31  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  MRT1.SEQ
31  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  MRT2.SEQ
31  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  MRT3.SEQ
31  . . . . . . . . . . . . . . . . . . . . . . . . . G . . . .  NM_001101831
    A A A A G T                                                  Majority
    ------
    ------
61  . . . . . .                                                  MRT1.SEQ
61  . . . . . .                                                  MRT2.SEQ
61  . . . . . .                                                  MRT3.SEQ
61  . . . . . .                                                  NM_001101831
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Fig. 2. Nucleotide sequence alignment of *DMRT1* gene by MegAlign in right testes of chicken.

(Bratus *et al.* 2011). SNP observed in the third intron of the *DMRT1* gene in Chinese tongue sole fish (*Cynoglossus semilaevis*) Z chromosome is strongly linked to sex reversal (Cui *et al.* 2018). In addition, the SNPs C → A at position 8,566,940 and C → T at location 8,566,916 in the exon 2 resulted in Alanine → Serine and Valine → Isoleucine amino acid substitutions, respectively suggesting that *DMRT1* is essential in sex reversal (Cui *et al.* 2018). Non-coding SNPs can potentially affect a gene's expression level. In present study, SNP in the 3′ UTR of the *DMRT1* gene was identified, which indicate a possible effect of this SNP on mRNA structure and its expression.

The *FOXL2* 188 bp (Fig. 3) fragment has revealed a non-synonymous SNP (c.289C>G) at 295thand 289th position (Fig. 4) which has showed an amino acid change from Histidine (H) (CAC) to Aspartic acid (D) (GAC). SNPs can result in two or more allelic variants of mRNA which might show an impact on mRNA splicing (Coleman and Rosser 1998), processing (Allain *et al.* 1996), translational control (Shen and Tinoco 1995) and regulation (Addess *et al.* 1997). Further, SNPs can alter a protein's binding site, which can affect interactions with partner proteins, ligands and other molecules and can affect both stability and protein assembly (Von Bullow *et al.* 2002; Denayer *et al.* 2008). In accordance to present results, a non-synonymous SNP (A238G) in the coding region of the *FOXL2* gene was identified with the amino acid altered from isoleucine to valine in native Chicken of china (Qin *et al.* 2015). Similarly, in Brown Local Iraqi chickens, a non-synonymous SNP C919T substituted the amino acid threonine (ACG) to methionine (ATG) (Abu-Rekaiba *et al.* 2021). Similarly, in female Japanese flounders (*Paralichthys olivaceus*), the SNP1 [c.540ANC (p. Asn102His) and c.591ANG (p. Asn119Asp)] was discovered in the forkhead domain, while the SNP2 [c.864GNA (p. Lys210Glu) and c.875GNA] was found in the downstream (Shi *et al.* 2009).

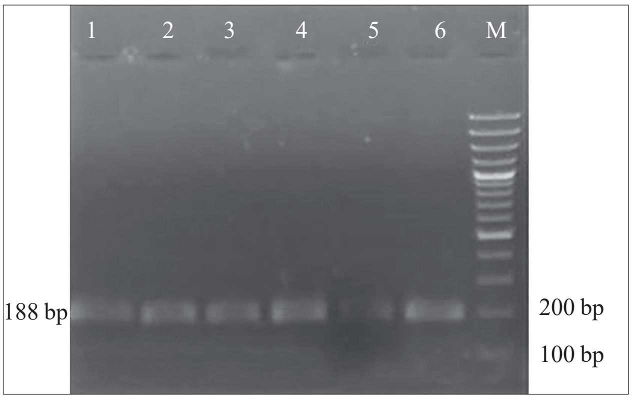
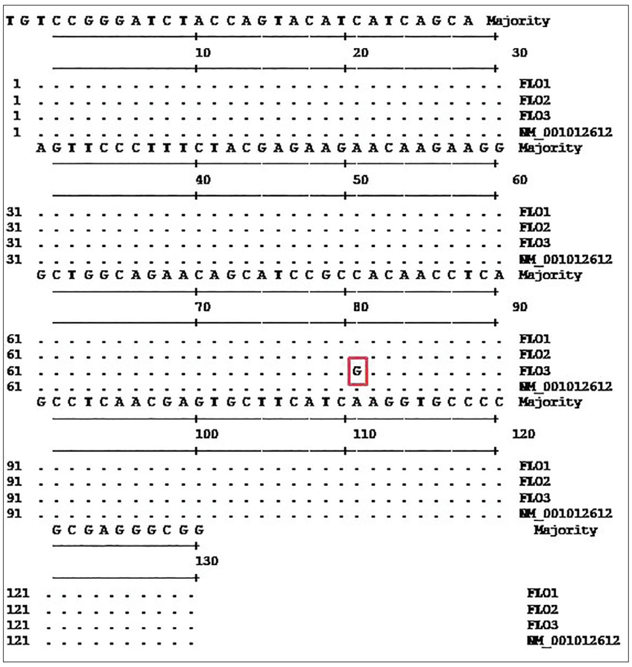


Fig. 3. Agarose gel electrophoresis showing PCR amplified product of *FOXL2* gene. Lane: 1-6: Ovaries; Lane M: 100 bp Ladder.



```
T G T C C G G G A T C T A C C A G T A C A T C A T C A G C A  Majority
    ---------+---------+---------+
             10        20        30
    ---------+---------+---------+
 1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL01
 1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL02
 1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL03
 1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  NM_001012612
    A G T T C C C T T T C T A C G A G A A G A A C A A G A A G G  Majority
    ---------+---------+---------+
             40        50        60
    ---------+---------+---------+
31  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL01
31  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL02
31  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL03
31  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  NM_001012612
    G C T G G C A G A A C A G C A T C C G C C A C A A C C T C A  Majority
    ---------+---------+---------+
             70        80        90
    ---------+---------+---------+
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL01
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL02
61  . . . . . . . . . . . . . . . . . . . . G . . . . . . . . .  FL03
61  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  NM_001012612
    G C C T C A A C G A G T G C T T C A T C A A G G T G C C C C  Majority
    ---------+---------+---------+
             100       110       120
    ---------+---------+---------+
91  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL01
91  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL02
91  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  FL03
91  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  NM_001012612
    G C G A G G G C G G                                            Majority
    ---------+
             130
    ---------+
121 . . . . . . . . . .                                            FL01
121 . . . . . . . . . .                                            FL02
121 . . . . . . . . . .                                            FL03
121 . . . . . . . . . .                                            NM_001012612
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Fig. 4. Nucleotide sequence alignment of *FOXL2* gene by MegAlign in left ovaries of chicken.

In conclusion, the *DMRT1* gene expression in right testes compared to left and right ovaries were not significant. The expression level of *FOXL2* was significantly higher in the left ovary compared to the right testes but not significant when compared to the right ovary. Both *DMRT1* and *FOXL2* genes have polymorphic variants and the polymorphism in the *DMRT1* gene was identified in the non-coding region, which may have the ability to change the mRNA structure that needs to be further explored. In the secondary structure of the *FOXL2* gene, the difference in the α helix of protein was identified between the reference and polymorphic SNP sample which needs to be further studied. The differences in the gene expression, genetic polymorphism and protein structure of *DMRT1* and *FOXL2* genes in the gonads of chicken provide further information about the gonadal development in chicken.

ACKNOWLEDGEMENTS

The authors extends their gratitude to the Dean, PVNRTVU, Hyderabad, Director and ICAR- Directorate of Poultry Research, Hyderabad for the support and facilities to carry out present research work.

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