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MOLECULAR CLONING AND QUANTITATIVE mRNA EXPRESSION OF *SOX9* GENE IN GONADAL DEVELOPMENTAL PERIOD OF *HYPOATHERINA TSURUGAE*

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ABSTRACT

Sox9 is a transcription factor of high mobility group (HMG) box family DNA binding domain. It plays a crucial role in gonadogenesis during embryonic developmental period. 1454bp of *sox9* mRNA transcript was cloned and sequenced. It consists of open reading frame (ORF) of 1436 bp, that encodes a 479 aa protein and it found identical to the HMG box of other fish species. Phylogenetic tree was constructed by comparing the mRNA sequence of 50 different fishes across various taxa available in NCBI database and taking out group as *Xenopus laevis*. The tree shows a high homology of *H. tsurugae sox9* with *Maelanotaenia boesemani sox9* forming a single clad. The expression of *sox9* was studied that reveals in *amhy+* male, the expression begins from baseline from 0 wah and it expresses in an increasing fashion whereas in *amhy-* female individual highly expressed at beginning stage (0 wah) and the expression reaches peak at 2 wah then decline which indicates the low expression needed for differentiation of female sex organ. The histological sections of gonads are studied in different stages of biweekly collected larva during sex determination/differentiation period and it showed that differentiation of gonads male/female decided at 6 wah. In this stage the primary oocytes are clearly recognized and it correlated with expression of *sox 9* genes. These finding added an extra knowledge for better understanding of molecular mechanisms of sex determination and differentiation period in fish.

Keywords: Atheriniformes, *Sox9*, *Hypoatherina tsurugae*, Gonadal development

INTRODUCTION

It has been reported that *amhy* gene has critical role in male sex determination of an old world Silverside, *Hypoatherina tsurugae* species [1]. Many other genes/transcription factors have also been described as master sex determining genes in various fishes [2-6]. From these references, it is clear that genetic machinery of fish which control gonadal development is very much diverse and is not limited to a particular gene/transcription factors as more interestingly reported *sdY* an immune related gene can be crosstalk as sex determining genes in Salmonids [3].

Sox (Sry related high mobility group box) a gene family of transcription factors that posses an important role in reproduction and development of gonads [7]. Sox9 is a member of Sox-family that serves a crucial role in testis formation besides other function like cartilage formation [8-9]. This is a potential candidate gene in fish that may drive downstream development of gonads after triggered by master sex determining gene during gonadal differentiation period. This gene believed to be the main effector of *Sry* gene [10]. It has been studied that the mutation in sox9 gene results abnormal bone formation and even sex reversal [9, 11].

Hypoatherina tsurugae commonly called Cobaltcap silverside which has very little information about its reproductive biology and sex differentiation. In this species besides *amhy* gene [1], the expression of other genes has not been studied during gonadal determination/differentiation period. So, objective of this paper is to study the expression pattern of *Sox9* gene in this particular species and its role in during gonadal development period.

MATERIALS AND METHODS

About 100 matured wild cobaltcap silversides were collected by hand net and it was reared in a 500 liter tank to obtain gametes and offsprings for experiments. The tanks were supplied with

filtered natural sea water at a rate of 100 ml/min. Larvae were fed rotifers *Branchionus rotundiformis* and *Artemia* nauplii from the first day to satiation twice daily and gradually weaned into powdered marine fish food (AQUEON, Franklin, WI).

Genomic DNA was isolated from caudal fin tissue following protocol described by Aljanabi and Martinez [12]. The genotyping of larvae to know male / female are performed by primer Amh 613 F and Amh 35 R [1].

Cloning of Sox9 gene

For cloning, total RNA was isolated *Amhy+* individual testis by using TRIzol (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instruction. 1 µg of total RNA per sample was reverse transcribed using SuperScript III (Thermo Fisher Scientific) with Oligo-(dT) primers (Merk Milipore, Darmstadt, German) in 20 µl reactions. The PCR was performed amplifications were done according to the following conditions: 3 min at 94 ºC, 30 cycles of 30 sec at 94 ºC, 45 sec at 60 ºC and 2.5 min at 72 ºC, then followed by a final elongation for 5 min at 72 ºC. PCR products were electrophoresed in 1% agarose gel, purified, and sequenced in an ABI PRISM 3100 capillary sequencer (Life Technologies, Carlsbad, CA) using the BigDye Terminator method. Sequences were analyzed with GENETYX version 11.0 (GENETYX, Tokyo, Japan). All primers are listed in Table 1.

Real-Time/Quantitative PCR (qRT-PCR)

For expression studies, total RNA was isolated *amhy+* and *amhy-* individual at the interval 2 week after hatch (wah) like 0wah, 2wah, 4wah, 6wah, 8wah and 10wah. The expression level of mRNA transcripts was analyzed by qRT-PCR using specific RT primers designed for Sox9 loci

using conditions of previous study [1]. The β-*actin* gene was taken as an endogenous control because of its stability during sex determination/differentiation period.

Sequence analysis

The multiple alignment software Clustal X was used for alignment of nucleotide sequences and their deduced amino acid sequences. The phylogenetic tree was constructed using MEGAX with Neighbour-Joining method, Maximum Likelihood and BioNJ Algorithm to a matrix of pairwise distances estimated in Tamura-Nei model with bootstrap value 10,000 replicates each to determine confidence of tree topology. The neckwick file export to iTOL (https://itol.embl.de) an interactive Tree of Life webserver.

Statistical analysis

The differences in gene expression between groups were analyzed by ANOVA followed by Tukey test using GraphPad prism (v.6.0; GraphPad software, San Diego, CA). Differences in gene expression were considered as statistically significant at $P < 0.05$.

Data Accessibility

DNA sequences: GenBank accessions; *Hypoatherina tsurugae sox*9 [PP108745], *Acanthochromis polyacanthus sox9a* [XM_022211835.2], *Amphiprion ocellaris* sox9a [XM_023286694.3], *Anabas testudineus sox9a* [XM_026358727.1], *Anarrhichthys ocellatus sox9* [XM_031867589.1], *Anoplopoma fimbria sox9a* [XM_054606768.1], *Astatotilapia calliptera sox9a* [XM_026164918.1], *Chelmon rostratus sox9* [XM_041957848.1], *Cololabis saira sox9* [XM_061712632.1], *Cottoperca gobio sox9* [XM_029437533.1], *Dicentrarchus labrax sox9* [XM_051389381.1], *Epinephelus fuscoguttatus sox9* [XM_049561363.1],

Etheostoma cragini sox9 [XM_034894690.1], *Etheostoma spectabile sox9* [XM_032544694.1], *Haplochromis burtoni sox9a* [XM_005936187.2], *Hippoglossus hippoglossus sox9a* [XM_034593479.1], *Kryptolebias marmoratus sox9a* [XM_017425448.3], *Larimichthys crocea Sox9b* [MH996432.1], *Lates calcarifer sox9* [KR492508.1], *Mastacembelus armatus sox9b* [XM_026325302.2], *Maylandia zebra sox9* [XM_004559402.2], *Melanotaenia boesemani sox9* [XM_041970542.1], *Micropterus salmoides sox9a* [XM_038701099.1], *Morone saxatilis sox 9* [XM_035655588.1], *Nematolebias whitei sox9a* [XM_037689002.1], *Neolamprologus brichardi sox9* [XM_006791412.2], *Odontesthes bonariensis sox9* [AY319415.4], *Oncorhynchus mykiss sox9* [AB006448.1], *Oreochromis niloticus sox9* [XM_003450119.4], *Oryzias latipes sox9* [NM_001105086.1], *Oryzias melastigma sox9a* [XM_024272555.2], *Paralichthys olivaceus sox9a* [KY924902.1], *Perca fluviatilis sox9* [XM_039825773.1], *Plectropomus leopardus sox9a* [XM_042504339.1], *Poecilia formosa sox9* [XM_007556363.2], *Pseudoliparis swirei sox9* [XM_056407289.1] , *Pundamilia nyererei sox9a* [XM_005744343.1], *Sander lucioperca sox 9a* [XM_031312361.2], *Scatophagus argus sox9* [XM_046415919.1], *Seriola dumerili sox9* [XM_022752333.1], *Simochromis diagramma sox9* [XM_040049522.1], *Siniperca chuatsi sox9* [XM_044181768.1], *Solea senegalensis sox9* [XM_044024558.1], *Stegastes partitus sox9* [XM_008303357.1], *Thunnus albacares sox9b* [XM_044332285.1], *Thunnus maccoyii sox9b* [XM_042393607.1], *Toxotes jaculatrix sox9* [XM_041062045.1], *Trematomus bernacchii sox9a* [XM_034143142.1], *Xiphias gladius sox9a* [XM_040123617.1] and *Xenopus laevis sox9* [BC170060.1]

RESULTS AND DISCUSSION

Sequence analysis of sox9

The isolated Sox9 cDNA was 1454 bp with an open reading frame (ORF) of 1436 bp, encoding a 479 aa protein (GenBank Accession number – PP108745) (Fig. 1). It is identical to the HMG box of Sox9 gene of *Melanotaenia boesemani* (96.42%), *Stegastes partitus* (92.43%), *Odontesthes bonariensis* (92.42%), *Xiphias gladius* (91.01%), *Seriola dumerili* (90.98%), *Lates calcarifer* (90.52%), *Dicentrarchus labrax* (90.51%), *Oreochromis niloticus* (89.40%). By using Clustal W software, the 479 amino acid sequence of *H. tsurugae* with 9 other fish species was aligned. The results showed that the homology was *Melanotaenia boesemani* (98.54%), *Odontesthes bonariensis* (96.66%), *Lates calcarifer* (96.86%), *Xiphias gladius* (96.25%), *Dicentrarchus labrax* (95.82%), *Plectropomus leopardus* (95.41%), *Perca flavescens* (95.2%) and *Oreochromis niloticus* (92.99%) (Fig. 2).

A phylogenetic tree was constructed by comparing the mRNA sequence of 50 different fishes across various taxa available in NCBI database and taking out group as *Xenopus laevis* (Fig. 3). The tree shows a high homology of *H. tsurugae sox9* with *Maelanotaenia boesemani sox9* forming a single clad as they are belonging to the same order Atheriniformes.

Gene expression analysis

The expression level of mRNA transcript was analyzed by RT-PCR using specific RT primers of *sox9* loci. The β-actin gene was taken as an endogenous control because of its stability during sex determination/differentiation period. The result of qRT-PCR displays that in *amhy*- female individual the expression of *sox9* gene is quite high at 2wah then abruptly decreases. In contrast,

amhy+ male individual it begins from baseline at 0 wah and gradually increases in an exponential fashion until complete differentiation of gonads occur (Fig. 4).

The histological sections of gonads in different larval stages showed that differentiation of gonads male/female decided at 6 wah. In this stage the primary oocytes are clearly recognized (Fig. 5) which is also correlated with expression of *sox 9* genes.

In the present study, the *H. tsurugae sox*9 gene has been successfully cloned and sequenced. The *sox*9 mRNA is 1454 bp encoding a 479 aa protein. The *sox*9 gene has very close similarity with *sox*9 gene of *Melanotaenia boesemani*, *Stegastes partitus*, *Odontesthes bonariensis*, *Xiphias gladius*, *Seriola dumerili*, *Lates calcarifer*, *Dicentrarchus labrax*, and *Oreochromis niloticus*. Different number of *sox*9 subtypes exists in different species [13]. It is presumed that there may be two types of *sox*9 genes in *H. tsurugae* but only one type cloned in this study. There is only one type of *sox*9 gene found in higher vertebrates but usually two subtypes are found in teleosts. It is may be due to genome duplication [7, 14]. Phylogenetic analysis revealed that it forms single clad with another Atheriniformes *Maelanotaenia boesemani*.

In this study, focus is given in the expression pattern of *sox*9 gene in gonads of *H. tsurugae*. From the qRT-PCR result, the expression of *sox*9 gene is correlated with the expression of *amhy* gene that significantly reached peak at 6 wah than decreases [1]. The expression of *amhy* was detected from before the appearance of first signs of histological differentiation in presumptive sertoli cells surrounding germ cells in the undifferentiated gonads. Similarly, the *sox9* in *amhy*+ male the expression begins from baseline at 0 wah and it expresses in an increasing fashion that needs for male developmental pathway for testis differentiation whereas in *amhy*- female individual though highly expressed at beginning stage (0 wah) and the expression reaches peak at

2 wah then decline which indicates the low expression needed for differentiation of female sex organ ovary. It has been reported in expression profile of Zebra fish that the *sox9* gene reaches peak at 18 days post hatch which is significantly different from its opposite male sex individual and after 18 days after hatch it decline abruptly [15]. The *sox9a* and *sox9b* expression reported in medaka fish and it is expressed in testis and ovary during developmental period of gonads [16]. However, it is seen in medaka fish the expression of *sox9* gene is provide inconsistent results [17-18]. In rice field eel, *Monopterus albus*, the *sox9* gene is also expressed in both testis and ovary during developmental period [19]. The *sox9* gene is over expressed in testicles as compare to ovary in *Acipeneser sturio* and *Acipenser fulvescens* [20-21]. It has been reported that *sox9* is involved in many signaling pathways during sex determination/differentiation period and in gonad development of embryo and adult. In mammal, the primordial germ cells of gonad are controlled by antagonistically *Wnt4* and *Fgf9* genes but this two gene transcription is effected by *sox9* expression [22].

From above study, it may conclude that after trigger of sex determining gene *amhy* switch on, the next cascade is performed by *sox9* gene and other sex related genes to differentiate the gonad during sex differentiation period for the *H. tsurugae* species. However, more functional experiments are necessary to understand the mechanisms of downstream pathways of gene regulation during gonadal differentiation period of this species.

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FIGURE LEGENDS

Fig. 1 Sox9 gene with complete CDS

Fig. 2 Deduced Amino acids sequence of *H. tsurugae Sox9* gene aligned with other orthologs species

Fig. 3 Phylogenetic analysis constructed with mRNA sequence of 50 different species along with *H. tsurugae*.

Fig. 4 Quantitative mRNA expression of *sox9* gene in *amhy*+ individual (male) and *amhy*- individual (Female).

Fig. 5 Histological differentiation of gonad *amhy*+ male and *amhy*- female

Table 1. List of Primers used in cloning and qRT-PCR

```
aatttttttcgcatgaatctcctcgacccatacctgaagatgacagaagaacaggagaagtgt
   F  F  R  M  N  L  L  D  P  Y  L  K  M  T  E  E  Q  E  K  C
cactctgacgctcccagcccaagcatgtctgaggactccgcaggctcgccgtgcccgtcc
 H  S  D  A  P  S  P  S  M  S  E  D  S  A  G  S  P  C  P  S
ggctccgggtcggacactgaaaacacccggccgtccgacaaccacctcctcggaggtcct
 G  S  G  S  D  T  E  N  T  R  P  S  D  N  H  L  L  G  G  P
gactacaagaaggagaacgaagaagaaaagtttcccgtgtgcatcagagacgcggtgtcc
 D  Y  K  K  E  N  E  E  E  K  F  P  V  C  I  R  D  A  V  S
caggtattgaagggttatgactggacgctggtgcccatgccggtgcgcgtcaacggttca
 Q  V  L  K  G  Y  D  W  T  L  V  P  M  P  V  R  V  N  G  S
agcaaaagcaaacctcacgtcaaaagacccatgaacgcgttcatggtgtgggctcaagca
 S  K  S  K  P  H  V  K  R  P  M  N  A  F  M  V  W  A  Q  A
gctcggaggaaactggcagatcaataccctcatttgcacaacgcagaactcagcaaaacc
 A  R  R  K  L  A  D  Q  Y  P  H  L  H  N  A  E  L  S  K  T
ctgggaaaactttggagattgctcaatgaggtagagaagcgaccgtttgtggaggaagct
 L  G  K  L  W  R  L  L  N  E  V  E  K  R  P  F  V  E  E  A
gagcgactgagagtgcaacataagaaggatcaccccgactacaaatatcagccaaggcga
 E  R  L  R  V  Q  H  K  K  D  H  P  D  Y  K  Y  Q  P  R  R
agaaaatcagtcaagaacggtcagagcgagtccgaggacggcgagcaaactcacatctct
 R  K  S  V  K  N  G  Q  S  E  S  E  D  G  E  Q  T  H  I  S
ccaaatgcgatcttcaaggctctgcagcaggccgactctccggcctccagcatgggcgag
 P  N  A  I  F  K  A  L  Q  Q  A  D  S  P  A  S  S  M  G  E
gttcactcaccaggagaacattcaggtcaatcacagggcccgccaacacccccaaccacc
 V  H  S  P  G  E  H  S  G  Q  S  Q  G  P  P  T  P  P  T  T
cccaagacagatctcccttccagcaaagctgacctaaaacgtgaggggcgccccatgcag
 P  K  T  D  L  P  S  S  K  A  D  L  K  R  E  G  R  P  M  Q
gagggctccagccgccagctcaacatagactttggagctgtggacatcggtgagctgagc
 E  G  S  S  R  Q  L  N  I  D  F  G  A  V  D  I  G  E  L  S
agcgaggtcatctccaacatgggaagcttcgatgttgatgagtttgatcagtacctgccc
 S  E  V  I  S  N  M  G  S  F  D  V  D  E  F  D  Q  Y  L  P
cctcacagccatgccggggtgactggcgcagcccccgctggctacactggcagctacggt
 P  H  S  H  A  G  V  T  G  A  A  P  A  G  Y  T  G  S  Y  G
atcaacagctcctcggttggccaggcagccaacgttggagcccacgcctggatgtccaaa
 I  N  S  S  S  V  G  Q  A  A  N  V  G  A  H  A  W  M  S  K
cagcagcagcagcagcattctctgaccaccctgggtggagcaggagaacaaggccagcag
 Q  Q  Q  Q  Q  H  S  L  T  T  L  G  G  A  G  E  Q  G  Q  Q
ggtcagcagagagccacccagattaagacagagcagctgagccccagtcactacagcgag
 G  Q  Q  R  A  T  Q  I  K  T  E  Q  L  S  P  S  H  Y  S  E
cagcagggctccccacagcatgtcacctacggctccttcaacctgcagcactacagcacc
 Q  Q  G  S  P  Q  H  V  T  Y  G  S  F  N  L  Q  H  Y  S  T
tcctcttacccctccatcacaagagcacagtatgactattcagaccaccaaagtggtgcc
 S  S  Y  P  S  I  T  R  A  Q  Y  D  Y  S  D  H  Q  S  G  A
aactcatactacagccatgcagctggtcagggctccagcctgtactccaccttcagctat
 N  S  Y  Y  S  H  A  A  G  Q  G  S  S  L  Y  S  T  F  S  Y
atgagccccagccagaggccgatgtacaccccgattgctgacagcaccggggtgccctct
 M  S  P  S  Q  R  P  M  Y  T  P  I  A  D  S  T  G  V  P  S
gtgccgcagacccacagtccgcagcactgggagcagcagcccatttacacacaactgtcc
 V  P  Q  T  H  S  P  Q  H  W  E  Q  Q  P  I  Y  T  Q  L  S
aggccctgagga
 R  P  -  -
```

Fig. 1. *Sox9 gene of Hypoatherina tsurugae with complete CDS*

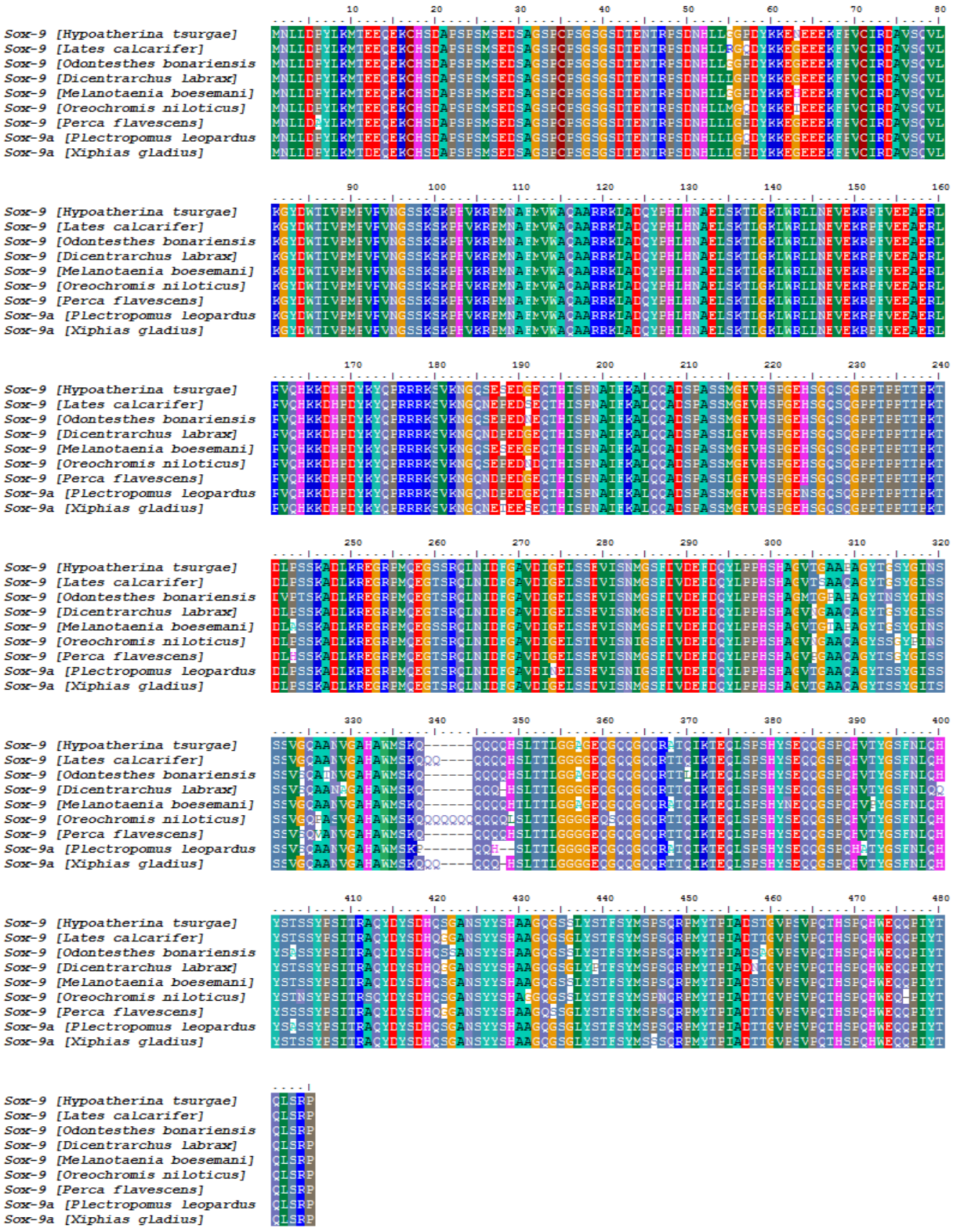

Fig. 2*. Deduced Amino acids sequence of H. tsurugae Sox9 gene aligned with other orthologs species*

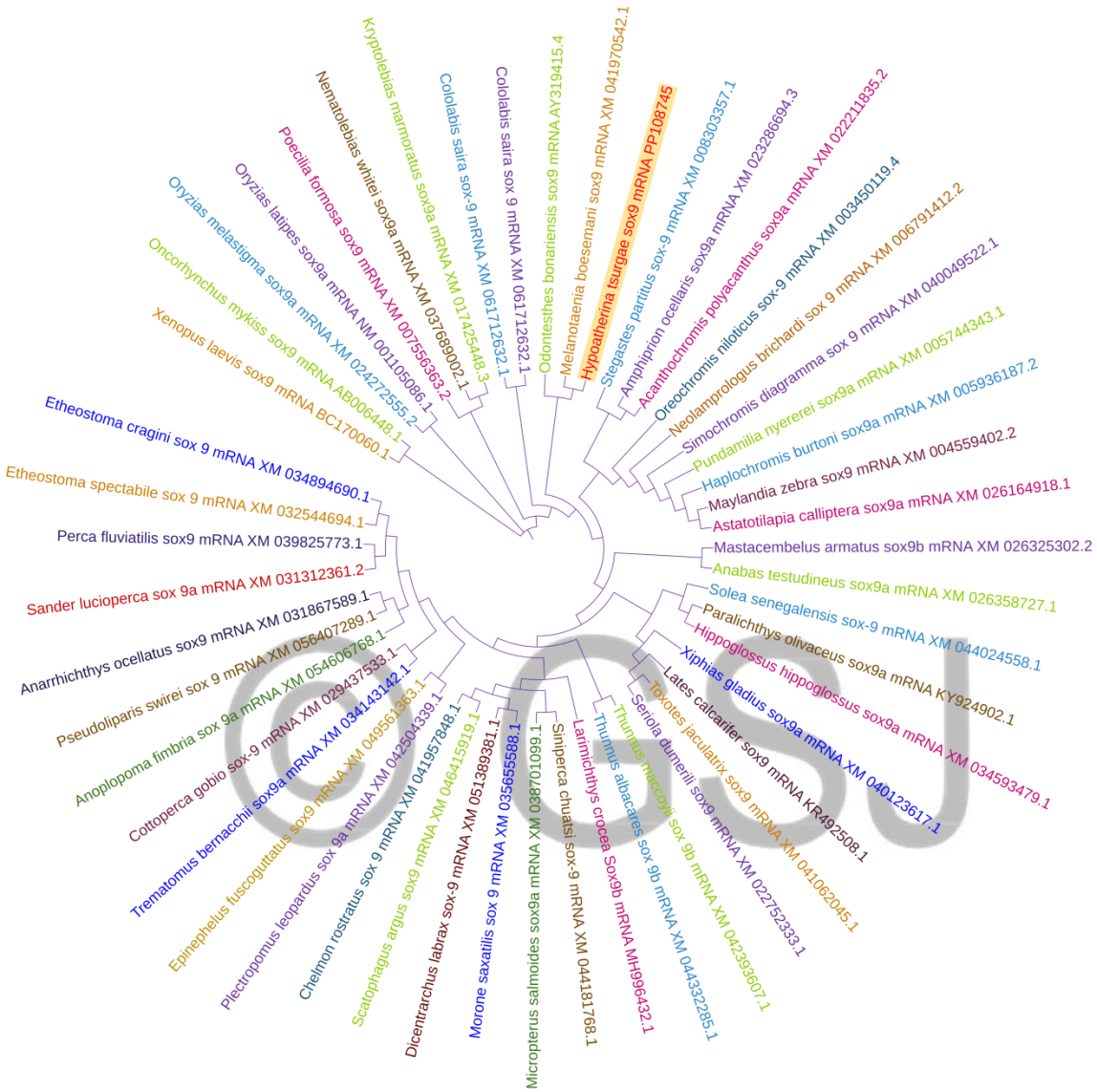

Fig. 3. *Phylogenetic analysis constructed with mRNA sequence of 50 different fish species along with H. tsurugae.*

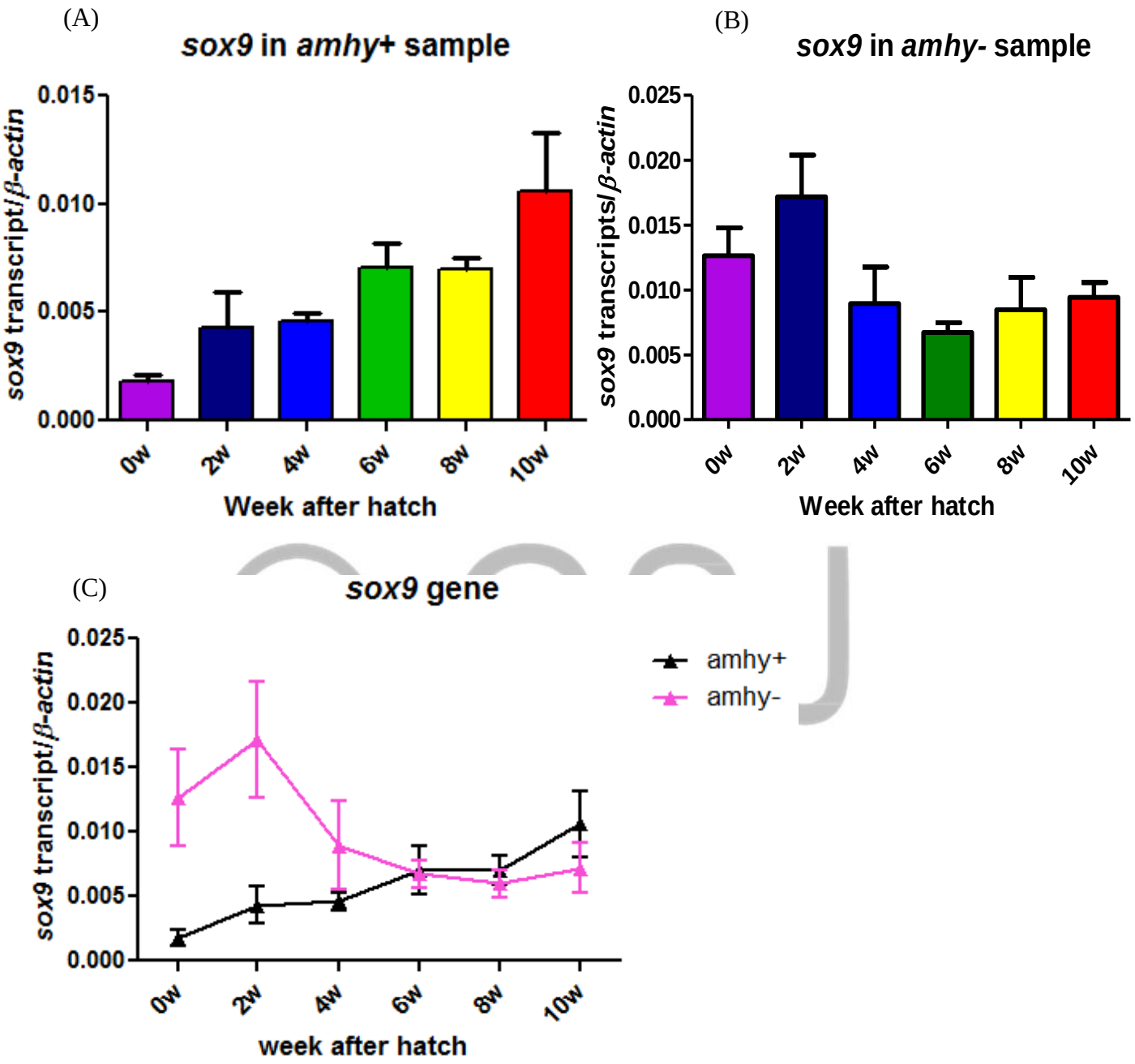

Fig. 4. *Quantitative mRNA expression of sox9 gene (a) in amhy+ individual (male) (b) amhy- individual (Female) (c) Both in amhy+ and amhy- plot in same graph. Values represent the mean ± SEM of 5-7 fish per time point. Symbols with the same letter indicate groups that are not significantly different between time points.*

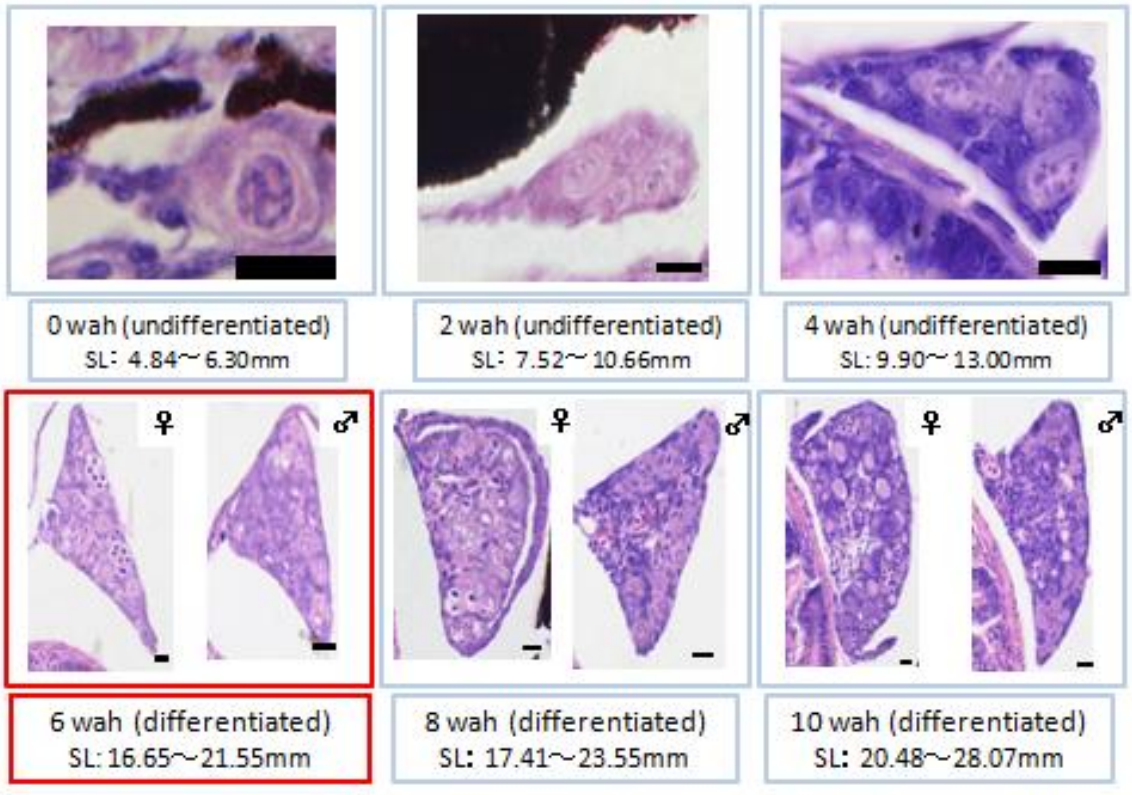

Fig. 5. *Histological differentiation of gonad amhy+ male and amhy- female at different week after hatch*

Table 1. List of Primers used in cloning and qRT-PCR

Sl.No	Name of Primers	Sequences
1	Sox1 F	5'-TTCGCATGAATCTCCTCGACC-3'
2	Sox last R	5'-TCCTCAGGGCCTGGACACAG -3'
3	*sox* RT 809 F	5'-GGTGAGCTGAGCA GCGAGGT-3'
4	*sox* RT 935 R	5'-TGCAGGTTGAAGGAGCCGTA-3'
5	*β-actin*Fw17	5'- GCCTGAAACCGGTTCCCTT-3'
6	*β-actin*Rv1838	5'-TTTTCGGAACACATGTGCACT-3'
7	*β-actin* RT F	5'-GTGCTGTCTTCCCCTCCATC-3'
8	*β-actin* RT R	5'-TCTTGCTCTGGGCTTCATCA-3'