E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2020; 8(3): 49-54

www.fisheriesjournal.com
Received: 24-03-2020
Accepted: 25-04-2020

Rose Gregoria PJ
Endocrinology Unit, Department of Zoology, Madras Christian College, Tambaram, Chennai, Tamil Nadu, India

Dr. Moses Inbaraj R
Endocrinology Unit, Department of Zoology, Madras Christian College, Tambaram, Chennai, Tamil Nadu, India

Corresponding Author:
Dr. Moses Inbaraj R
Endocrinology Unit, Department of Zoology, Madras Christian College, Tambaram, Chennai, Tamil Nadu, India

Identification, expression and phylogenetic analysis of the gene *sox9a* in the testes of Indian major carp *Catla catla* throughout a reproductive cycle and in the extragonadal tissues

Rose Gregoria PJ and Dr. Moses Inbaraj R

Abstract
Sox9 is a transcriptional factor which is a member of the *sox* gene family of SRY-related high mobility group (HMG) box genes. *Catla catla* like other vertebrates, express *sox9a* gene mainly in the testes, responsible for the sex determination and male gonadal development. The abundance of the mRNA encoding Sox9a was determined by rtqRT-PCR in the tissues of the testes of catla fish throughout the annual reproductive cycle. The expression of *sox9a* has seen its peak level during the preparatory and previtellogenic period and the expression was very law during the time of resting period or postvitellogenic period. The expression level was prominent in the vitellogenic period, but it is lesser than preparatory and previtellogenic period. The expression of *sox9a* in the testes was also expressed in other tissues of brain, kidney, heart, muscle and gill. There was no expression of *sox9a* in the female gonad. This reveals the significant role of this gene in the testes differentiation and in the different phases of reproductory cycle of *C. catla*.

Keywords: *sox9a*, SRY-related high mobility group (HMG), vitellogenic, hpf (hour post fertilization)

1. Introduction
Sox9 is a transcriptional factor which is a member of the Sox gene family of SRY-related high mobility group (HMG) box genes [1]. Researchers indicate that Sox9 plays a very important role in the male gonadal development of many vertebrates [2], [3]. These findings suggest that the role of *sox9* in male gonad development appears to be evolutionally conserved in many vertebrates. SOX9 was initially recognized as the gene accountable for autosomal XY sex reversal linked with campomelic dysplasia, a human skeletal malformation condition [1], and in the majority of XY cases, partial or complete sex reversal was discovered [4]. The expression of *sox9* is detected in chicken, turtle, and alligator with the developing testis [5], [6], [7]. *Sox9* is the straight target of the testicular sex-determining gene, Sry [8]. *Sox9* transcription is up-regulated in the presence of Sry in developing pre-sertoli cells during male development [9]. However, *sox9* over expression in the genital ridges resulted in normal male development in transgenic mice lacking Sry [10]. During gonad differentiation, *sox9* is up-regulated in testes and down-regulated in ovaries [11]. This expression pattern, along with male sex reversal in SOX9-deficient patients, implies that SOX9 is important in the male developmental pathway. So far, the *sox9* gene has been recognized in a number of vertebrate species together with mammals [1], birds [12], reptiles [13], amphibians [14], and fishes [15], [16]. *Anti-Müllerian hormone* (*amh*) is one of the original functional genes expressed for the duration of testicular differentiation. It is confirmed that a critical mechanism underlying the direct synergy of androgen signalling and *sox9a* in the regulation of *amh* transcription [17].
Sox9 was demonstrated in the testis of rainbow trout [18] and in zebrafish, *sox9a* was present in the testis [15]. Two *sox9a* genes (*sox9a1* and *sox9a2*) were seen in rice field eel [19] and in medaka, *sox9a2* was expressed in adult testis [20]. In adult allotetraploids *Sox9b* was highly expressed in the testis while no expression of *Sox9b* mRNA was discovered in the ovary [21]. In Nile tilapia *Sox9a* is expressed in mature gonads of males but not in females [22]. The gene *sox9b* was present in the testis of common carp [23]. But in triploid crucian carp *sox9a* was predominantly expressed in the testis [24]. The *sox9a* was isolated from the testis of catfish and *sox9b* from its ovary [25]. These results put forward that the appearance of *sox9* in gonads is diversified in fish species.

Research screening the influence of the gene *sox9a* throughout the reproductive cycle is very rare. In the present work we are trying to analyse the gene expression of *sox9a* in the testes of the Indian major carp *C. catla* and its changes in the annual reproductive cycle. Present studies disclose that *sox9a* is involved in various aspects like vitellogenesis, gonadal development, reproduction and reproductive behaviors. So, we can conclude that *sox9a* plays a potent role in preserving the continuity of life in diverse vertebrates including fishes by sustaining important physiological process, reproduction. As we have seen above, *sox9a* is having significant role in breeding season; we can improve the quantity and quality of this edible fish by manipulating the gene.

2. Materials and methods

2.1. Sample collection

The fish samples like gonads and other tissues of the Indian major carp, *Catla catla* were collected from the Tamil Nadu Fisheries Department Corporation (TNFDC), Sathanoor Dam, situated about 200 km south of Chennai. Monthly samples were collected from the matured live fishes and the tissues were pooled in eppendorf tubes with RNA later and stored in $-20°C$ until RNA extraction.

2.2. Total RNA extraction

Total RNA was isolated from the 100 mg. of tissue using TRIzol method. The quantity and quality of RNA were determined by UV absorbance at 260 and 280 nm wavelength. Agarose gel electrophoresis has done and the clear 18S and 28S RNA bands has visualised by UV illuminator and gel documentation has done with the computer.

2.3. cDNA synthesis

One microgram of total RNA from the sample tissue was reverse transcribed using M-Mu LV RT- PCR Kit according to manufacturer's instruction. The RT-PCR products were quantified and qualified by using spectrophotometer and gel electrophoresis.

2.4. Real-Time Quantitative RT-PCR

Suitable primers were constructed by appropriate software for the gene sox9a and the housekeeping gene beta actin.
sox 9a F: 5′-TGAAGRGCTACGAYTGGACG-3′
sox 9a R: 5′-CCCTCTCGYYTCAGATCAACTT-3′
Beta Actin F: 5′- CGGTTATCGTTGTAGGCACG-3′
Beta Actin R: 5′-CACTGCCTGCACAAAGAACT-3′

Transcript abundance of *sox9a* was quantified by rt qRT-PCR of total RNA isolated from the ovaries of catla fish throughout the year to findout the variation of the gene expression in the annual breeding cycle. rt qRT-PCR cycle conditions were 40 cycles with $95^{\circ}C$ for 10 minutes, $95^{\circ}C$ for 15 seconds and $60^{\circ}C$ for 1 minutes. Rt qRT-PCR was carried out with SYBR Green fluorescent label using beta actin as an endogenous control.

2.5. Sequencing and Phylogenetic analysis

The PCR product was send for sequencing and sequence data is submitted to Genbank. Accession number received is KJ699356. According to the sequence data multiple sequence alignment and phylogenetic analysis has done by using the software Mega5.

3. Results

The abundance of the mRNA encoding *sox9a* was determined by rtqRT-PCR in the tissues of the testes of catla fish all through the annual reproductive cycle. The expression of *sox9a* has seen its max out during the preparatory period and the expression was near to the ground during the time of resting period. A steady decline can see in the gene expression from the preparatory phase to post spawning phase. Although the expression was low down in the last month of post spawning phase, a sudden elevate in the gene expression is obtained in the preparatory phase. And also a steady boost up in the expression level can be seen right through the preparatory phase (Fig.2). From this expression data we can understand that *sox9a* has a significant role in the reproductory function.

The onset of gonadal recrudescence was evident by the month of January and following two months includes the preparatory period. Then a steady diminish can be seen from the month of April, the beginning period of previtellogenic phase. But there is a prominent expression all through the previtellogenic phase revealing that *sox9a* has an important role in spermatogenesis. Then a steady decline can be seen in the following month upto December which is the closing stages of postvitellogenic period. Again, the preparatory phase start from the month of January and expression level is suddenly getting higher in this period from the post spawning phase (Fig.1). This shows the significant role of sox9a throughout the annual reproductive cycle of *C. catla*.

The expression of *sox9a* is found in other tissues too. Samples of brain, kidney, heart, muscle, intestine, liver, ovary and gill were processed to detect the expression of *sox9a*. There was no expression in the samples of liver, intestine and ovary. But *sox9a* is obviously expressed in the tissues of brain, kidney, heart, muscle and gill. This result shows that *sox9a* is not only present in the testes alone but also in many other different tissues (Fig.3).

Multiple Sequence Alignment was done with the sequence of *sox9a* in *Catla catla* (KJ699356) with those of other species. The sequences used for analysis were downloaded from NCBI, and have following accession numbers : *Carassius carassius* (DQ777836), *Carassius auratus* (EF219274), Triploid red crucian carp hybrid (EF370035), *Carassius carassius* hybrid (EU312055), Triploid red crucian carp hybrid (EF370038), Diploid improved black crucian carp hybrid (EU337023), Diploid improved red crucian carp hybrid (EU337020), *Carassius carassius* hybrid (FJ854550), Diploid *Xenocypris davidi* hybrid (GU981682), Triploid *Megalobrama amblycephala* (GU981683), *Xenocypris davidi* (GU981685), *Hypophthalmicthys nobilis* (KC847694), *Megalobrama amblycephala* (EF219277), Diploid *Megalobrama amblycephala* hybrid (HQ651063), Triploid *Megalobrama amblycephala* (HQ651064), *Mylopharyngodon picius* (EU180210), *Ctenopharyngodon idella* (EU180211), Weather loach (GU166139), Pig (KF422601), Chicken (AB012236), Mouse (NM011448), Frog (BC170060), Human (Z4662). Phylogenetic tree was constructed based on the multiple sequence alignment (Fig.4). These results can be summarizing as that the gene *sox9a* is evolutionally conserved throughout the vertebrate phyla.

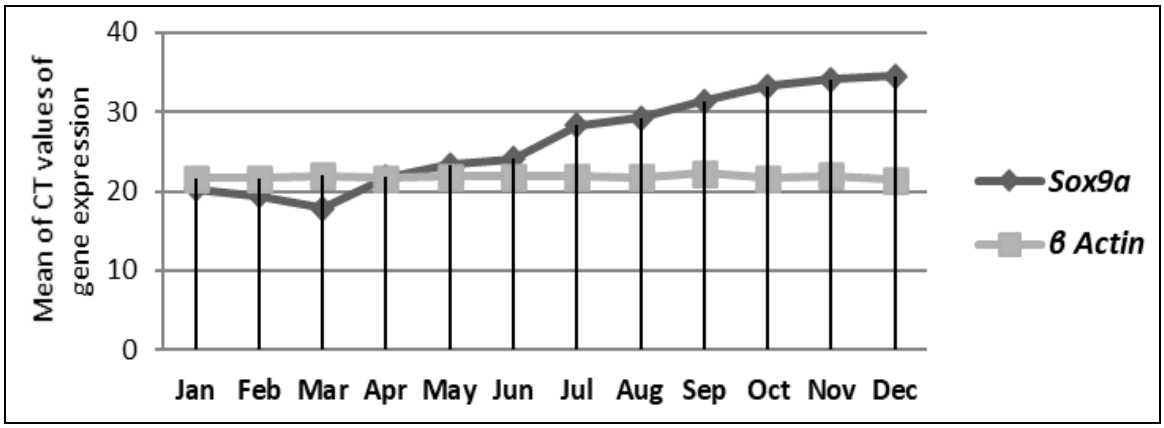


Fig 1: Seasonal Changes in the expression of *sox9a* in testes of an annual reproductive cycle of *Catla catla*

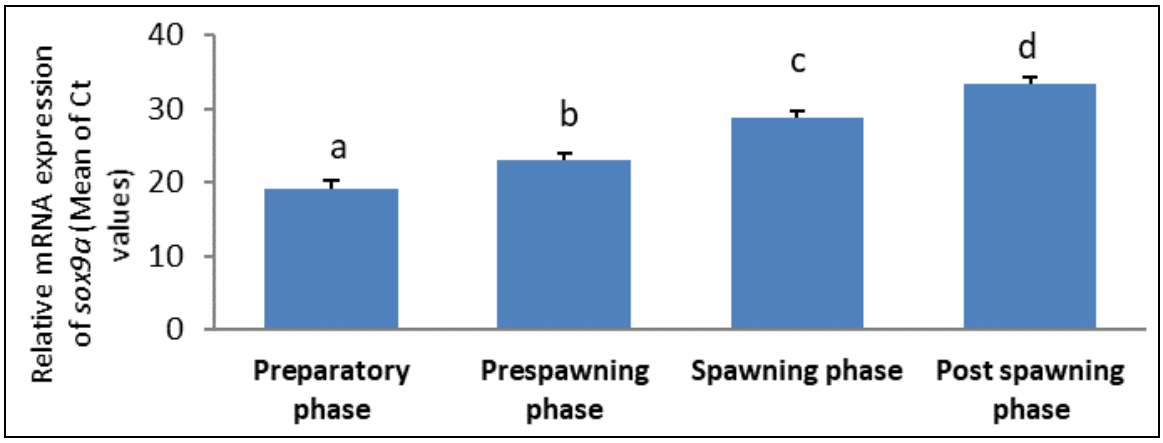
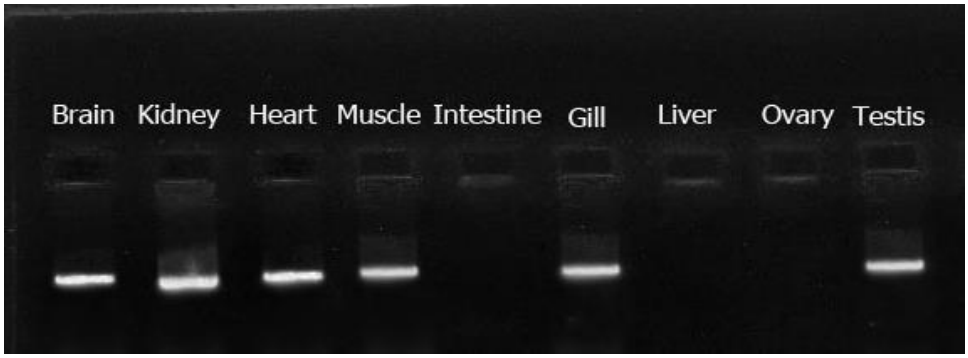
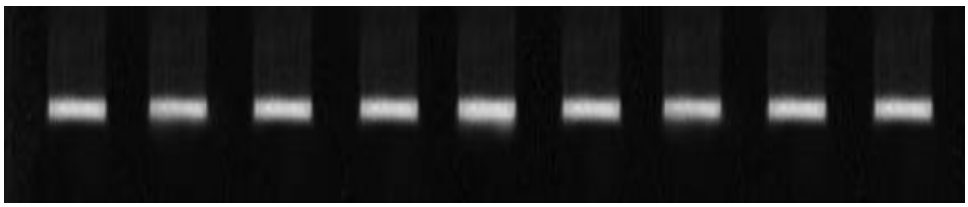


Fig 2: The expression of *sox9a* of *Catla catla* in different reproductory phases of testes (b differ from a and d: $p<0.01$; b differ from a and c: $p<0.01$; c differ from a and d: $p<0.01$)



sox9a



β actin

Fig 3: Bands showing the expression of *sox9a* and *β actin* in different tissues of *Catla catla*

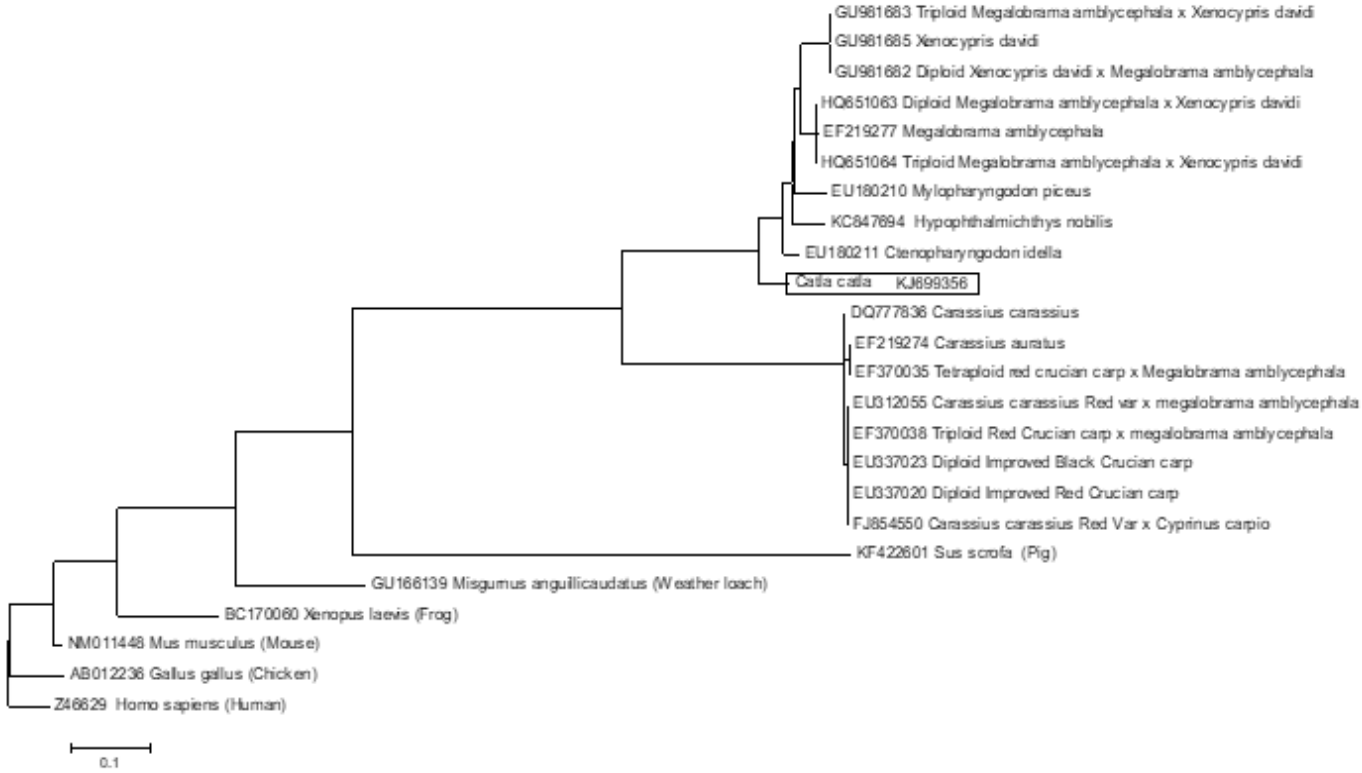


Fig 4: Evolutionary relationships of taxa in reference to *sox9a* gene of *Catla catla* (KJ699356).

The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 2.54200026 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm [26] at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. The analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 387 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [27].

4. Discussion and conclusion

The purpose of this study was to determine systematically, for the first time, the expression profiles of the *sox9a* in the testes of a lower vertebrate *C. catla*, throughout an annual reproductive cycle. This gene was well expressed in all the important time of reproductory phases except post spawning period. This result correlate with the findings in some vertebrates that the expression of *sox9* in chicken, turtle, and alligator is associated with the developing testis [5], [6], [7]. Some other previous studies have too indicated that *sox9* plays a very important role in the male gonadal development of many vertebrates [2], [3]. These findings suggest that the role of *sox9* in male gonad development appears to be evolutionally conserved in many vertebrates.

It is observed from this study that there is a clear variation in the expression of *sox9a* gene in different reproductive phases in an annual cycle. It is proved that activity of *sox9a* is much high during the time of preparatory and previtellogenic phases. In a previous study it is revealed that *sox9a* expression in catfish was drastically higher during preparatory and pre-spawning phases [25]. Findings clearly indicate that *sox9* genes play essential role during seasonal variation of gonads [28]. These three results point out that *sox9a* transcripts are abundantly expressed during active spermatogenesis, i.e. in preparatory and pre-spawning phases, in which spermatogonia and spermatocytes are differentiated more [29] and its expression decreased gradually thereafter during spermiation and post-spawning phases. This elevated expression for the duration of spermatogenesis is due to the upper number of germ cells. *Sox9* expression was down regulated in mammals and birds during ovarian differentiation, but its expression was high in increasing testis and all through adulthood [5], [12]. The fate of *sox9* in different phases of adult gonads during the reproductive cycle has been studied in detail so far is very few. In this study, we quantified *sox9* transcript abundance in different stages of testicular cycle.

Current study shows that the *sox9a* expression is not only in testes but also in many other tissues like brain, kidney, heart, muscle and gill. It was not present in intestine, liver and ovary. In addition to the testicular expression, *sox9a* was seen in many other tissues in triploid crucian carp, signifying that *sox9* may have exclusive functions in some specific tissues all through development [24]. Analysis of the tissue distribution pattern of *sox9* in orange-spotted grouper revealed that *sox9* was ubiquitously expressed in tissues such as brain, kidney, heart, liver, spleen, muscle, stomach, intestine, testis and ovary and the expression was relatively high in stomach and testis, while it was low in the kidney and heart [30]. The expression of *sox9a* was restricted to few tissues like brain, heart, gill, liver, kidney, and testis in catfish [25]. The bone development through *sox9a* expression during osteogenesis in zebra fish is reported [31].

The two *sox9* genes have been observed in stickleback, zebra fish, medaka, triploid crucian carp and rice field eel [20], [15], [16],

[32], [24]. But here reporting only one gene, termed *sox9a*. However, only one *sox9* gene has been found in the genome of mammals and birds, indicating that the Sox9 gene was duplicated during the evolution of some fish lineages. The previous studies showed that rainbow trout *sox9* is expressed in the testis [18], medaka *sox9* is observed in the ovary [16], and zebra fish *sox9a* in the testis, and *sox9b* in the ovary [15].

Above observations recommended that the role of the *sox9* gene for the duration of gonad differentiation is quite different among fish. In effect, the present study revealed that *sox9a* is expressed in the testis of *C. catla*. Together with the present finding and previous observations, it is reasonable to assume that *sox9* genes differentially contribute to sex differentiation among fish and it has a significant role in the reproductive cycle of *C. catla*.

5. Acknowledgements

The authors acknowledge the institutional support of Madras Christian College, Chennai.

6. References

1. Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M *et al*. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature. 1994; 372:525-530.
2. Pask AJ, Harry JL, Graves JA, O'Neill RJ, Layfield SL, Shaw G *et al*. SOX9 has both conserved and novel roles in marsupial sexual differentiation. Genesis. 2002; 33:131-139.
3. Chaboissier MC, Kobayashi A, Vidal VI, Lutzkendorf S, van de Kant HJ, Wegner M, *et al*. Functional analysis of Sox8 and Sox9 during sex determination in the mouse. Development. 2004; 131:1891-1901.
4. Tommerup N, Schemp W, Meinecke P, Pedersen S, Bolund L, Brandt C *et al*. Assignment of an autosomal sex reversal locus (SRA1) and campomelic dysplasia (CMPD1) to 17q24.3-q25.1. Nat Genet. 1993; 4:170-174.
5. Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P. A male-specific role for SOX9 in vertebrate sex determination. Development. 1996; 122:2813-2822.
6. Spotila LD, Spotila JR, Hall SE. Sequence and expression analysis of WT1 and Sox9 in the red-earedslider turtle, Trachemys scripta. J Exp Zool. 1998; 281:417-427.
7. Western PS, Harry JL, Graves JAM, Sinclair AH. Temperature dependent sex determination, upregulation of Sox9 expression after commitment to male development. Developmental Dynamics. 1999; 214:171-177.
8. Sekido R, Lovell-Badge R. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature. 2008; 453:930-934.
9. Sekido R, Bar I, Narvaez V, Penny G, Lovell-Badge R. SOX9 is up regulated by the transient expression of SRY specifically in Sertoli cell precursors. Developmental Biology. 2004; 274:271-279.
10. Qin Y, Bishop CE. Sox9 is sufficient for functional testis development producing fertile male mice in the absence of Sry. Human Molecular Genetics. 2005; 14:221-1229.
11. Moreno-Mendoza N, Harley VR, Merchant-Larios H. Differential expression of SOX9 in gonads of the sea turtle *Lepidochelys olivacea* at male- or female-promoting temperatures. Journal of Experimental Zoology. 1999; 284:705-710.
12. Morais da Silva S, Hacker A, Harley V, Goodfellow P, Swain A, Lovell- Badge R. Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nature Genetics. 1996; 14:62-68.
13. Choudhary B, Ganesh S, Raman R. Evolutionary conservation of the gene Cvsox9 in the lizard, Calotes versicolor, and its expression during gonadal differentiation. Development Genes and Evolution. 2000; 210:250-257.
14. Takase M, Noguchi S, Nakamura M. Two Sox9 messenger RNA isoforms, isolation of cDNAs and their expression during gonadal development in the frog *Rana rugosa*. FEBS Letters. 2000; 466:249-254.
15. Chiang EF, Pai CI, Wyatt M, Yan YL, Postlethwait J, Chung B. Two sox9 genes on duplicated zebra fish chromosomes, expression of similar transcription activators in distinct sites. Developmental Biology. 2001; 231:149-163.
16. Yokoi H, Kobayashi T, Tanaka M, Nagahama Y, Wakamatsu Y, Takeda H *et al*. Sox9 in a Teleost Fish, Medaka (*Oryzias latipes*): evidence for diversified function of Sox9 in gonad differentiation. Molecular Reproduction and Development. 2002; 63:5-16.
17. Gang Zhai, Tingting Shu, Zhan Yin. Androgen signalling regulates the transcription of anti-Mullerian hormone via synergy with SRY-related protein SOX9A. Science Bulletin. 2017; 62:3.
18. Takamatsu N, Kanda H, Ito M, Yamashita A, Yamashita S, Shiba T. Rainbow trout SOX9, cDNA cloning, gene structure and expression. Gene. 1997; 202:167-170.
19. Zhou R, Liu L, Guo Y, Yu H, Cheng H, Huang X *et al*. Similar gene structure of two Sox9a genes and their expression patterns during gonadal differentiation in a teleost fish, rice field eel (*Monopterus albus*). Molecular Reproduction and Development. 2003; 66:211-217.
20. Nakamoto M, Suzuki A, Matsuda M, Nagahama Y, Shibata N. Testicular type Sox9 is not involved in sex determination but might be in the development of testicular structures in the medaka, *Oryzias latipes*. Biochemical and Biophysical Research Communications. 2005; 333:729-736.
21. Liu Jifang, Shaojun Liu, Min Tao, Wei Li, Yun Liu. Isolation and Expression Analysis of Testicular Type Sox9b in Allotetraploid Fish. Marine Biotechnology. 2007; 9:329-334.
22. Kobayashi T, Hiroko K, Guijun G, Nagahama Y. Sexual Dimorphic Expression of DMRT1 and Sox9a During Gonadal Differentiation and Hormone-Induced Sex Reversal in the Teleost Fish Nile Tilapia (*Oreochromis niloticus*). Dev. Dyn. 2008; 237:297-306.
23. Du QY, Wang FY, Hua HY, Chang ZJ. Cloning and study of adult tissue- specific expression of Sox9 in *Cyprinus carpio*. Journal of Genetics. 2007; 86:85-91.
24. Guo Xinhong, Jinpeng Yan, Shaojun Liu, Bing Xiang, Yun Liu. Isolation and expression analyses of the Sox9a gene in triploid crucian carp. Fish Physiol Biochem. 2010; 36:125-133.
25. Raghuveer K, Senthilkumaran B. Isolation of sox9 duplicates in catfish: localization, differential expression pattern during gonadal development and recrudescence, and hCG-induced up-regulation of sox9 in testicular slices. Society for Reproduction and Fertility. 2010; ISSN:1470-1626.

26. Nei M, Kumar S. Molecular Evolution and Phylogenetics. Oxford University Press, New York, 2000.
27. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution. 2011; 28:2731-2739.
28. Bhat IA, Rather MA, Saha R, Pathakotta GB, Pavan-Kumar A, Sharma R. Expression analysis of Sox9 genes during annual reproductive cycles in gonads and after nanodelivery of LHRH in *Clarias batrachus.* Research in Veterinary Science. 2016; 106:100-6.
29. Swapna I, Rajasekhar M, Supriya A, Raghuveer K, Sreenivasulu G, Rasheeda MK *et al*. Thiourea-induced thyroid hormone depletion impairs testicular recrudescence in the air-breathing catfish, *Clarias gariepinus*. Comparative Biochemistry and Physiology. 2006; 144:1-10.
30. Luo Yu-Shan, Wei Hu, Xiao-Chun Liu, Hao-Ran Lin, Zuo-Yan Zhu. Molecular cloning and mRNA expression pattern of Sox9 during sex reversal in orange-spotted grouper (Epinephelus coioides). Aquaculture. 2010; 306:322-328.
31. Kalaiarasi Sivaji, Rajaretinam Rajesh Kannan, Soundarapandiyan Nandhagopal, Carlton Ranjith. Exogenous human beta amyloid peptide interferes osteogenesis through Sox9a in embryonic Zebra fish. Molecular Biology Reports. 2019; 46(5).
32. Cresko WA, Yan YL, Baltrus DA, Amores A, Singer A, Rodriguez-Mari A *et al*. Genome duplication, subfunction partitioning, and lineage divergence: Sox9 in stickleback and zebrafish. Devlopmental Dynamics. 2003; 22:480-489.