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**RESEARCH ARTICLE**

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# Cloning, Characterization and Expression Pattern of the Ovarian Cytochrome P450 *Cyp19a1a* Gene in Gonadal Developmental Period of Cobaltcap Silverside *Hypoatherina tsurugae*

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**ABSTRACT**
The upregulation of *cyp19a1a* transcription factor required for granulosa cell differentiation and ovarian maintenance. 1630 bp of *cyp19a1* mRNA transcript of *Hypoatherina tsurugae* was cloned and sequenced. It consists of open reading frame (ORF) of 1551 bp that encodes a 517 aa protein, found to be identical to the sequence of other fish species. A phylogenetic tree was constructed by comparing the mRNA sequence of 41 different fishes across various taxa available in the NCBI database and using as outgroup as *Acipenser sinensis*. The tree shows a high homology of *cyp19a1a* from *H. tsurugae* with *cyp19a1a* of *Maelanotaenia boesemani*, the two forming a single clade. The qRT expression of *cyp19a1a* was studied in both *amhy*+ (male) and *amhy*- (female) individuals. In *amhy*- (female) individuals, the expression was begins from 0 wah and peak at 6 wah then sharply decreases whereas in *amhy*+ (male) individuals expression was very low and it is in base line. The histological sections of gonads were studied in different stages of biweekly collected larvae during the sex determination/differentiation period and it showed that differentiation of gonads male/female was decided at 6 wah. In this stage the primary oocytes are recognized. These finding add to the knowledge for a better understanding of molecular mechanisms of sex determination and differentiation period in fishes.

**Key words:** Atheriniformes, *cyp19a1a*, *Hypoatherina tsurugae*, Gonadal development

## Introduction

The marine atherinid fish *Hypoatherina tsurugae* commonly called Cobaltcap silverside (Jordan & Stark, 1904) belongs to the family Atherinidae and order Atheriniformes has an unique significance concerning its temperature-dependent sex determination (TSD) for that it serves as a good model to study in the issue of global warming and climate change. Our previous research reported that the *amhy* gene (Y chromosome-linked anti-Müllerian hormone) has a critical role in male sex determination of an old world Silverside, *Hypoatherina tsurugae* (Bej et al., 2017). The plasticity of the sex-determination mechanism observed in fish is very high. It is determined by a hierarchical gene network and is considered to be one of the most variable and complicated processes in evolution (Schartl et al., 2018). Till now, many other genes/transcription factors have also been reported as master sex-determining genes in various fishes (Hattori et al., 2013, Yano et al., 2012, Takehana et al., 2014, Matsuda et al., 2002, Myosho et al., 2012). From these references, it is clear that the genetic machinery of fishes that control gonadal development is very diverse and is not limited to a particular gene/transcription factor as most interestingly reported for the *sdY*, an immune-related gene that can crosstalk as a sex-determining gene in Salmonidae (Yano et al., 2012).

All teleost fish need estrogen for ovarian differentiation which is the expression of gonadal aromatase gene *cyp19a1a*, cytochrome P450, family 19, subfamily A, polypeptide 1a (Guiguen et al., 2010). The *cyp19a1a* aromatase is a key steroidogenic catalyzing the estrogen biosynthesis process and thus controls many physiological processes of females (Simpson et al., 2002). Most teleost possess two different *cyp19a1* genes - one predominantly expressed in the gonad ovary (*cyp19a1a*) that play a crucial role in sex differentiation and gonadal development and other highly

expressed in the brain (*cyp19a1b*) for neuroprotection and neurogenesis (Blázquez and Piferrer 2004, Guiguen et al., 2010). Administration of estrogen can cause sex reversal from male to female in marsupials, birds, reptiles, and teleosts (Conveney et al., 2001, Scheib, 1983, Piferrer, 2001, Kobayashi et al., 2003). Similarly, treatment with aromatase inhibitors results in production of phenotypic females to males (Hudson et al., 2005, Belaid et al., 2001). The ovarian differentiation pathway is triggered by the upregulation of the *cyp19a1a* gene and downregulated in testicular differentiation (Guiguen et al., 2010, Piferrer et al., 2012). Thus, the *cyp19a1a* gene has a pivotal role in ovarian differentiation in most of the species.

*Hypoatherina tsurugae* has very little information about its reproductive biology and sex differentiation. In this species besides the *amhy* gene (Bej et al., 2017), the expression of other genes has not been studied yet during the gonadal determination/differentiation period. So, this paper aims to study the potential candidate gene *cyp19a1a* in this particular species.

## Materials and Methods

Approximately 100 matured wild cobaltcap silversides were collected using a hand net and were then successfully reared in a 500-liter tank to acquire gametes and offsprings for experiments. The tanks were supplied with filtered natural seawater 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 dissuade into powdered marine fish food (AQUEON, Franklin, WI).

Genomic DNA was extracted from caudal fin tissue following a protocol described by Aljanabi and Martinez (1997). The genotyping of larvae to know their sex (male/female) was accomplished using primers Amh 613 F and Amh 35 R (Bej et al., 2017).

### *Cloning of cyp19a1a gene*

The total RNA was isolated from *amhy*+ individual's testis for cloning 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 Millipore, Darmstadt, German) in 20 µl reactions. The PCR was performed 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). 5' and 3' RACE was used to obtain a full-length cDNA sequence of *cyp19a1*. All primers are listed in Table 1.

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

| Sl. No | Name of Primers | Sequences Description |
|---|---|---|
| **1** | *aro1 F* | 5'-ATGGAACTGATCTCTGCTTGCGT-3' |
| **2** | *aro330F* | 5'-GACCCTCATACTCAGCGGTGCATC-3' |
| **3** | *aro710F* | 5'-TGGCAGACTGTACTGATCAAACCTG-3' |
| **4** | *aro1030 F* | 5'-CTGCTGCAGGAAATAGACACGGTTG-3' |
| **5** | *aro855R* | 5'-CAGCTTATCTGCCTGCTCCA-3' |
| **6** | *aro1280R* | 5'-TCCAGACTAAATTCATTGGCT-3' |
| **7** | *aro last R* | 5'-TTGTACAAACATTAGATCATAT -3' |
| **8** | *aro RT 100 F* | 5'-AAGTCTTGTAGAACAGAAGAGGAGAGA-3' |
| **9** | *aro RT 257 R* | 5'-AAGAAGAGGCTGATGGACAGAGT-3' |
| **10** | *β-actinFw17* | 5'-GCCTGAAACCGGTTCCCTT-3' |
| **11** | *β-actinRv1838* | 5'-TTTTCGGAACACATGTGCACT-3' |
| **12** | *β-actin RT F* | 5'-GTGCTGTCTTCCCCTCCATC-3' |
| **13** | *β-actin RT R* | 5'-TCTTGCTCTGGGCTTCATCA-3' |

### *Real-Time/Quantitative PCR (qRT-PCR)*

For expression studies, total RNA was isolated from *amhy*+ and *amhy*- individuals of every two stages post-hatching, measured in weeks (wah), namely 0wah, 2wah, 4wah, 6wah, 8wah, and 10wah. The expression level of mRNA transcripts was analyzed by qRT-PCR using specific RT primers designed for the *cyp19a1a* locus. The length of qRT-PCR amplicon was 180 bp. The final reaction mix consisted of 5 µL FastStar Universal SyBR green Master (Roche), 1.5 µL forforward/reverse primer mix (1 µM), 2.5 µL cDNA template and 1 µL of water (total reaction volume: 10 µL). The qRT-PCR was performed on a StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific), and the reaction progress was monitored by fluorescence detection.


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The thermal cycling program was: 95 ºC for 5 min, 40 cycles of 95 ºC for 30 sec, 60 ºC for 15 sec and 72 ºC for 30 sec.

The β-*actin* gene was taken as an endogenous control due to its stability during the sex determination/differentiation period, using the primers (Chapman et al., 2015). In relative qRT-expression studies of the genotype of *amhy*- and *amhy*+ individuals, the *amhy*- individuals (females) were taken for reference control.

***Sequence analysis***

The multiple alignment software Clustal W was used for the alignment of nucleotide sequences and their deduced amino acid sequences. The phylogenetic tree was constructed using MEGA11 with Maximum Likelihood, the initial tree inferred with Neighbour-Joining method, and the BioNJ Algorithm and Tamura-Nei model**.** The model was determined also using MEGA11. The Confidence in the tree topology was assessed with bootstrap 1,000 replicates.

***Statistical analysis***

In qRT-PCR expression studies, per each time point three to five samples were taken. The differences in gene expression between groups were analyzed by ANOVA followed by a Tukey test using GraphPad prism (v.6.0; GraphPad software, San Diego, CA). Differences in gene expression were considered as statistically significant at $p < 0.05$.

***Histological analysis of gonadal sex differentiation***

First, trunk samples were dehydrated through an ascending ethanol series (70%, 90%, 99%, and 100%), then cleared in xylene and embedded in Paraplast Plus (McCormick Scientific, St. Louis, MO), sectioned serially with a thickness of 5 µm, and stained with hematoxylin and eosin. Various stages of gonadal sex differentiation were determined by light microscopy using histological criteria for another atheriniform, the pejerrey *O. bonariensis* (Ito et al., 2005).

***Data Accessibility for cyp19a1 gene***

DNA sequences: GenBank accessions; *Hypoatherina tsurugae cyp19a1* [PP129528], *Acanthopagrus schlegelii cyp19a1* [AY273211.1], *Acipenser schrenckii cyp19a1* [KC417317.1], *Amphiprion ocellaris cyp19a1* [AB918721.1], *Anoplopoma fimbria cyp19a1* [XM_054616276.1], *Archocentrus centrarchus cyp19a1* [XM_030718411.1], *Centropristis striata cyp19a1* [XM_059350864.1], *Chelmon rostratus cyp19a1* [XM_041940280.1], *Chrysophrys major cyp19a1* [AB051290.1], *Cololabis saira cyp19a1* [XM_061734829.1], *Cottoperca gobio cyp19a1* [XM_029455858.1], *Cromileptes altivelis cyp19a1* [AY684255.1], *Dicentrarchus labrax cyp19a1* [XM_051380240.1], *Epinephelus coioides cyp19a1* [AY510711.1], *Etheostoma cragini cyp19a1* [XM_034865636.1], *Fundulus heteroclitus cyp19a1* [AY428665.1], *Girardinichthys multiradiatus cyp19a1* [XM_047363720.1], *Larimichthys crocea cyp19a1* [NM_001303347.1], *Lateolabrax maculatus cyp19a1* [KP335158.1], *Lates calcarifer cyp19a1* [AY684256.1], *Melanotaenia boesemani cyp19a1* [XM_041982561.1], *Micropogonias undulatus cyp19a1* [DQ184486.1], *Micropterus salmoides cyp19a1* [XM_038702087.1], *Monopterus albus cyp19a1* [EU252487.1], *Morone saxatilis cyp19a1* [XM_035682371.1], *Mugil cephalus cyp19a1* [AY859425.1], *Nibea mitsukurii cyp19a1* [LC317122.1], *Nothobranchius furzeri cyp19a1* [XM_015941214.2], *Odontesthes bonariensis cyp19a1* [EF030342.1], *Odontesthes hatcheri cyp19a1* [EF051123.1], *Oryzias luzonensis cyp19a1* [LC121908.1], *Pennahia argentata cyp19a1* [LC317123.1], *Perca fluviatilis cyp19a1* [XM_039795266.1], *Plectropomus leopardus cyp19a1* [XM_042492203.1], *Poeciliopsis prolifica cyp19a1* [XM_055045253.1], *Sander lucioperca cyp19a1* [XM_031306306.2], *Sebastes schlegelii cyp19a1* [FJ594995.2], *Siniperca chuatsi cyp19a1* [XM_044194289.1], *Tilapia mossambica cyp19a1* [AF135851.1], *Toxotes jaculatrix cyp19a1* [ XM_041067101.1], *Xiphophorus maculatus cyp19a1* [XM_005799744.2].

## Results

***Sequence analysis of cyp19a1a***

The isolated *cyp19a1a* cDNA was 1630 bp with an open reading frame (ORF) of 1551 bp, encoding a 517 aa protein (GenBank Accession number – PP129528). It shows identical at nucleotide level to the *cyp19a1a* gene of *Melanotaenia boesemani* (93.99%), *Odontesthes bonariensis* (87.62%), *Epinephelus coioides* (87.04%), *Dicentrarchus labrax* (86.71%), *Lates calcarifer* (86.62%), *Plectropomus leopardus* (86.01%), *Oryzias latipes* (84.59%) and *Oreochromis aureus* (83.85%) (Fig. 1).

By using the Clustal W software, the 517 amino acid sequence of *H. tsurugae* was aligned with nine other fish species. The results showed that the homology was high: *Melanotaenia boesemani* (94.20%), *Odontesthes hatcheri* (88.35%), *Plectropomus leopardus* (88.01%), *Siniperca chuatsi* (87.62%), *Odontesthes bonariensis* (86.85%), *Lates calcarifer* (86.85%), *Perca fluvialitis* (86.24%), *Oryzias latipes* (85.30%), and *Dicentrarchus labrax* (85.11%) (Fig. 2).


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 R  F  I  W  T  G  I  G  T  A  S  N  Y  Y  N  T  K  Y  G  D
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 I  V  R  V  W  I  N  R  E  E  T  L  I  L  S  G  A  S  A  V
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 H  H  V  L  K  N  G  N  Y  T  S  R  F  G  S  K  Q  G  L  S
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 C  I  G  M  N  E  K  G  I  I  F  N  N  N  V  A  L  W  K  K
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 P  K  G  T  N  I  I  L  N  T  G  R  M  H  R  T  E  F  F  H
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 E  H  L  S  M  T  F  L  P  R  Q  R  G  R  W  Q  T  -
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**Figure 1.** *Aromatase cyp19a1 gene of Hypoatherina tsurugae complete CDS.*


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**Figure 2.** *Deduced amino acids sequence of H. tsurugae cyp19a1 gene aligned with other ortholog sequences.*

The phylogenetic trees were constructed separately by comparing the mRNA sequences of 41 different fishes across various taxa available in the NCBI database and taking outgroup as *Acipenser sinensis* which is belongs to order Acipenseriformes and family Acipenseridae. The phylogenetic tree of mRNA sequence of *cyp19a1* was presented (Fig. 3). The trees shows a high homology of *H. tsurugae cyp19a1* with *Maelanotaenia boesemani cyp19a1*, the two being sister taxa as they belong to the same order Atheriniformes.

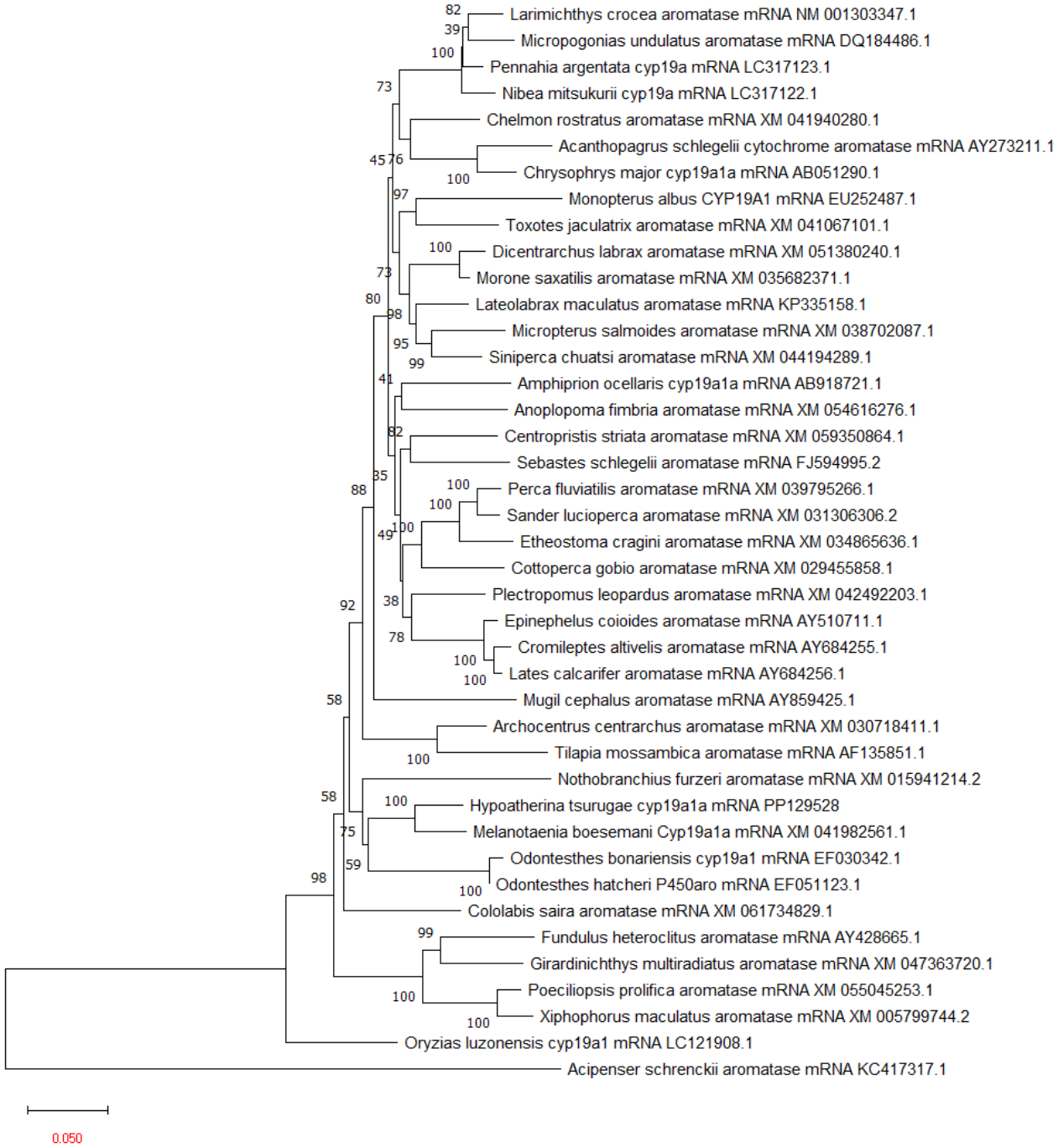


**Figure 3.** *Phylogenetic analysis constructed with the cyp19a1 mRNA sequence of 41 different fish species along with H. tsurugae.*

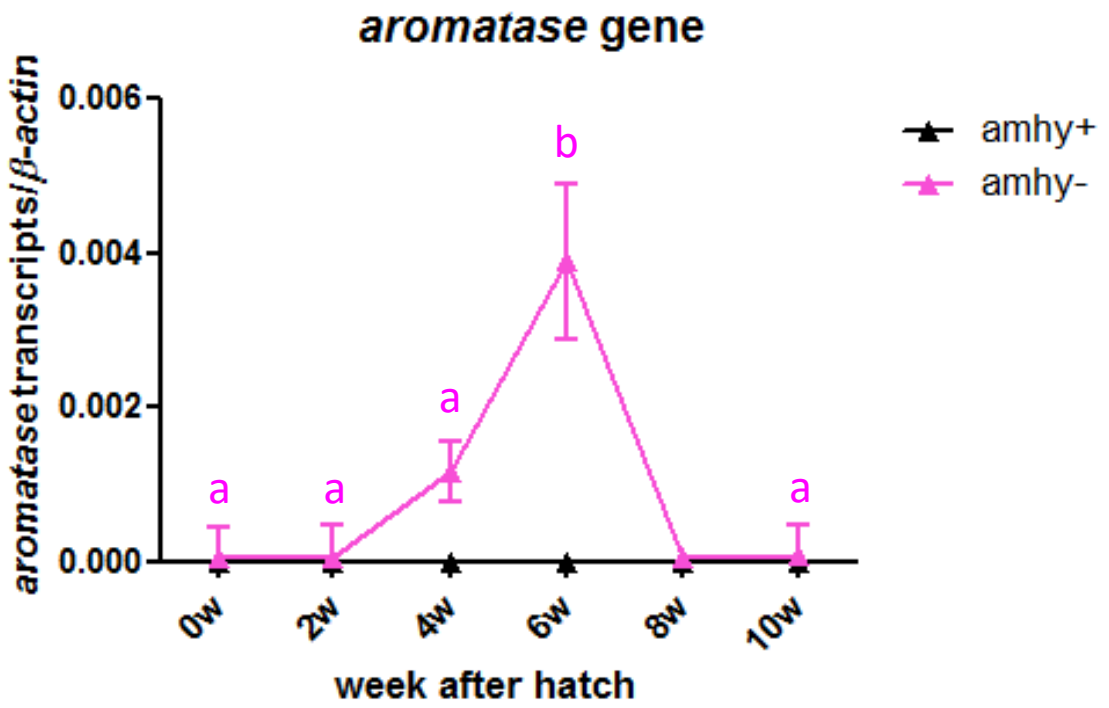


**Figure 4.** *Quantitative mRNA expression of aromatase transcript of amhy- individual (Female) and in amhy+ individual (male) during sex differentiation. Values represent the mean ± SEM of 3-5 fish per time point.*

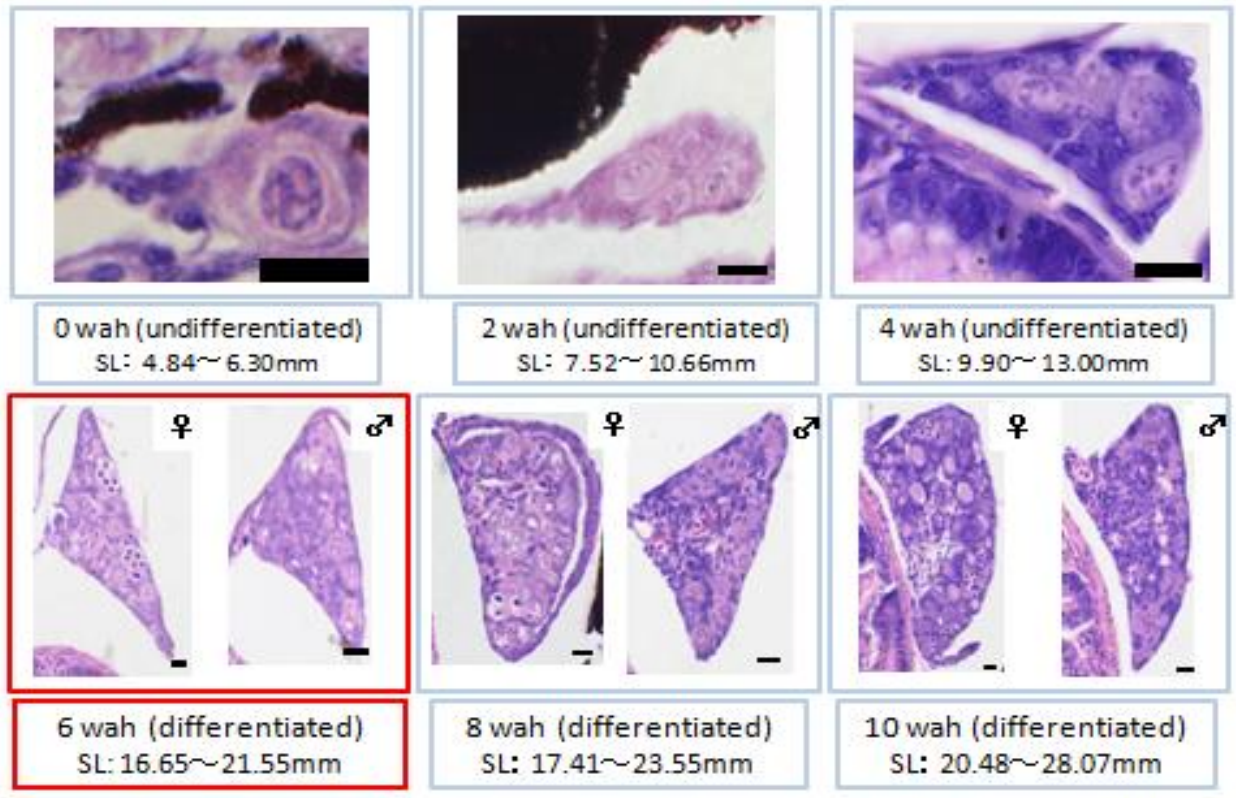


**Figure 5.** *Histological differentiation of gonad amhy+ (male) and amhy- (female) analyzed every two weeks after hatch.*

***Gene expression analysis***

The result of qRT-PCR of the *cyp19a1* aromatase gene illustrated that in *amhy-* (female) individuals, the expression begins from 0wah and peaks at 6wah then sharply decreases whereas in *amhy+* (male) individuals the expression is very low and it is in baseline (Fig. 4).

The histological sections of gonads in different larval stages showed that differentiation of gonads male/female is decided at 6wah. In this stage the primary oocytes are recognized (Fig. 5) which is also correlated with expression of *cyp19a1* aromatase gene.

## Discussion

In the present study, the *H. tsurugae cyp19a1a* gene has been successfully cloned and sequenced. The *cyp19a1a* mRNA is 1630 bp encoding a 517 aa protein. The *cyp19a1a* gene exhibits very close similarity with the *cyp19a1* gene of *Melanotaenia boesemani*, *Odontesthes bonariensis*, *Epinephelus coioides*, *Dicentrarchus labrax*, *Lates calcarifer*, *Plectropomus leopardus*, *Oryzias latipes*, and *Oreochromis aureus*.

As mentioned above, most teleosts possess two distinct *cyp19a1* aromatase genes with distinct tissue distribution. Both are the orthologs of *cyp19a1* of tetrapods and reveal different paralogous clad in the fish lineage (Blázquez and Piferrer 2004). In this paper, emphasis is given to the *cyp19a1a* gene isolated from gonads only. The phylogenetic analysis revealed that our sequence forms a clade with another Atheriniformes, *Maelanotaenia boesemani*.

In this study, the focus is given to the expression pattern of the *cyp19a1a* gene in the gonads of *H. tsurugae*. From the qRT-PCR result, the expression of the *cyp19a1a* gene is correlated with the expression of the *amhy* gene significantly reached a peak at 6 wah, then decreased (Bej et al., 2017). The expression of *amhy* was detected before the appearance of the first signs of histological differentiation in presumptive Sertoli cells surrounding germ cells in the undifferentiated gonads. This increasing pattern of *amhy* gene expression is needed for the male developmental pathway for testis differentiation but in females, the expression of the *cyp19a1* gene begins from 0wah and become peak at 6wah then declines which is needed for the formation and differentiation of the ovary. However, the expression of *foxl2* revealed that it was highly expressed before the expression *cyp19a1* (our unpublished data). This study indicates a race between *amhy* gene and *foxl2* in the developmental period of the fish gonad. If the *foxl2* gene wins the race, it will lead to female gonad differentiation, and/or if the *amhy+* gene is highly expressed in the initial period of development then, it will shift towards the formation of male gonad. It has been reported that the *foxl2* gene up-regulates the *cyp19a1* gene and acts as a repressor of the male pathway during female gonad development (Pannetier et al., 2006; Wang et al., 2007). If the *foxl2* gene is disrupted, there is a drastic reduction in *cypa19a1* gene expression in Goat (Pailhoux et al., 2002). Transcription factors *foxl2* and *foxl3* promoted ovarian differentiation by directly up-regulating the expression of aromatase gene *cyp19a1*and *cyp11b* in orange-spotted grouper fish *Epinephelus coioides* (Zhang et al., 2022). The *cyp19a1* gene is the direct target of *foxl2* and it binds to the putative forkhead element of the aromatase promoter and regulates its function (Flemming et al., 2010). The mutation in the *foxl2* or *cyp19a1* gene results in female XX to male sex reversal in Nile Tilapia (Zhang et al., 2017).

It is observed that if organism is develop to female, the *cyp19a1* gene is highly expressed during ovarian differentiation in teleost fish like rainbow trout (Guiguen et al., 1999, Vizziano et al., 2007), European sea bass (Blázquez et al., 2008), Japanese medaka (Nakamoto et al., 2006) and Southern flounder *paralichthyes lethostigma* (Luckenbach et al., 2005). It is consistent with the expression of the *cyp19a1* gene in *H. tsurugae* species. The correlation between *foxl2* expression and *cyp19a1* revealed that *foxl2* is expressed during the undifferentiated period that induces the aromatase to shift the formation of the female gonad pathway.

## Conclusion

From the above study, it may be concluded that expression of *cyp19a1a* is highly essential for ovarian differentiation. In this species, it is highly expressed at 6 wah during the gonadal differentiation period in females. Such dimorphic expression of *cyp19a1a* aromatase leads to an estrogen synthesis pathway for the development and maintenance of the ovary. However, more functional experiments are necessary to understand the mechanisms of downstream pathways of gene regulation during the gonadal differentiation period of this species.

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