Studies on the molecular mechanism of sex determination in the cobaltcap silverside Hypoatherina tsurugae

Doctoral Dissertation

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Hypoatherina tsurugae

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Graduate School of Marine Science and Technology

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Declaration

I hereby declare that this thesis has been composed by myself and is a result of my own investigations. It has neither been accepted, not submitted for any other degrees. All sources of information have been duly acknowledged.

Dilip Kumar Bej

Index

General Abstract

The sex-determining gene *amhy* (Y chromosome-linked Anti-Müllerian hormone) has been confirmed in Atherinopsid species of the genus *Odontesthes* (*O. hatcheri* and *O. bonariensis*) which occur naturally in inland waters of Argentina, Brazil and Uruguay. The presence of *amhy* in other families of Atheriniforms besides Atherinopsidae is unknown. In order to understand the distribution of *amhy* gene in this order, I selected the cobaltcap silverside *Hypoatherina tsurugae*, which inhabits the coastal waters of Japan and the Korean peninsula, as a model. The cobaltcap silverside belongs to the family Atherinidae, which is phylogenetically and geographically the most distant family from the Atherinopsidae where *amhy* has been discovered. In addition to the insight on the evolution of *amhy*, this study aims to understand the genetic mechanism of sex determination in *H. tsurugae*, in particular to clarify the role(s) of *amhy* in gonadal sex determination/differentiation and its relation to other sex related key genes (*sox9*, *cyp19a1a*, etc) in these processes.

In the 1st chapter, I identified the *amhy* gene from *H. tsurugae*. The complete gene structure of *amhy* and its somatic homologue *amha* (autosomal amh, *amha*) were obtained and compared. The *amha* gene is composed of 2,015 nucleotide bases and seven exons. The TGF-β (Transforming growth factor-beta) domain is present in Exon 7 as in other species. The *amhy* gene is composed of 1,838 nucleotide bases and has only 4 exons. Exons 2 and 3 are completely lacking in the *amhy* gene structure. A specific insertion of 195 nucleotide bases is present at the between exons 1 and 4. The exon 5 sequence is found in genomic sequence but it is not translated. The nucleotide identity between exons of *amha* and *amhy* was 87%. The deduced amino acid sequence of Amha (511 aa) and Amhy (340 aa) shared 91% identity. The exon 7 which contains the TGF-β domain shared 93% amino acid identity with *amha* and it contains 7

canonical cysteine residues that form disulfide bonds to make cysteine knots during dimer formation. The linkage between *amhy*+ genotype (individual possess *amhy* gene) and sex phenotype was analyzed in wild samples as well as in laboratory reared fish that were raised from hatching at the average temperature of the spawning season of *H. tsurugae*. The PCR analysis yielded that *amhy* gene linkage with male sex in 95% and 85% of the wild adults and the laboratory-reared progeny, respectively. The temporal expression of *amha* and *amhy* gene was studied by qRT-PCR. In *amhy*+/+ or *amhy*+/-fish, the *amhy* gene was highly expressed during early sex differentiation period while the *amha* gene expression maintained low until the early juvenile stage. The spatial expression of *amha* and *amhy* was studied by *in situ* hybridization (ISH). This analysis showed that at 4 week after hatch (wah) (undifferentiated period), *amhy* was expressed in somatic cells surrounding germ cell. In contrast, signals of *amha* could not be detected at this time. As the *amhy* gene in *H. tsurugae* is tightly linked to the phenotypic sex and was expressed in the gonads of *amhy*+ individuals during the during the sex determination/differentiation period, *amhy* can be considered as a strong candidate for sex determining gene in this species.

In the 2nd chapter, to gain better understanding of the sex determination/differentiation mechanisms in *H. tsurugae*, I studied the gene expression profile of sex-related genes that could be adjuvants to *amhy* gene in sex determination/differentiation. The expression of six key sex differentiation genes (*sox9, dmrt1, gsdf, foxl2, cyp19a1a* and *scp3*) in *amhy*+ and *amhy*- individuals of *H. tsurugae* were studied during the early stages of gonadal development. Most of the genes show a dimorphic expression related to sex genotype (*amhy*+/*amhy*-) with exception of *sox9*. The reason for the lack of sex dimorphism in *sox9* expression during this period may be

that this gene is necessary for proliferation of germ cells in both sexes, as shown for other species.

In conclusion, I successfully isolated the *amhy* gene in *H. tsurugae.* This gene is tightly linked with the male phenotype and highly expressed during early gonadal sex determination /differentiation. The sex related key genes *dmrt1, foxl2, cyp19a1a* and *scp3* showed dimorphic expression and an apparent synchronization with *amhy* gene expression. Future studies should look in more detail about their relations with as well as their regulation by *amhy* in order to corroborate the status of sex determining gene for *amhy* in *H. tsurugae*.

General Introduction

Sex determination and differentiation in fish are highly diverse and plastic developmental processes. The term of "sex determination" can be used to describe the genetic or environmental cues that ultimately determine the fate of the gonad of an individual. For example, in most of mammals, the sex chromosome (Y-chromosome) determines the sex of male individual (Koopman et al., 1991). But in some reptiles, temperatures regulate the fate of gonad during embryonic development, rather than its genetic constituents (Devlin and Nagahama 2002). On the other hand, it is obvious that in teleost fish, the molecular mechanisms of sexual development are evolutionarily flexible (Heule et al., 2014) and require complex regulatory pathways that are governed by the balance of genetic and environmental cues (Baroiller and Cotta 2001)

Determination of sex governed by the inheritance of sex determining genes located on specific sex chromosomes found in the some fish (Budd et al., 2015). The sex determining genes may be considered as either upstream "master" switches, or downstream differentiators, depending on their relative roles in sex determination and/or differentiation. Sex determining gene first identified in fish was *Dmy* (DM-domain gene on the Y chromosome) in medaka *Oryzias latipes* (Matsuda et al., 2002). This gene is a transcription factor expressed in the somatic cells surrounding germ cells and is involved in germ cell proliferation and development of pre-sertoli cells into sertoli cells (Matsuda et al., 2002). After the discovery of *Dmy*, several master sex determining genes have been identified in fish including *amhy* (Y-linked anti-müllerian hormone) in pejerrey *Odontesthes hatcheri* and in Tilapia (*Oreochromis niloticus*), *sdY* (sexually dimorphic on the Y chromosome) in rainbow trout *Oncorhynchus mykiss*, $Gsdf^{y}$ (gonadal soma derived growth factor) and *sox3* in medaka *O. luzonensis* and *O. dancena*, respectively, and *amhr2* (anti-müllerian hormone receptor type 2) in pufferfish *Takifugu rubripes*

(Hattori et al., 2012, Yano et al., 2012, Myosho et al., 2012, Kamiya et al., 2012, Takehana et al., 2014). Thus, not like in mammals, sex determining genes in teleost are highly diverse and therefore it is an interesting topic to understand the molecular basis of sex determination mechanism and the distribution and evolution of sex determining genes in teleost.

Although sex is determined initially by genotypic or environmental cues, the variety of gonadal genes are expressed downstream of the cues and have critical roles for the ovaries and testes formation in vertebrates. For instance, male sex differentiation can be achieved through up-regulation of a highly conserved transcription factor, doublesex and mab-3 related transcription factor 1 (*dmrt1*), which acts in combination with transcription factor *sox9* (*sox9*) to promote testis formation (Smith et al., 2009). Alternatively, female sex differentiation is stimulated by cytochrome P450 aromatase (*cyp19a*) through a positive feedback loop involving the female-associated transcription factor known as forkhead box protein L2 (*foxl2*) (Guigen et al., 2010). *Cyp19a* encodes for gonadal aromatase, which catalyzes the conversion of androgens into estrogens and is seem to play a pivotal role in sex differentiation and sex change in fish (Guigen et al., 2010. Other genes, such as r-spondin 1 **(***rspo*) and wnt-signalling protein (*wnt*) in the ovary, and *amh* and *gsdf* in the testis are also thought to play roles in β-catenin and TGF-β signaling pathways, respectively, to promote sexual differentiation and subsequent gonadal development (Budd et al., 2015). These sex differentiation-related genes have been characterized in mammals and some model fish species, but the expression profiles of these genes in relation to sex determining gene during sex determination/differentiation period are still largely unknown in marine fishes.

The Atheriniformes, an order of ray-finned fishes, comprises major six families including Atherinopsidae, Notocheiridae, Melanotaeniidae, Atherionidae, Phallostethidae and Atherinidae

(Dyer 2006). As described in a previous paragraph, master sex determining gene *amhy* was found in two atherinopsids, *Odontesthes hatcheri* (Hattori et al., 2012) and *O. bonariensis* (Yamamoto et al., 2014). To date, all studies on sex determination of Atheriniforms have dealt with atherinopsid species (genera *Odontesthes*, *Menidia*, and *Chirostoma*) (Strüssmann and Patiño 1999; Corona-Herrera et al., 2016) and therefore little is known on the sex determination mechanism in general and on the distribution and evolution of *amhy* in particular in other Atheriniform families. In this study, I selected cobaltcap silverside *Hypoatherina tsurugae*, belongs to a family of Atherinidae, as a model. Phylogenetically, *Odontesthes* and *Hypoatherina* are placed in Atherinopsidae and Atherinidae, respectively, the farthest related families in the Atheriniformes order (Bloom et al., 2012). In order to understand the mechanism of sex determination and distribution of sex determining genes among the different families of Atheriniforms, first I searched *amhy* gene in *H.tsurugae*. Then, I identified sex differentiation-related genes in *H.tsurugae* and examined its expression profiles during early sexual developmental period.

Chapter 1

Identification of two *amh* homologues and its expression profiles during gonadal sex differentiation of cobaltcap silverside *Hypoatherina tsurugae*, an atherinid fish from the Northwest Pacific Ocean

Introduction

A growing number of sex-determining genes are being identified in teleosts (Kikuchi et al., 2013; Hattori et al., 2013; Takehana et al., 2014; and other references below). It is becoming evident that sex-determining genes in fishes are not restricted to transcription factors, as demonstrated by examples with members of the TGF-beta superfamily and even an immune-related gene. Also, the degree of conservation of these genes varies according to the taxonomic group. For instance, in the genus *Oryzias*, there seems to be a rewiring of sex-determining genes as shown by *dmy/dmrt1bY* (Matsuda et al., 2002), *gsdfY* (Myosho et al., 2012), and *sox3Y* (Takehana et al., 2014) genes. On the other hand, the *sdY* gene (Yano et al., 2012) shows high conservation across Salmonid species (Yano et al., 2013).

Recently, a homologue of the Y chromosome-linked duplication of the anti-Müllerian hormone gene (hence *amhy*), firstdiscovered in Patagonian pejerrey (*Odontesthes hatcheri*; Atherinopsidae; Hattori et al., 2012), was found to be present also in the sister species *O. bonariensis* (Yamamoto et al., 2014). Like in *O. hatcheri*, this gene was shown to play an important role in testis determination of *O. bonariensis* at an intermediate temperature (Yamamoto et al., 2014). To date, all studies on sex determination of Atheriniforms have dealt with atherinopsid species (genera *Odontesthes*, *Menidia*, and *Chirostoma*) (Strüssmann and Patiño 1999; Corona-Herrera et al., 2016) and therefore little is known on the sex determination mechanism in general and on the distribution and evolution of *amhy* in particular in other Atheriniform families.

The phylogenetic relationships of Atheriniformes are still controversial but it is considered to include between six and nine families (Sparks and Smith 2004; Nelson 2006; Froese et al.,

2012). The Atherinopsidae (also known as New World silversides) and the Atherinidae (Old World silversides), which previously were united as one family (Atherinidae), include numerous species inhabiting near-shore marine, estuarine, and freshwater environments. These species represent important forage fishes that form large schools (Bloom et al., 2012). The cobaltcap silverside *Hypoatherina tsurugae* is a near-shore marine atherinid from the Indo-Pacific region. In this study, the presence of the *amhy* gene and its role in testis determination were examined in a population of *H. tsurugae* from the Northwest Pacific Ocean.

Materials and Methods

Collection of wild specimens

Sexually-mature adult cobaltcap silversides were collected by hand net on July 2014 in Tokyo Bay (Chiba, Japan). The gonadal sex of 81 individuals was assessed by careful stripping of gametes and eight fish of each sex were randomly selected for cloning of *amh* genes (see details below). The remaining fish (48 females and 17 males) were stocked in 500-liter circular tanks at the Tateyama Station, Field Science Center of Tokyo University of Marine Science and Technology (Chiba, Japan) and used as broodstock fish to obtain gametes and offspring for further experiments (see below).

Cloning of autosomal *amh* (*amha*) and Y chromosome-linked *amh* (*amhy*)

Genomic DNA was extracted from the caudal fin tissue of a mature male following the protocol described by Aljanabi and Martinez (1997) and subjected to PCR amplification using

degenerate primers designed based on *Odontesthes hatcheri amha*. To determine the complete open reading frame for cobaltcap silverside *amha,* total RNA was isolated from adult testis using TRIzol (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instruction and 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. RT-PCR, Genome Walking, and 5'- and 3'-RACE PCRs were then performed using Smart RACE cDNA amplification kit (Takara bio, Shiga, Japan) according to the manufacturer's protocol.

Based on the *amha* full sequence, several primer sets flanking intronic sequences were designed in coding regions and used to amplify a Y-chromosome-linked *amh*, hence *amhy*, in this species. This strategy was based on the differences between *amhy* and *amha* genes in *O. hatcheri* and *O. bonariensis*, whereby an insertion of approximately five hundred bases specific to *amhy* is found in the third intron. Genomic DNA was isolated from the caudal fin of 16 adult fish, eight females and eight males, following the protocol described above and subjected to PCR amplification. One set of the primers designed in the first and fifth exons (Table S1; Amh 613 F and Amh 35 R) amplified two fragments. The larger fragment was present in both sexes whereas the smaller one, a putative *amhy*, was present only in males. The smaller fragment was purified, cloned, and sequenced as described above. To obtain the full genomic and cDNA sequences of the putative *amhy*, genome walking and RACE PCR were conducted according to the same protocols as *amha* cloning.

The specific amplicons from each PCR reaction were purified, cloned, and sequenced in an ABI PRISM 3100 capillary sequencer (Thermo Fisher Scientific) using BigDye Terminator method. Sequences were then analyzed by GENETYX version 11.0 (GENETYX, Tokyo, Japan)

software. The PCR conditions and specific primers used in each reaction are listed in Tables S1 and S2.

Phylogenetic analysis of *amh* sequences

The predicted amino acid sequences of *Hypoatherina tsurugae* Amha and Amhy (GenBank accession numbers KU664386 and KU664387, respectively) were compared with Amh sequences of other species available in GenBank using the software GENETYX version 11.0. Multiple alignments were performed using Clustal W in the MEGA software version 5.2 (Tamura *et al*. 2011). The sequences for *Odontesthes bonariensis* Amha (AHG98063.1) and Amhy (AAV31752.2), *Odontesthes hatcheri* Amha (AEE60845.1) and Amhy (ABF47515.2), *Dicentrarchus labrax* Amh (CAJ78431.1), *Oreochromis niloticus* Amh (ABS58513.1), and *Danio rerio* Amh (NP001007780.1) were used in the comparison and *Xenopus laevis* Amh (BAO04196.1) was used as outgroup. Phylogenetic trees were generated by MEGA software version 5.2 using the Neighbour-Joining tree method (Saitou and Nei 1987) and 10,000 bootstrap replicates to determine confidence.

Sex linkage analysis by *amhy* amplification in wild specimens

All wild-caught fish were screened for the presence of *amhy* by PCR analysis using the same primers (Table S1; Amh 613 F and Amh 35 R**)** and conditions described previously. Animals carrying the *amhy* gene (*amhy* positives) were represented by *amhy*+ when the exact genotype could not be determined and by *amhy*+/+ or *amhy*+/− (homozygous or heterozygous,

respectively) when the exact genotype was determined by progeny testing. The representation *amhy*− was used for the *amhy* negative fishes.

Testing of Mendelian inheritance and determination of parental genotype

For testing the Mendelian inheritance of *amhy* and determining the exact parental genotype, four single-pair crosses between four *amhy*− females and four *amhy*+ males were performed. Fertilized eggs were obtained by artificial insemination and incubated as described below. Randomly-chosen eyed-egg stage embryos (n= 38-45) from each cross were analyzed by *amhy* amplification following procedures described above.

Rearing of larvae for gene expression analysis and gonadal histology

Fertilized eggs were obtained from natural spawning of the captive-reared wild broodstock mentioned above. Approximately 500 hatchlings (10 to 13 days post fertilization) were stocked in two 30-liter tanks kept at 22ºC, the average temperature during the spawning season of *H. tsurugae* in Tateyama Bay, and reared for up to 12 weeks. 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 weaned into powdered marine fish food (AQUEON, Franklin, WI) from the fifth week of the experiment.

Fish were sampled biweekly from 0 to 10 weeks after hatching (wah) for gene expression analysis and gonadal histology. The remaining larvae were sampled at the end of the rearing experiment to determine the sex ratio. The trunks of the fish were stored in RNA later (Thermo

Fisher Scientific) (n=8) or in Bouin's solution (n=8) for gene expression analysis and gonadal histology, respectively, at each time point. Samples in RNA later were stored at −80°C until use. Bouin-fixed samples were rinsed three times with phosphate-buffered saline (PBS), transferred into 70% ethanol, and stored at 4°C until use. All larvae were fin-clipped for *amhy* genotyping as described above.

Histological analysis of gonadal sex differentiation and sex ratio

Trunk samples were dehydrated through an ascending series of ethanol (70%, 90%, 99%, and 100%), cleared in xylene, embedded in paraffin wax, sectioned serially (thickness, 5 µm), and stained with hematoxylin and eosin. Stages of gonadal sex differentiation were determined by light microscopy using histological criteria for another Atheriniform, the pejerrey *O. bonariensis* (Ito *et al*. 2005; Strüssmann and Ito 2005).

Expression analysis by qRT-PCR and *in situ* hybridization

Total RNA extraction and cDNA synthesis were performed following previous studies (Yamamoto *et al.* 2014). The expression of mRNA transcripts was analyzed by qRT-PCR using specific primers designed for *amha* and *amhy loci*. The *β-actin* gene was taken as an endogenous control because of its stability during sex determination/differentiation period (Figure S1). All primer sets and their respective conditions are listed in Tables S1 and S2.

The *in situ* hybridization (ISH) analysis used adult gonads and trunks of larvae collected before (4 wah) and after (8 wah) the onset of histological differentiation of the gonads. Samples

were fixed and processed as per the protocol mentioned above. We were not able to develop an *amhy*-specific probe so hybridizations were conducted using a 775 bp *amhy* probe (nucleotides +207 to +982; exons VI to VII; 93.5% identity with the respective sequence for *amha*) that recognized both *loci* and a 523 bp *amha*-specific probe designed in the *amha*-specific region (nucleotides -22 to +501; exons I to III; 17.2% of identity with the *amhy*). This *amha*-specific probe did not produce any signals in larvae but its binding ability to *amha* mRNA was confirmed using ovaries from adult *amhy*- specimens (data not shown). Thus it was possible to ascertain that the positive signals in *amhy*+ larvae were specific to *amhy*. NBT/BCIP was used for signal detection according to the recommendation of the manufacturer (Roche Diagnostics, Basel, Schweiz).

Statistical analysis

The significance of the differences between groups was determined by the $\chi 2$ method for sex ratios and by ANOVA followed by the Tukey test for gene expression using GraphPad Prism (v.6.0; GraphPad Software, San Diego, CA, USA). Differences were considered as statistically significant at $P<0.05$.

Results

Isolation of *amh* paralogues in *H. tsurugae*

Two *amh* genes were cloned and isolated in *Hypoatherina tsurugae*. One was detected in all individuals regardless of sex (Figure 1) and for this reason was named *Hts-amha*, for *H. tsurugae amh* on autosomes. The cDNA sequence has 2,015 nucleotides (nt) and 7 exons (Figure 1.1B). The other was detected only in phenotypic males (Figure 1A) and was named *Hts-amhy* for its possible linkage to the Y chromosome as in *Odontesthes hatcheri* (Hattori et al., 2012) and *O. bonariensis* (Yamamoto et al., 2014). The full length *Hts-amhy* cDNA sequence comprises 1,838 nt and only four exons (Figure 1B). The homologues of *amha* exons II and III were absent in *amhy*. In contrast, an insertion of 195 bp was detected between exons I and IV when compared to the *amha* gene structure. The homologue of *amha* exon V was detected in genomic DNA sequence but not in cDNA sequence. The lowest and highest nucleotide identity values were found for exons I and IV, respectively (Figure 1C). The deduced amino acid sequences of Amha (511 aa) and Amhy (340 aa) shared 91% of identity. Both the *amha* and *amhy* genes contained the TGF-β domain with seven canonical cysteine residues, which form disulfide bonds necessary for dimer formation. Phylogenetic analysis of Amha and Amhy amino acid sequences of *H. tsurugae* and other species available in the NCBI database using *Xenopus laevis* as out group revealed that *H. tsurugae amhy* and *amha* form a clade different from that of *Odontesthes* species *amhy* and *amha* (Figure 2).

Linkage of *amhy* genotype and phenotypic sex in wild and laboratory-reared fish

Adult specimens of *H. tsurugae* collected from Tokyo Bay showed high concordance between phenotypic sex and the presence/absence of *amhy*. For instance, 96% of the fish bearing testes (males) and 91.1% of the fish bearing ovaries (females) were *amhy*+ and *amhy*-, respectively (Table 1). Laboratory-reared fish kept at 22°C during the period of gonadal sex differentiation also showed a high linkage between phenotypic and genotypic sex (Table 2). In the progeny test of four single-pair crosses, the ratios of *amhy*- and *amhy*+ in the progeny did not deviate significantly from 1:1 in any of the crosses (Table 3), supporting the Mendelian inheritance of *amhy* gene and indicating that all males used for single-pair crosses were heterozygous (*amhy*+/−) for the *amhy* gene.

Expression analysis of *amha* and *amhy* during gonadal sex differentiation

The results of qRT-PCR revealed that in *amhy*+ individuals, transcripts of *amhy* expression were detected between 2 and 10 wah with a significant peak at 6 wah (Figure 3B). In contrast, the levels of *amha* expressions were extremely low in both genotypes (Figures 3A and 3C). ISH signals with the *amhy* probe that potentially detects also *amha* were detected in undifferentiated gonads (4 wah) and differentiating testes (8 wah) only in individuals of the *amhy*+ genotype (Figure 4). Since ISH with an *amha*-specific probe did not detect any signal in the same samples (data not shown), it can be surmised that the signals obtained in larvae with the *amhy* probe represented only *amhy* transcripts. Signals in larvae were found in presumptive Sertoli cells surrounding germ cells at the ventral side of the gonads (Figure 4).

Discussion

In this study, I investigated the occurrence of two *amh* paralogs and their possible roles in sex determination of the atheriniform *Hypoatherina tsurugae*. One *locus* was termed *amha* for its occurrence in specimens of both sexes whereas the other was found predominantly in males (see discussion below) and for this reason was denominated as *amhy*. Although the amino acid sequences of both *loci* shared 91% identity, a comparative analysis revealed the absence of exons II, III and V in the cDNA sequence of *amhy*, resulting in a truncated gene structure. Interestingly, exon V was found in the genomic DNA sequence but for some reason was not transcribed together with other exons. The structure of TGF-β domain in *amhy locus* with seven cysteine knots, which form the disulfide bonds required for protein homodimerization (Vitt et al., 2001), was conserved and shared 93% amino acid identity with the same domain of *amha*. Therefore, even in the absence of three exons, the integrity of TGF-β domain suggests that *amhy* might be able to bind to the *amhrII* (*Amh* receptor type II) and thus activate the downstream pathway of testis differentiation (Vitt et al., 2001).

Sex linkage analysis using wild adults and juveniles reared from hatching at the average temperature during the spawning season of *H. tsurugae* (22ºC) showed a high linkage between the presence and absence of *amhy* with maleness and femaleness, respectively. The relatively few mismatches between phenotypic and genotypic sex could be due to environmental effects on sex determination, in particular temperature-dependent sex determination (TSD) which is common in atheriniforms (Strüssmann and Patiño 1999; Corona-Herrera et al., 2016). It is important to note that in one of such species, the pejerrey *O. bonariensis*, both TSD and genotypic sex determination coexist and which system prevails depends on the temperature during early development (Yamamoto et al., 2014). If a similar situation exists in *H. tsurugae*, it could explain

the observed non-complete linkage between gender and *amhy* genotype (but see following discussion on gene expression patterns).

The analysis of mRNA expression during larval development showed that *amhy* transcripts were restricted to *amhy*+ individuals. The expression of *amhy* was detected from before the appearance of the first signs of histological sex differentiation in presumptive Sertoli cells surrounding germ cells in the undifferentiated gonad and was maintained during testis differentiation. In contrast, *amha* showed low, basal expression levels in both genotypes during the same period. This is similar to the pattern described for *O. hatcheri* (Hattori et al., 2012) and different to that of *O. bonariensis*, where *amha* is expressed during the critical period of sex determination (Yamamoto et al., 2014). It has been reported that sex determination in *O. bonariensis* shows higher temperature sensitivity than that of *O. hatcheri* (Strüssmann et al., 1997). The high thermosensitivity at both high and low temperatures in the former species could be related to the profile of *amha*, which increases before the appearance of sex-specific histological differences not only in XY genotypes but also during masculinization of XX individuals (Yamamoto et al., 2014). If this is true, we could expect only moderate effects of temperature on sex ratios in *H. tsurugae* as described for *O. hatcheri*. Studies on the effects of temperature on the sex ratios of *H. tsurugae* are being currently conducted using the *amhy* gene as a genotypic sex marker.

Although these results suggest that *H. tsurugae amhy* is a strong candidate for sex determination gene, whether it is an orthologue of the *O. hatcheri amhy* is still unclear (Figure 2). It is currently impossible to ascertain this because a comparative analysis with coding and non-coding gene sequences could not reveal any clear characteristics shared by *H. tsurugae* and *O. hatcheri amhys*. It is important to emphasize that the former species' *amhy* is a truncated gene

with only four exons while the latter *amhy* has seven exons. Phylogenetically, *Odontesthes* and *Hypoatherina* are placed in Atherinopsidae and Atherinidae, respectively, the farthest related families in the Atheriniformes order (Bloom et al., 2012), whose origin is estimated in 71 million of years ago (Campanella et al., 2015). It is known that genes located on sex chromosomes have evolutionary rates much higher than their autosomal paralogues (Mawaribuchi et al., 2012). Thus, the *amhy* in *H. tsurugae* and *O. hatcheri* could have accumulated enough structural changes as to make them lose the characteristics they once had in common. Nevertheless, we cannot ignore the possibility of a coincidental *de novo* appearance of *amhy* in *H. tsurugae*, since sex-determining genes are known to show repeated and independent evolution in teleosts (e.g. as exemplified in medaka species; Matsuda et al., 2002; Myosho et al., 2012; Takehana et al., 2014). The hypothesis of *de novo* evolution is supported by recent reports in the Nile tilapia *Oreochromis niloticus* (Eshel et al., 2014; Li et al., 2015) and a marsupial mammal *Ornithorhynchus anatinus* (Cortez et al., 2014) that indicate *amh/AMH* as candidate sex-determining gene and suggest that this gene has a high probability of being recruited as a key genetic player of sex determination. Screening for *amhy* in other atheriniform families besides Atherinopsidae and Atherinidae should provide critical evidence supporting either conservation or *de novo* evolution of *amhy* and clarifying the evolution of sex chromosomes in this order.

In conclusion, this study showed the presence of a duplicated Y-linked *amh* gene in *H. tsurugae*, as in *Odontesthes hatcheri* and *O. bonariensis* (Hattori et al., 2012; Yamamoto et al., 2014). The high expression of *amhy* early in larval development and the high linkage with maleness in captive-reared and wild animals make *H. tsurugae amhy* a strong sex-determining gene candidate. Further studies in other atheriniforms will contribute to our knowledge on the evolutionary processes shaping sex-determining genes in teleost fish.

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Figure Legends

Figure 1. Isolation, cloning and characterization of *amha* and *amhy* in *H. tsurugae*.

(A) PCR-amplified *amha* in both male and female wild specimens (upper band) and *amhy* amplified only in males (lower band). (B) Comparison of full length gene structure of *amha* and *amhy* in *H. tsurugae* with reference to *O. hatcheri*. Compared to *amha*, the *amhy* gene of *H. tsurugae* is shorter, lacks exons II and III, and contains a specific insertion of 195 bp at the position of exons II and IV. Exon V is present in the genomic sequence but it is not transcribed. (C) Identity values of nucleotide sequence between *amha* and *amhy* exons, UTRs, and TGF-β domain.

Figure 2. Phylogenetic analysis (Neighbour-joining tree) of the amino acid sequences of *H. tsurugae* Amha and Amhy in relation to other species. Numbers indicate bootstrap values based on 10,000 replicates.

Figure 3. Expression profiles of *amha* (A) and *amhy* (B) in *amhy*+ genotype and *amha* (C) in *amhy*- genotype during gonadal sex differentiation. Values represent the mean ± SEM of 3-6 fish per time point. Symbols with the same letter indicate groups that are not significantly different between time points.

Figure 4. Localization of *amha* and *amhy* mRNAs by ISH in undifferentiated (4 wah, A) and differentiated (8 wah, C) gonads of *H. tsurugae*. Corresponding adjacent sections were stained

with hematoxylin and eosin (4 wah, B; 8 wah, D). Scale bars represent 10 μm (A, B) and 20 μm (C, D).

Supplementary Figure S1. Expression profiles of *actb* in *amhy*+ (A) and *amhy*- (B) genotypes during gonadal sex differentiation. Values represent the mean ± SEM of 3-6 fish per time point. Symbols with the same letter indicate groups that are not significantly different between time points.

Table 1 Relationship between genotype (presence/absence of *amhy*) and the phenotypic sex in wild-caught *Hypoatherina tsurugae*.

Phenotypic sex	**Genotype***		**Total**
	amhy+	*amhy-*	
Testis*	24 (96.0%)	1 (4.0%)	25 (31.9%)
Ovary*	5 (8.9%)	51 (91.1%)	56 (69.1%)
Total	29 (35.8%)	52 (64.2%)	81

*The proportion of genotypes deviates significantly from 1:1.

Table 2 Relationship between genotype (presence/absence of *amhy*) and the phenotypic sex in laboratory-reared *Hypoatherina tsurugae* (rearing at 22°C during the period of sex determination).

Phenotypic sex	**Genotype**		**Total**
	amhy+	*amhy-*	
Testis*	26 (96.0%)	1 (4.0%)	27 (57.4%)
Ovary*	4 (20.0%)	16 (80.0%)	20 (42.6%)
Total	30 (63.8%)	17 (36.2%)	47

*The proportion of genotypes deviates significantly from 1:1.

Table 3 Frequency of *amhy*$^{+/-}$ and *amhy*$^{-/-}$ genotypes in progenies from four single-pair crosses of *amhy*− females and *amhy*+ males.

Cross*	**Genotype**		**Total**
	amhy+/-	*amhy-/-*	
A	21 (46.7%)	24 (53.3%)	45
B	16 (35.6%)	29 (64.4%)	45
C	21 (51.2%)	20 (49.8%)	41
D	20 (52.6%)	18 (47.4%)	38
Total	78 (46.2%)	91 (53.8%)	169

*The sex ratios of all progenies do not deviate significantly from 1:1

Table S1 Sequence of primers used for isolation and analysis of *amha* and *amhy* genes in *Hypoatherina tsurugae*.

Purpose	Primers	Sequence	Primer order
amha Degenerate PCR	Amh 208 F	5'-ACGGTGCTCTCCTTCACTT-3'	Sense
	Amh 2R	5'-GTCTKCAGVGCCTTCAGCAG-3'	Antisense
amha Genome walking / 5' RACE	5end Amha Race R1 (1st PCR)	5'-GACATCCACACTCCCTTGCTA-3'	Antisense
	5end Amha Race R2 (nested)	5'-CCACCTCTTCCTCATTTATCAACTCC-3'	Antisense
amha 3' RACE	3end Amha Race F1	5'-AGACACATCAAGGGTT-3'	Sense
	3 end Amha Race F2	5'-CCCCACTATCTTCTCCTTCAC-3'	Sense
amhy Genotyping	Amh 613 F	5'-CTCACAGCCCTGCAGTGT-3'	Sense
	Amh 35 R	5'-AGAAGGTCTTTCAGGTTTTGCT-3'	Antisense
amhy Genome walking	GW Amhy F1	5'-CGAGGACGCAGGTTACATTGG-3'	Sense
	GW Amhy F2	5'-TTTGCAACATCTATGGAATATATTGTTG-3'	Sense
	GW Amhy R1	5'-ACAACTTCACAAATCACTCTAAGAAATG-3'	Antisense
	GW Amhy R2	5'-ACTTTCACCATAAACAGATTTCTTTGGA-3'	Antisense

amhy 5’ RACE	5 end Amhy Race R1	5’-CACCGTCTGCAGGGCCTTCAGCA-3’	Antisense
	5 end Amhy Race R2	5’-AGCCTCTCTACGGCTTTCTG-3’	Antisense
amhy 3’ RACE	3 end Amhy Race F1	5’-GAGCACGGCATGGATTTCGG-3’	Sense
	3 end Amhy Race F2	5’-AGTCTCAGCTGATACAGGTGGACT-3’	Sense
amha qRT-PCR	AmhaRT355F	5’-AACAGCAGTACTGGTGTCAG-3’	Sense
	AmhaRT607R	5’-CCATGTCTGCTCCACGTTTCC-3’	Antisense
amhy qRT-PCR	AmhyRT236F	5’-CCAGTTTGGACACATCAAGGGTT-3’	Sense
	AmhyRT394R	5’-CTGGAGGATAAACCGAGAGTCAA-3’	Antisense
actb qRT-PCR	Beta actin RT F	5’-GTGCTGTCTTCCCCTCCATC-3’	Sense
	Beta actin RT R	5’-TCTTGCTCTGGGCTTCATCA-3’	Antisense
amh ISH Probe	ISH_amh_Fw	5’- CCAGTTTGGACACATCAAGGGTT-3’	Sense
	ISH_amh_Rv	5’- TGGAGAGAAAGGCGCCTTGT-3’	Antisense
amha ISH Probe	Amha sp.1F	5’-GCATTCAAGCGGACAGCAA-3’	Sense
	Amha sp.523R	5’- GTGAGGCTGCAGACACTGAC-3’	Antisense

Table S2. PCR conditions and primer combinations used for isolation and analysis of *amha* and *amhy* genes in *Hypoatherina tsurugae*.

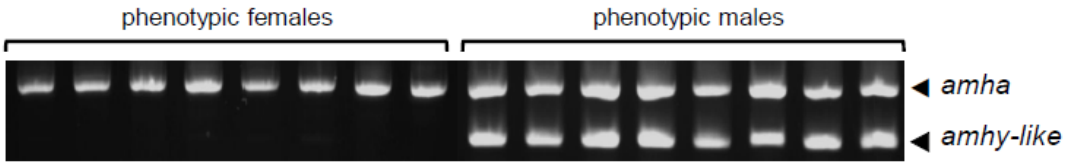
Purpose	Sense primer	Antisense primer	Denaturing temperature (time)	Annealing temperature (time)	Extension temperature (time)	Cycles
amha Degenerate PCR	Amh 208 F	Amh 2R	94°C (60s)	60°C (60s)	72°C (180s)	35
amha 5'Genome walking	AP1	5end Amha Race R1 (1st PCR)	94°C (60s)	60°C (60s)	72°C (180s)	35
	AP2	5end Amha Race R2 (nested)	94°C (60s)	68°C(180s)	72°C (300s)	35
amha 5' RACE	UPM	5end *amha* Race R1 (1st PCR)	94°C (60s)	60°C (60s)	72°C (180s)	35
	NUP	5end *amha* Race R2 (nested)	94°C (60s)	68°C (60s)	72°C (150s)	35
amha 3' RACE	3end *amha* Race F1 (1st PCR)	UPM	94°C (60s)	60°C (60s)	72°C (180s)	35
	3 end *amha* Race F2 (nested)	NUP	94°C (60s)	68°C (60s)	72°C (150s)	35
amhy Genotyping	*amh* 613 F	*amh* 35 R	94°C (60s)	60°C (60s)	72°C (180s)	35
amhy 3'Genome walking	GW Amhy F1 (1st PCR)	AP1	94°C (60s)	60°C (60s)	72°C (180s)	35
	GW Amhy F2 (nested)	AP2	94°C (60s)	68°C (180s)	72°C (300s)	35
amhy 5'Genome walking	AP1	GW Amhy R1 (1st PCR)	94°C (60s)	60°C (60s)	72°C (180s)	35
	AP2	GW Amhy R2 (nested)	94°C (60s)	68°C (180s)	72°C (300s)	35
amhy 5' RACE	UPM	5 end Amhy Race R1 (1st PCR)	94°C (60s)	60°C (60s)	72°C (180s)	35
	NUP	5 end Amhy Race R2 (nested)	94°C (60s)	68°C (60s)	72°C (150s)	35

amhy 3' RACE	3 end Amhy Race F1 (1st PCR)	UPM	94°C (60s)	60°C (60s)	72°C (180s)	35
	3 end Amhy Race F2 (nested)	NUP	94°C (60s)	68°C (60s)	72°C (150s)	35
amha qRT-PCR	AmhaRT355F	AmhaRT607R	94°C (30s)	60°C (30s)	68°C (90s)	30
amhy qRT-PCR	AmhyRT236F	AmhyRT394R	94°C (30s)	60°C (30s)	68°C (90s)	30
actb qRT-PCR	Beta actin RT F	Beta actin RT R	94°C (30s)	60°C (30s)	68°C (90s)	30
amh ISH Probe	ISH_amh_Fw	ISH_amh_Rv	94°C (30s)	60°C (30s)	68°C (90s)	35
amha ISH Probe	Amha sp.1F	Amha sp.523R	94°C (30s)	60°C (30s)	68°C (90s)	35

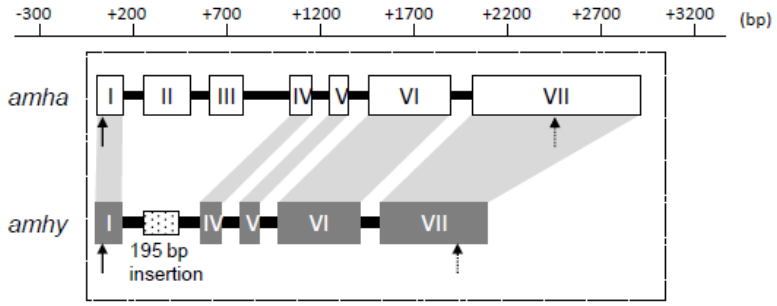
AP1, AP2, UPM, and NUP are universal primers supplied in the kits.

Figure 1

(A)



(B)



(C)

Region	Exon							5' UTR	3' UTR	TGF-β domain
	I	II	III	IV	V	VI	VII			
amha (bp)	125	237	168	111	99	410	834	22	426	284
amhy (bp)	134	–	–	109	–	410	535	29	761	284
identity (%)	71	–	–	99	–	95	81	78	62	93

Figure 2

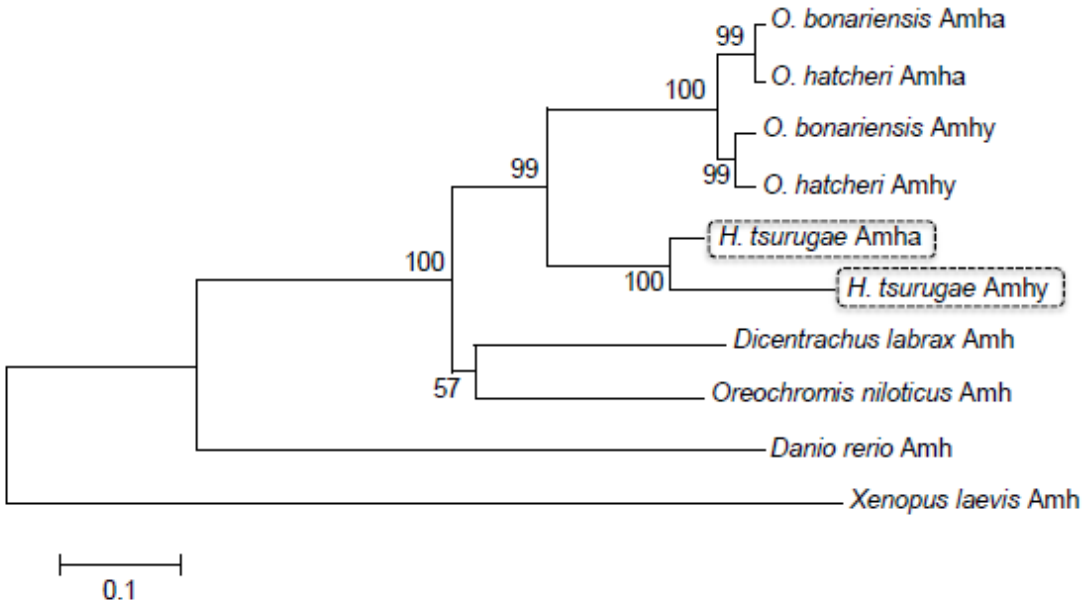
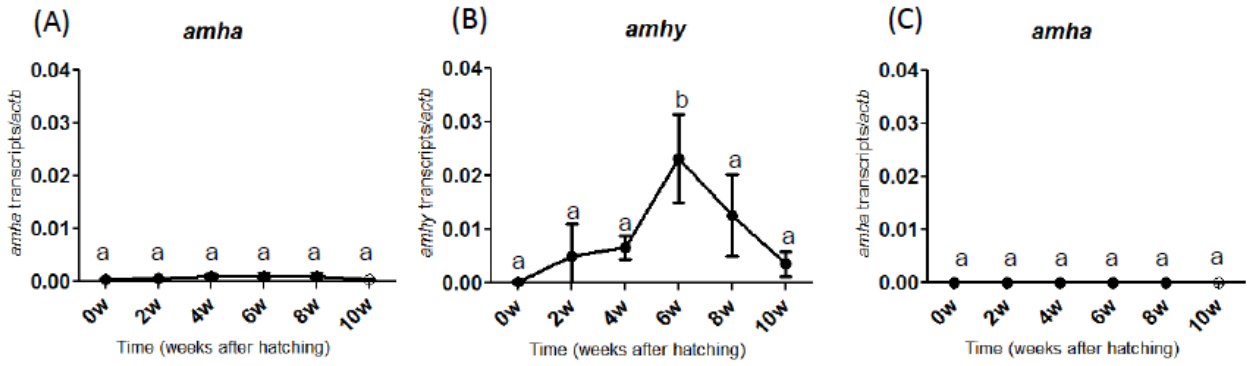

O. bonariensis Amha
O. hatcheri Amha
O. bonariensis Amhy
O. hatcheri Amhy
H. tsurugae Amha
H. tsurugae Amhy
Dicentrachus labrax Amh
Oreochromis niloticus Amh
Danio rerio Amh
Xenopus laevis Amh
99
100
99
99
100
100
57
0.1

Figure 3


(A)
amha
amha transcripts/actb
0.04
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0.01
0.00
a a a a a a
0w 2w 4w 6w 8w 10w
Time (weeks after hatching)
(B)
amhy
amhy transcripts/actb
0.04
0.03
0.02
0.01
0.00
a a a b a a
0w 2w 4w 6w 8w 10w
Time (weeks after hatching)
(C)
amha
amha transcripts/actb
0.04
0.03
0.02
0.01
0.00
a a a a a a
0w 2w 4w 6w 8w 10w
Time (weeks after hatching)

Figure 4

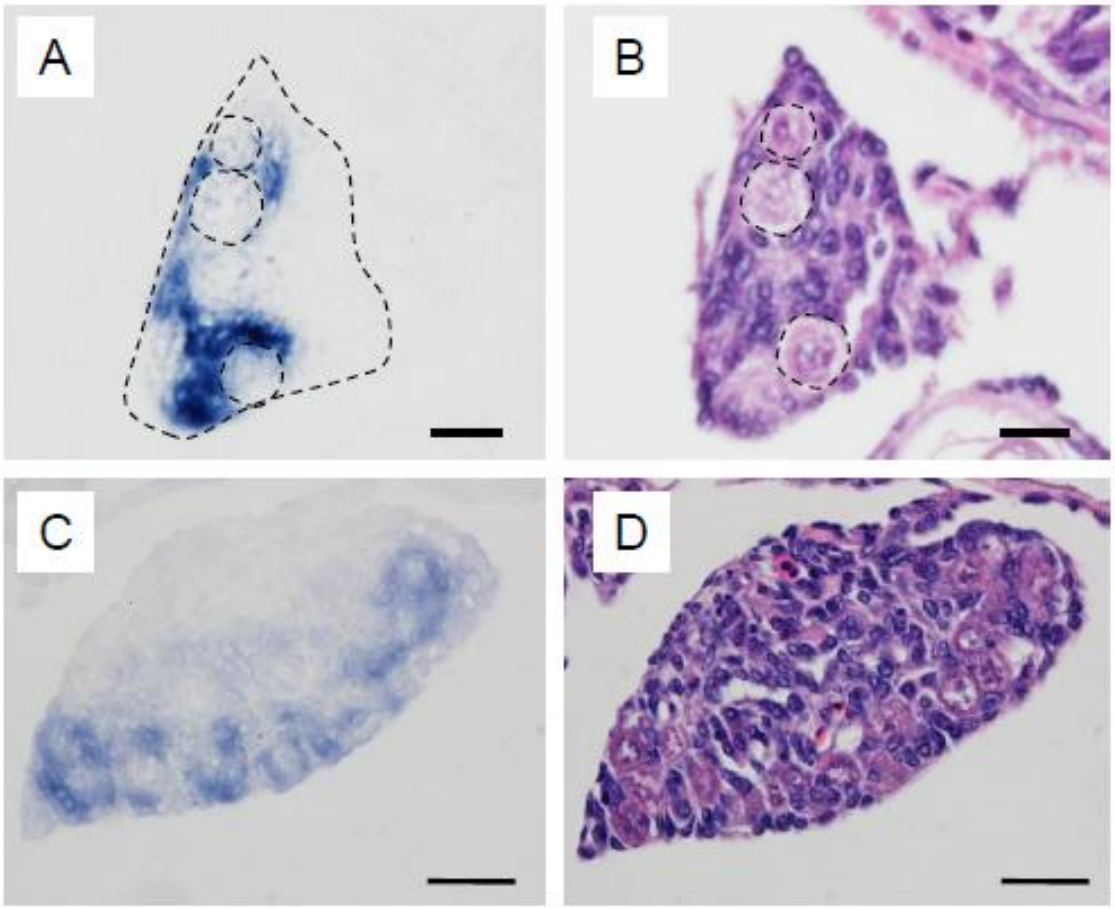
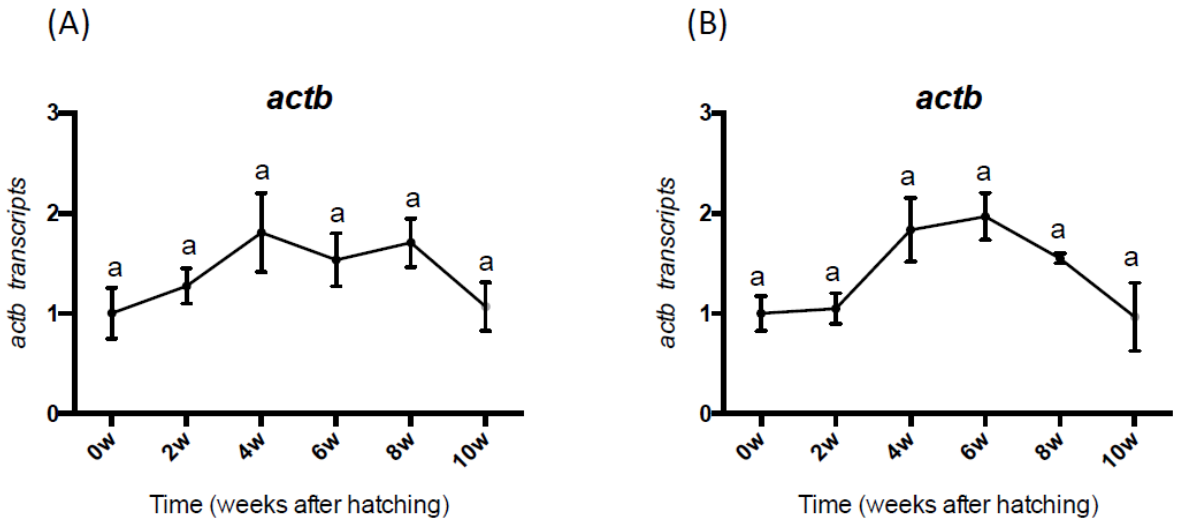


Figure S1


(A)
actb
actb transcripts
3
2
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Time (weeks after hatching)
(B)
actb
actb transcripts
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8w
10w
Time (weeks after hatching)

Chapter II

Molecular cloning, characterization and expression of six major sex differentiation-related genes in Old World atherinid, cobaltcap silverside *Hypoatherina tsurugae*

Introduction

The genetic machinery controlling gonadal development is very diverse specifically in fish (Smith et al., 2013). Some fish species demonstrate a strong genotypic sex determination and some rely on environmental, social/chemical signals to inducing the pathways that regulate the gonadal differentiation (Devlin and Nagahama 2002). However, whether sex determined by genetic or non-genetic factors in upstream, the gonadal genes expressed downstream of these cues are relatively conserved and critical for gonadal differentiation (Smith et al., 2013). For example, *dmrt1*, *amh* and *gsdf* are reported involved in testicular differentiation in many fish species (Ijiri et al., 2008). The *dmrt1* gene is required for testis differentiation in mammals (Raymond et al., 2000) and be master sex determining gene in birds (Smith et al., 2009). Also, the paralogues of *dmrt1* gene, *dmy* have a pivotal role in early sex differentiation in *Oryzias latipes* and serve as its sex determining gene (Matsuda et al., 2002). The *sox9* also actively involved in differentiation of testis in mammals as well as many other species (Kanai et al., 2005; Takada et al., 2006). In females, *foxl2* and *cyp19a1a* plays a pivotal role in ovarian differentiation in almost all nonmammalian vertebrates including fish (Devlin and Nagahama 2002). The *foxl2* gene encodes a transcription factor that binds the promoter of *cyp19a1a*, the gene that encodes P450 aromatase, and activates its transcription (Smith et al., 2013; Guiguen et al., 2010). Aromatase is a steroidogenic enzyme that is responsible for biosynthesis of estradiol-17β (E2), via conversion of testosterone to E2. Endogenous synthesis of E2 by the gonad is critical for ovarian differentiation and development in fishes (Kobayashi et al., 2003; Liu et al., 2010). Hence, *foxl2* and *cyp19a1a* are thought to be critical to these processes through their role in driving an E2-driven auto-regulatory loop (Guiguen et al., 2010; Liu et al., 2010).

In contrast, the roles of these genes in early gonadal sex determination/differentiation do not appear to always be the same among different vertebrate groups (Ijiri et al., 2008). For example, in eutherian mammals, estrogens do not play an important role in early ovarian differentiation (Ijiri et al., 2008). Also, it has been documented that *sox9* showed relatively strong expression at equivalent levels in both male and female gonads during early sex differentiation of fish (Nakamoto et al., 2005). In *Odontesthes hatcheri, dmrt1* mRNA is expressed significantly higher in adult testis than ovary but no dimorphic expression during gonadogenesis (Hattori et al., 2008). Thus, it is still controversial whether the functions of sex differentiation-related genes during early gonadogenesis are conserved across fishes.

In 1st chapter, I showed the presence of *amhy* gene in *H. tsurugae* and it is a strong sex-determining gene candidate in this species. To gain a better understanding of sex determination/differentiation mechanisms in this species, in this chapter, I first isolated and cloned six major sex-related genes (*sox9, dmrt1, gsdf, foxl2, cyp19a1a* and *scp3*) and examined expression profiles of these genes in relation to *amhy* during critical period of sex determination/differentiation in *H.tsurugae*.

Materials and methods

Identification of six sex differentiation-related genes in *H. tsurugae*

The total RNA was isolated from adult ovary and testis using TRIzol (Thermo Fisher Scientific) following the manufacturer's instruction and 1 µg of total RNA per sample was reverse transcribed using SuperScript III (Thermo Fisher Scientific) with Oligo-(dT) primers (Merk Millipore) in 20 µl reactions. RT-PCR was performed by using the specific gene primers

or degenerate primers designed based on other species. The specific amplicons from each PCR reaction were purified, cloned, and sequenced in an ABI PRISM 3100 capillary sequencer (Thermo Fisher Scientific) using BigDye Terminator method. Sequences were then analyzed by GENETYX version 11.0 (GENETYX) software. The PCR conditions and primers used in each reaction are listed in Tables 1 and 2.

Rearing of larvae for gene expression analysis

Fertilized eggs were obtained from natural spawning of the captive-reared wild broodstock as mentioned in 1st chapter. Briefly, approximately 500 hatchlings (10 to 13 days post fertilization) were stocked in two 30-liter tanks kept at 22ºC and reared for up to 12 weeks. 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) from the fifth week of the experiment.

Fish were sampled biweekly from 0 to 10 wah for gene expression analysis and gonadal histology. The remaining larvae were sampled at the end of the rearing experiment to determine the sex ratio. The trunks of the fish were stored in RNA later (Thermo Fisher Scientific) (n=8) or in Bouin's solution (n=8) for gene expression analysis and gonadal histology, respectively, at each time point. Samples in RNA later were stored at −80°C until use. Bouin-fixed samples were rinsed three times with PBS, transferred into 70% ethanol, and stored at 4°C until use. All larvae were fin-clipped for *amhy* genotyping as described in chapter 1. The expression of mRNA transcripts was analyzed by qRT-PCR using specific primers designed for *sox9*, *dmrt1*, *gsdf*,

foxl2, cyp19a1a and *scp3* are listed in Tables 3. The *β-actin* gene was taken as an endogenous control.

Statistical analysis

Results from *amh*y+ and *amhy*- groups at each time point were compared by unpaired t-tests (Prism 6). The time course differences within each group were subjected to one-way analysis of variance (ANOVA), followed by Tukey's multiple mean comparison tests. The minimum level of statistical significance was set at $P < 0.05$.

Results

Partial *sox9* mRNA and its expression during sex determination/differentiation period

The 2876 bp of *sox9* mRNA nucleotide sequence was obtained with an open reading frame (ORF) of 1215 bp encoding a 405 aa predicted protein (Figure 1). In NCBI_Blastp search reveal that protein identity of transcription factor *sox9* like *Stegastes partitus* [XP_008301579] 96%, *Odontesthes bonariensis* [AAP84605] 96%, *Poecilia Formosa* [XP_007556425] 95%, *Lates calcarifer* [AKI32580] 95%, *Poecilia maxicana* [XP_014856083] 94%, *Oreochromis niloticus* [XP_003450167] 92%, *Takifugu rubripes* [AAL32172] 92%, *Oryzias latipes* [AAX62151] 90%. Levels of *sox9* mRNA in both *amhy*+ and *amhy*- groups maintained over time and no significant difference was observed between time points (Figure 2). However, the

abundance of *sox9* transcripts was significantly higher in *amhy*- fish compared to those of *amhy*+ fish at 0wah.

Complete *dmrt1* mRNA and its expression during sex determination/differentiation period

The complete mRNA sequence of *H. tsurugae dmrt1* was 1569 bp with an ORF of 879 bp encoding 293 aa predicted protein (Figure 3). In NCBI_Blastp search showed that protein identity of *dmrt1* transcription factor of *Odontesthes bonariensis* [AAP84606] 78%, *Odontesthes hatcheri* [ACG69835] 79%, *Oreochromis niloticus* [AAF79931] 77%, *Oreochromis aureus* [ABA29161] 77%, *Lates calcarifer* [AKI32577] 74%, *Dicentrachus labrax* [CAQ52796] 69%, *Oryzias latipes* [AAL02165] 67% and *Oryzias curvinotus* [BAC65996] 66%. Levels of *dmrt1*mRNA in *amhy*+ fish increased from 2 wah and peaked at 6 wah and then declined, while levels in *amhy*- fish were maintained extremely low and unchanged over time. Level of *dmrt1* mRNA in *amhy*+ fish was significantly higher than those of *amhy*- fish at 6 wah (Figure 4).

Partial *gsdf* mRNA and its expression during sex determination/differentiation period

The partial 1391 bp mRNA sequence of *H. tsurugae gsdf* gene encoding 171 aa predicted protein was obtained (Figure 5). The NCBI_Blastp search showed the percent of protein identity of mRNA sequence with *Oreochromis niloticus* [BAJ78985.1] 66%, *Oreochromis mossambicus* [ALO18792] 66%, *Dicentrachus labrax* [AGA54135] 60%, *Oryzias latipes* [BAJ05045.1] 59% and *Takifugu rubripes* [AKP17236.1] 50%. The levels of *gsdf* in both *amhy*+ and *amhy*- individuals were maintained extremely low and unchanged over time (Figure 6).

Partial *foxl2* mRNA and its expression during sex determination/differentiation period

The partial 456 bp long mRNA sequence of *H. tsurugae foxl2* gene encoding 151 aa predicted protein was obtained (Figure 7). The NCBI_Blastp search showed the percent of protein identity of mRNA sequence with fork head protein L2 *Stegastes partitus* [XP_008301498] 99%, *Odontesthes hatcheri* [ACL80211] 99%, *Oreochromis niloticus* [NP_001266707] 99%, *Salmo solar* [XP_014018845] 98%, *Oryzias luzonensis* [BAH05020] 99%, *Oryzias latipes* [NP_001098358], *Dicentrachus labrax* [ACW83540] 98%, *Lates calcarifer* [AKI32579] 98% and *Odontesthes bonariensis* [ACG69834] 98%. Levels of *foxl2* mRNA in *amhy*+ fish maintained low and unchanged over time, while levels in *amhy*- fish rapidly increased at 2 wah and then declined. Level of *foxl2* mRNA in *amhy*- fish was significantly higher than those of *amhy*+ fish at 2 wah (Figure 8).

Partial *cyp19a1a* mRNA and its expression during sex determination/differentiation period

The 1638 bp of *cyp19a1a* mRNA nucleotide sequence was obtained in *H. tsurugae* with an open reading frame (ORF) of 1488 bp encoding a 496 aa predicted protein (Figure 9). In NCBI_Blastp search showed that protein identity of transcription factor with ovarian aromatase of *Melanotaenia fluviatilis* [AED99846] 93%, *Odontesthes hatcheri* [ABK41198] 88%, *Odontesthes bonariensis* [ABK30807] 87%, *Oryzias latipes* [NP_001265808] 86%, *Dicentrachus labrax* [CAC21712] 85%, *Oreochromis niloticus* [AA062625] 81%, *Oreochromis aureus* [ABB89869] 81% and *Takifugu rubripes* [NP_001266957] 81%. Levels of *cyp19a1a* mRNA in *amhy*+ fish maintained low and unchanged over time, while levels in *amhy*- fish

increased from 4 wah and peaked at 6 wah and then declined. Level of *cyp19a1a* mRNA in *amhy*- fish was significantly higher than those of *amhy*+ fish at 6 wah (Figure 10).

Complete *scp3* mRNA and its expression during sex determination/differentiation period

The complete mRNA sequence of *H. tsurugae scp3* was 979 bp with an ORF of 699 bp encoding 233 aa predicted protein (Figure 11). In NCBI_Blastp search showed that percent of protein identity of Synaptonemal complex protein3 *Oreochromis niloticus* [XP_003439417] 89%, *Xiphophorus maculatus* [XP_005795866] 84%, *Kryptolebias marmoratus* [XP_017295348] 88%, *Monopterus albus* [AJP00088] 83%, *Oncorhynchus mykiss* [NP_001117979] 69%, *Dicentrachus labrax* [AGC01375] 87% and *Oryzias latipes* [XP_011479284] 78%. The expression of *scp3* gene in *amhy*- individual was relatively low from 0 to 4 wah, but significantly increased at 6 wah and declined thereafter . In contrast, levels of transcript for *scp3*are maintained low. Levels of *scp3* mRNA in *amhy*- fish were significantly higher than those of *amhy*+ fish from 6 to 10 wah (Figure 12).

Discussion

In this study, I investigated the six sex differentiation-related genes that have pivotal roles in vertebrate sex determination were cloned and characterized in an atherinid species from the order Atheriniformes. Using qRT-PCR assays developed for each gene, I found that *foxl2*, *cyp19a1a* and *scp3* mRNAs were significantly increase in cobaltcap silverside (*H.tsurugae*) ovary compared to testis, and conversely, *dmrt1* mRNAs was significantly elevated in testis compared to ovary. The expression pattern of *dmrt1* observed in cobaltcap silverside corresponds well with proposed roles of *dmrt1* as a major player in the male sex determination and/or testis differentiation and development in fishes (Matsuda et al., 2002; Kobayashi et al., 2004). A recent study in Nile tilapia demonstrated that the DM-domain of *dmrt1* directly suppress *cyp19a1a* expression in vitro and inhibits both *cyp19a1a* expression and E2 synthesis in vivo (Wang et al., 2010). Gonadal *foxl2* and *cyp19a1a* expression is required for normal ovarian development, and gene mutations or knock-outs can lead to ovarian failure and partial or complete female-to-male sex reversal in mammals (Yao, 2005). In agreement with these genes playing a critical role in ovarian development, I found that *foxl2* and *cyp19a1a* mRNAs were significantly up regulated in cobaltcap silversides ovaries compared to testes.

Ontogenic assessments of sex differentiation-related genes in rainbow trout and Nile tilapia, sable fish (of known genetic sex) demonstrated that *foxl2* and *Cyp19a1a* mRNAs were elevated in ovaries relative to testes during early development, as I observed in cobaltcap silversides, but that these increases in ovaries began before gonadal differentiation was apparent by histology (Vizziano et al., 2007; Ijiri et al., 2008; Smith et al., 2013). This was also the case in some other Gonochoristic fish species, like flounder (*P. olivaceus* and *P. lethostigma*), Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic cod (*Gadus morhua*), and air breathing catfish

(*Clarias gariepinus*) (Kitano et al., 1999; Luckenbach et al., 2005; Matsuoka et al., 2006; Raghuveer et al., 2011; Haugen et al., 2012). The precise onset of gonadal *foxl2* and *cyp19a1a* expression will clearly important for us to determine in future studies with cobaltcap silversides and may lead to a better understanding of how these genes interact during ovarian differentiation.

Sox9 is a transcription factor that contains a sry-related high mobility group (HMG) box. Skeletal defects and male-to-female sex reversal are caused by mutation in *sox9* gene in human, signifies its important role in chondrogenesis and male gonad development. So, it recommended that the function of *sox9* gene in cartilage formation and testis development are conserved across vertebrates (Yokoi et al., 2002). In teleost fish, such as zebrafish, medaka and fugu, two subtypes of *sox9* genes were isolated (Chiang et al., 2001; Koopman et al., 2004; Klüver et al., 2005). One expressed in chondrogenic tissue and another in gonads. The expression of *sox9* gene in fish is to some extent diverse (Yokoi et al., 2002). The expression of *sox9* in *amhy*- individual of *H. tsurugae* species is comparatively high or more or less same as *amhy*+ individual can be explained, as the *sox9* gene involves in proliferation of germ cells in both testis and ovaries (Nakamura et al., 2012). Similar type of expression also observed in early developing gonad of Sablefish (Smith et al., 2013), Nile tilapia (Ijiri et al., 2008) and medaka (Nakamoto et al., 2005). In *H.tsurugae*, our data of *sox9* gene is not showing any significant dimorphic expression for male and female sex during earlier period of gonadal development.

Doublesex and mab-3 (DM) related transcription factor-1 (*dmrt1*) belongs to gene families that have a highly conservative zinc-finger DNA- binding motif (DM domain). It considered as first preserved gene in sex determination/differentiation cascade across phyla (Erdman and Burtis 1993; Raymond et al., 1998). It known to play a crucial role during early period of gonadal development in all metazoan, though it may work as an upstream or

downstream regulator of sex determining gene during the sex cascade, depending on the species. Till date, *dmrt1* gene or its paralogues identified as sex determining gene in three distantly related species - (1) *dmy* in *Oryzias latipes* (Matsuda et al., 2002; Nanda et al., 2002), (2) DM-W gene in *Xenopus* (Yoshimoto et al., 2008), (3) Z-linked dose based *dmrt1* gene in chicken (Smith et al., 2009). In *H.tsurugae* the expression of *dmrt1* significantly increases from 0 wah to 6wah in *amhy*+ individuals indicating its important role during the undifferentiation period of male gonad. Similarly, in zebrafish the expression of *dmrt1* gene in testis which is quite dominant than ovary (Guo et al., 2005). In medaka the *dmrt1* does not express in male and female embryo till 20 days of after hatch but *dmy* express very early, just before and after hatch in male gonad (Matsuyama et al., 2003). In tilapia, (*Oreochromis niloticus*) *dmrt1* express very earlier before the formation of gonad indicating *dmrt1* has an important role in early differentiation of male gonad (Kobayashi et al., 2008).

The gonadal soma-derived factor (*gsdf*) belongs to the transforming growth factor-β superfamily and it is only found in teleostean fish species. The amino acid sequnces of all fish *gsdf* share significant homology with the members of TGF-β superfamily, they lacked the glycine residue in the conserved cysteine knot motif (Vitt et al., 2001). Primarily the *gsdf* gene is expressed in gonads, and its expression is restricted to the granulosa and sertoli cells in fish like trout and medaka. The *gsdf* gene expression is correlated to early testis differentiation in medaka and was shown to stimulate primordial germ cell and spermatogonia proliferation in trout. The *gsdf* gene localizes to a syntenic chromosomal fragment conserved among vertebrates but, no *gsdf*-related gene is detected on the corresponding genomic region of tetrapods (Gautier et al., 2011). Also, the paralogue of *gsdf* gene, $gsdf^{Y}$ considered as male sex determining gene in Japaneese medaka (*Oryzias luzonensis*) (Myosho et al., 2012). In *H. tsurugae*, the expression of

gsdf in *amhy+* individual and *amhy-* individuals is very low. It may be highly express in later stage for the maintenance of gonad in this species.

The fork head family of transcription factors is conserved in evolution and play critical role in regulation of cellular differentiation and proliferation. Members of this family display tissue specific expression pattern and are involved in cell type determination and differentiation (Wang et al., 2004). *foxl2* is a putative transcription factor involved in ovarian development and function. Many number of ontogenic expression studies have been done in different species of vertebrates (Baron et al., 2005; Yao, 2005). From these studies, it revealed that *foxl2* is one of the earliest markers of ovarian differentiation in vertebrates. *foxl2* directly bind to the promoter region of the cytochrome P450 aromatase (P450arom) gene, resulting in the activation of P450arom transcription. The early expression of *foxl2* in *H.tsurugae* is correlated to aromatase gene; it may be to bind with the promoter of aromatase gene to activate its transcription which essential for the normal function and development of ovary. Similar type of high level of expression is also observed in ovary not in testis of Rainbow trout (Vizziano et al., 2007), medaka (Nakamoto et al., 2006), Nile tilapia (Wang et al., 2004).

Cytochrome P450 aromatase (P450aro, CYP19) is a member of the cytochrome P450 superfamily and it plays an important role in the sex-differentiation and ovary development in vertebrates. It encodes a P450 aromatase, the rate limited enzyme catalyzing the physiological process that synthesizes estrogen from androgen (Lange et al., 2002; Simpson et al., 2002). In teleosts, *cyp19a1* genes generally contain two subtypes- *cyp19a1a* predominantly expressed in ovary and *cyp19a1b* expressed in brain (Chiang et al., 2001; Kobayashi et al., 2004). In humans, *cyp19* transcript also extensively distributed in tissue like ovary, placenta, adipose and brain (Simpson et al., 1994). In fish, *cyp19* is expressed in vitellogenic follicles during oogenesis that

consistent with the function of estrogen in fish for ovarian development (Tanaka et al., 1995; Fukada et al., 1996; Chang et al., 1997). In *H. tsurugae*, the *Cyp19a1a* is significantly expressed during undifferentiated period in *amhy*- individual. High expression of *aromatase* gene was also observed in ovary of rainbow trout, tilapia and medaka (Tanaka et al., 1992; Fukada et al., 1996; Chang et al., 1997). Despite the pivotal role of *cyp19a1a* in female sexual differentiation, how the expression of *cyp19a1a* is regulated within the developing gonads remain to be determined.

In cell division, meiosis is unique to germ cells. During meiosis, the pairing of homologous chromosome (Synapsis) takes place leads to the formation of chiasmata and exchange of chromosomal parts following the recombination of genes and then, segregation of chromosome occurs. This process is necessary for generating an unique type of genetically distinct haploid cells. This type of unique behavior of meiotic chromosome that associated with the meiosis-specific supramolecular proteinaceous structure, the Synaptonemal complex (SC) which is generally observed in most sexually active meiotically dividing cells (Wettstein et al., 1984; Heyting 1996; Roeder 1997). At pachytene stage of meiosis prophase-I, the SC extended along with chromosome to make a bivalent structure. The SC is composed of two lateral elements, to which the chromatin of homologous chromosomes is attached and the central region is located between the lateral elements (Heyting 1996). The *scp3* is a structural component of lateral element. It has an important role in normal progression of meiosis. Protein scp3 is the primary determinant of SC (Yuan et al., 2000). In $scp3^{-/-}$ mice, the disruption of spermatogenesis occurs during meiosis leading to infertility in males and an increased aneuploidy rate during oogenesis and frequently followed by embryonic death (Meuwissen et al., 1992; Yuan et al., 2000, 2002). The expression of *scp3* gene in *amhy*- individual of *H. tsurugae* was significantly higher than the *amhy*+ individual during early differentiation period. But, in Zebrafish and

rainbow trout highly expressed and served as marker for spermatogonia (Yano et al., 2008) Thus, *scp3* gene may have a critical role in development of both gonad.

From the above expression profile of six sex related genes (*sox9, dmrt1, gsdf, foxl2, cyp19a1a* and *scp3*) with relation to *amhy* gene, it can summarize that the expression of the *amhy* gene is significantly high at 6wah. The same fashion of expression also observed in the *dmrt1* gene, though 1000 times in lesser concentration than the gene *amhy*. On the other hand, the ovarian gene *foxl2* highly expressed at 2wah to bind with the promoter of *cyp19a1a* directly through its fork head domain and activates its transcription to give the fate of gonad as ovarian determination. Further studies are required to determine whether *foxl2* is involved in the transcriptional regulation of other steroidogenic enzymes that are expressed during early sex differentiation period. Here, it can be estimate that there may be interaction or contention of genes between *foxl2* and *amhy* during early sex differentiation period to decide the gonadal fate of female or male respectively. When one of the genes is activated another pathway is continuously repressed. Although, there may be the network of transcription factors that tightly regulates the initiation and maintenance of these distinct pathways.

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Figure Legend

Figure 1 Partial *sox9* mRNA nucleotide sequence of 2876 bp was obtained with an open reading frame (ORF) of 1215 bp encoding a 405 aa predicted protein

Figure 2 Expression profile of *sox9* gene in *amhy*+ genotype and in *amhy*- genotype during gonadal sex differentiation*

Figure 3 Complete mRNA sequence of 1569 bp of *dmrt1* nucleotide with an ORF of 879 bp encoding 293 aa predicted protein

Figure 4 Expression profile of *dmrt1*gene in *amhy*+ genotype and in *amhy*- genotype during gonadal sex differentiation*

Figure 5 Partial 1391 bp mRNA sequence of *gsdf* gene encoding 171 aa predicted protein

Figure 6 Expression profile of *gsdf* gene in *amhy*+ genotype and in *amhy*- genotype during gonadal sex differentiation*

Figure 7 partial 456 bp long mRNA sequence of *foxl2* gene encoding 151 aa predicted protein

Figure 8 Expression profile of *foxl2* gene in *amhy*+ genotype and in *amhy*- genotype during gonadal sex differentiation*

Figure 9 Partial 1638 bp of *cyp19a1a* mRNA nucleotide sequence with an open reading frame (ORF) of 1488 bp encoding a 496 aa predicted protein

Figure 10 Expression profile of *cyp19a1a* gene in *amhy*+ genotype and in *amhy*- genotype during gonadal sex differentiation*

Figure 11 Complete mRNA sequence of *scp3* gene was 979 bp with an ORF of 699 bp encoding 233 aa predicted protein

Figure 12 Expression profile of *scp3* gene in *amhy*+ genotype and in *amhy*- genotype during gonadal sex differentiation*

*Values represent the mean ± SEM of 3-6 fish per time point. Symbols with the same letter indicate groups that are not significantly different between time points.

Table 1 List of primers used for isolation of sex related genes in *Hypoatherina tsurugae*

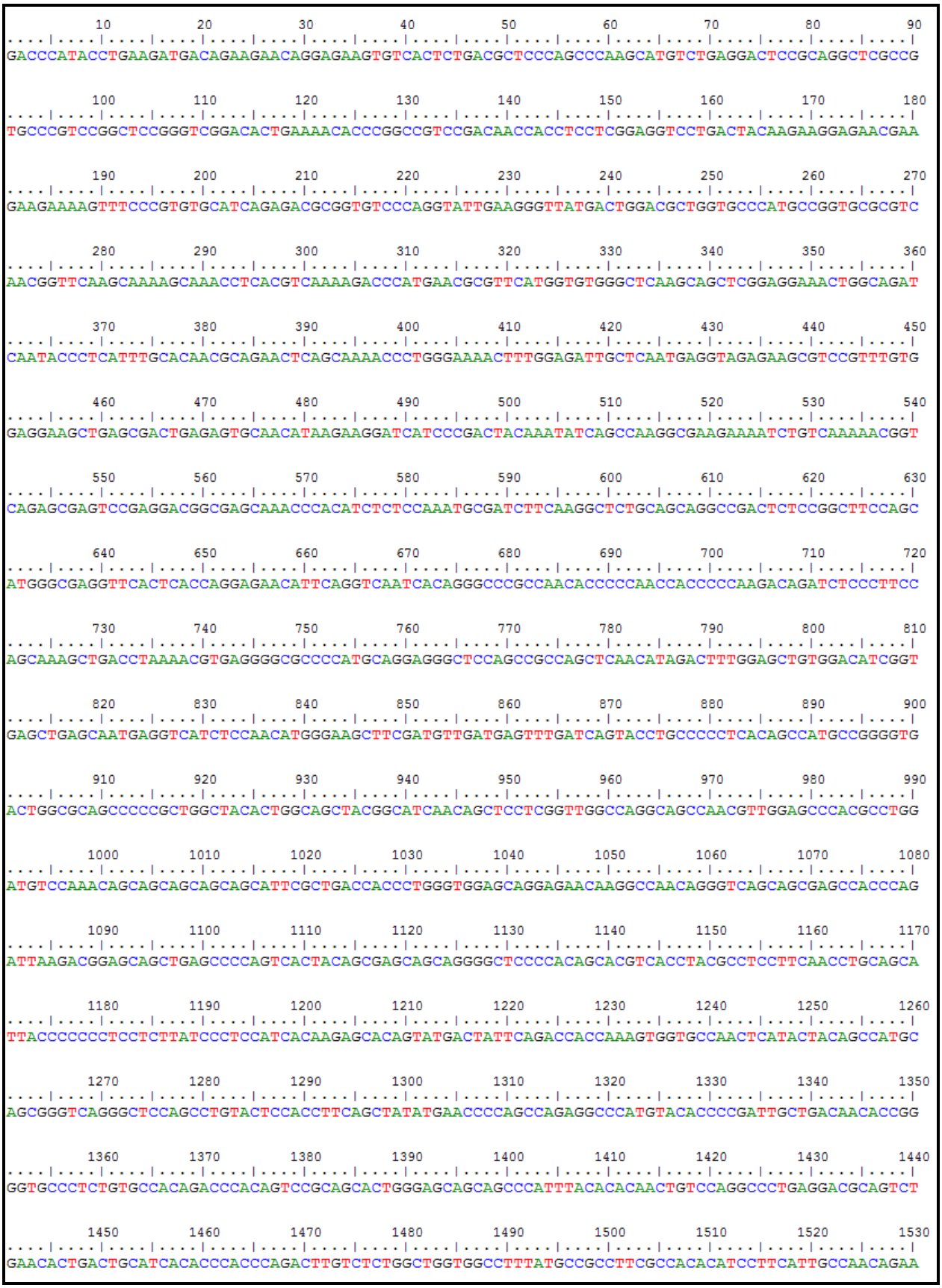
Genes	Primers	Sequence	Primer order
sox9	*sox9*Fw347	5'-CTGACCTAAAGCGTGAGGGA-3'	sense
	*sox9*R1448	5'-GAAGTAAGGCCACTGAGGGT-3'	antisense
dmrt1	*dmrt*Fw17	5'-GCAAATTGTAACAAAACTCCGAGTGTAG-3'	sense
	*dmrt*Rv1405	5'-ATAAGTGCTTTAGGAAAGAAGAGACTGA-3'	antisense
gsdf	*gsdf*Fw256	5'-GGTGYCARGRTGAGTCATKGCAGTCC-3'	sense
	*gsdf*Rv515	5'-CCAGCCCAGATCTTTCATGAAGAYCTC-3'	antisense
foxl2	*fox*Fw1	5'-CCCAGAAACCACCGTACTCTTACGTC-3'	sense
	*fox*Rv428	5'-CTGGCCTAACGACCAGGAGT-3'	antisense
cyp19a1a	*aro*Fw9	5'-GTGGCAACATCAAATGCTACAACTG-3'	sense
	*aro*Rv1447	5'-CTGAGGTGTTCAGAGTCTGGATGA-3'	antisense
scp3	*scp3*Fw1	5'-GAGAWGATGAAACTCCRATMRTKGACAAGCT-3'	sense
	*scp3*R680	5'-GARCATGGWCTKCAGGGA-3'	antisense
β-actin	*actin*Fw17	5'- GCCTGAAACCGGTTCCCTT-3'	sense
	*actin*Rv1838	5'- TTTTCGGAACACATGTGCACT-3'	antisense

Table 2 PCR conditions and primer combinations used for isolation of sex related genes in *Hypoatherina tsurugae*

Genes	**Sense primer**	**Antisense primer**	**Denaturing temperature (time)**	**Annealing temperature (time)**	**Extension temperature (time)**	**Cycles**
sox9	*sox9*Fw347	*sox9*R1448	94°C (30s)	60°C (30s)	72°C (90s)	35
dmrt1	*dmrt*Fw17	*dmrt*Rv1405	94°C (30s)	60°C (30s)	72°C (90s)	35
gsdf	*gsdf*Fw256	*gsdf*Rv515	94°C (30s)	60°C (30s)	72°C (90s)	35
foxl2	*fox*Fw1	*fox*Rv428	94°C (30s)	60°C (30s)	72°C (90s)	35
cyp19a1a	*aro*Fw9	*aro*Rv1447	94°C (30s)	60°C (30s)	72°C (90s)	35
scp3	*scp3*Fw1	*scp3*R680	94°C (30s)	60°C (30s)	72°C (90s)	35
β-actin	*actin*Fw17	*actin*Rv1838	94°C (30s)	60°C (30s)	72°C (90s)	35

Table 3 List of primers used in expression profile of *sox9, dmrt1, gsdf, foxl2, cyp19a1a, scp3* and *β-actin*

Genes	**Primers**	**Sequence**	**Primer order**
sox9	*sox* RT 809 F	5'-GGTGAGCTGAGCAATGAGGT-3'	sense
	sox RT 935 R	5'-GAGGAGCTGTTGATGCCGTA-3'	antisense
dmrt1	*dmrt* RT 234 F	5'-TGGTCCTGAGGTGACGGTTAAG-3'	sense
	dmrt RT 300 R	5'-GGATCGTCCCTCCACAGAA-3'	antisense
gsdf	*gsdf* RT 93 F	5'-CTGGACAGTATCCAGGAGCAGT-3'	sense
	gsdf RT 231 R	5'-CCAGCCCAGATTTTCATGAAGATCTC-3'	antisense
fox	*fox* RT 181 F	5'-TCATCAAGGTTCCACGCGAA-3'	sense
	fox RT 308 R	5'-TCTGAAAGGCCGCTTCATCC-3'	antisense
cyp19a1a	*aro* RT 100 F	5'-AAGTCTTGTAGAACAGAAGAGGAGAGA-3'	sense
	aro RT 257 R	5'-AAGAAGAGGCTGATGGACAGAGT-3'	antisense
scp3	*scp3* RT F	5'-GGAGGAGAAGCTCAATAACCTGTTC-3'	sense
	scp3 RT R	5'-ACAAACTGCTCGTACAGCTCTCT-3'	antisense
β- actin	*β-actin* RT F	5'-GTGCTGTCTTCCCCTCCATC-3'	sense
	β-actin RT R	5'-TCTTGCTCTGGGCTTCATCA-3'	antisense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100 110 120 130 140 150 160 170 180
TGCCCGTCCGGCTCCGGGTCGGACACTGAAAACACCCGGCCGTCCGACAACCACCTCCTCGGAGGTCCTGACTACAAGAAGGAGAACGAA
190 200 210 220 230 240 250 260 270
GAAGAAAAGTTTCCCGTGTGCATCAGAGACGCGGTGTCCCAGGTATTGAAGGGTTATGACTGGACGCTGGTGCCCATGCCGGTGCGCGTC
280 290 300 310 320 330 340 350 360
AACGGTTCAAGCAAAAGCAAACCTCACGTCAAAAGACCCATGAACGCGTTCATGGTGTGGGCTCAAGCAGCTCGGAGGAAACTGGCAGAT
370 380 390 400 410 420 430 440 450
CAATACCCTCATTTGCACAACGCAGAACTCAGCAAAACCCTGGGAAAACTTTGGAGATTGCTCAATGAGGTAGAGAAGCGTCCGTTTGTG
460 470 480 490 500 510 520 530 540
GAGGAAGCTGAGCGACTGAGAGTGCAACATAAGAAGGATCATCCCGACTACAAATATCAGCCAAGGCGAAGAAAATCTGTCAAAAACGGT
550 560 570 580 590 600 610 620 630
CAGAGCGAGTCCGAGGACGGCGAGCAAACCCACATCTCTCCAAATGCGATCTTCAAGGCTCTGCAGCAGGCCGACTCTCCGGCTTCCAGC
640 650 660 670 680 690 700 710 720
ATGGGCGAGGTTCACTCACCAGGAGAACATTCAGGTCAATCACAGGGCCCGCCAACACCCCCAACCACCCCCAAGACAGATCTCCCTTCC
730 740 750 760 770 780 790 800 810
AGCAAAGCTGACCTAAAACGTGAGGGGCGCCCCATGCAGGAGGGCTCCAGCCGCCAGCTCAACATAGACTTTGGAGCTGTGGACATCGGT
820 830 840 850 860 870 880 890 900
GAGCTGAGCAATGAGGTCATCTCCAACATGGGAAGCTTCGATGTTGATGAGTTTGATCAGTACCTGCCCCCTCACAGCCATGCCGGGGGTG
910 920 930 940 950 960 970 980 990
ACTGGCGCAGCCCCCGCTGGCTACACTGGCAGCTACGGCATCAACAGCTCCTCGGTTGGCCAGGCAGCCAACGTTGGAGCCCACGCCTGG
1000 1010 1020 1030 1040 1050 1060 1070 1080
ATGTCCAAACAGCAGCAGCAGCAGCATTCGCTGACCACCCTGGGTGGAGCAGGAGAACAAGGCCAACAGGGTCAGCAGCGAGCCACCCAG
1090 1100 1110 1120 1130 1140 1150 1160 1170
ATTAAGACGGAGCAGCTGAGCCCCAGTCACTACAGCGAGCAGCAGGGGCTCCCCACAGCACGTCACCTACGCCTCCTTCAACCTGCAGCA
1180 1190 1200 1210 1220 1230 1240 1250 1260
TTACCCCCCCTCCTCTTATCCCTCCATCACAAGAGCACAGTATGACTATTCAGACCACCAAAGTGGTGCCAACTCATACTACAGCCATGC
1270 1280 1290 1300 1310 1320 1330 1340 1350
AGCGGGTCAGGGCTCCAGCCTGTACTCCACCTTCAGCTATATGAACCCCAGCCAGAGGCCCATGTACACCCCGATTGCTGACAACACCGG
1360 1370 1380 1390 1400 1410 1420 1430 1440
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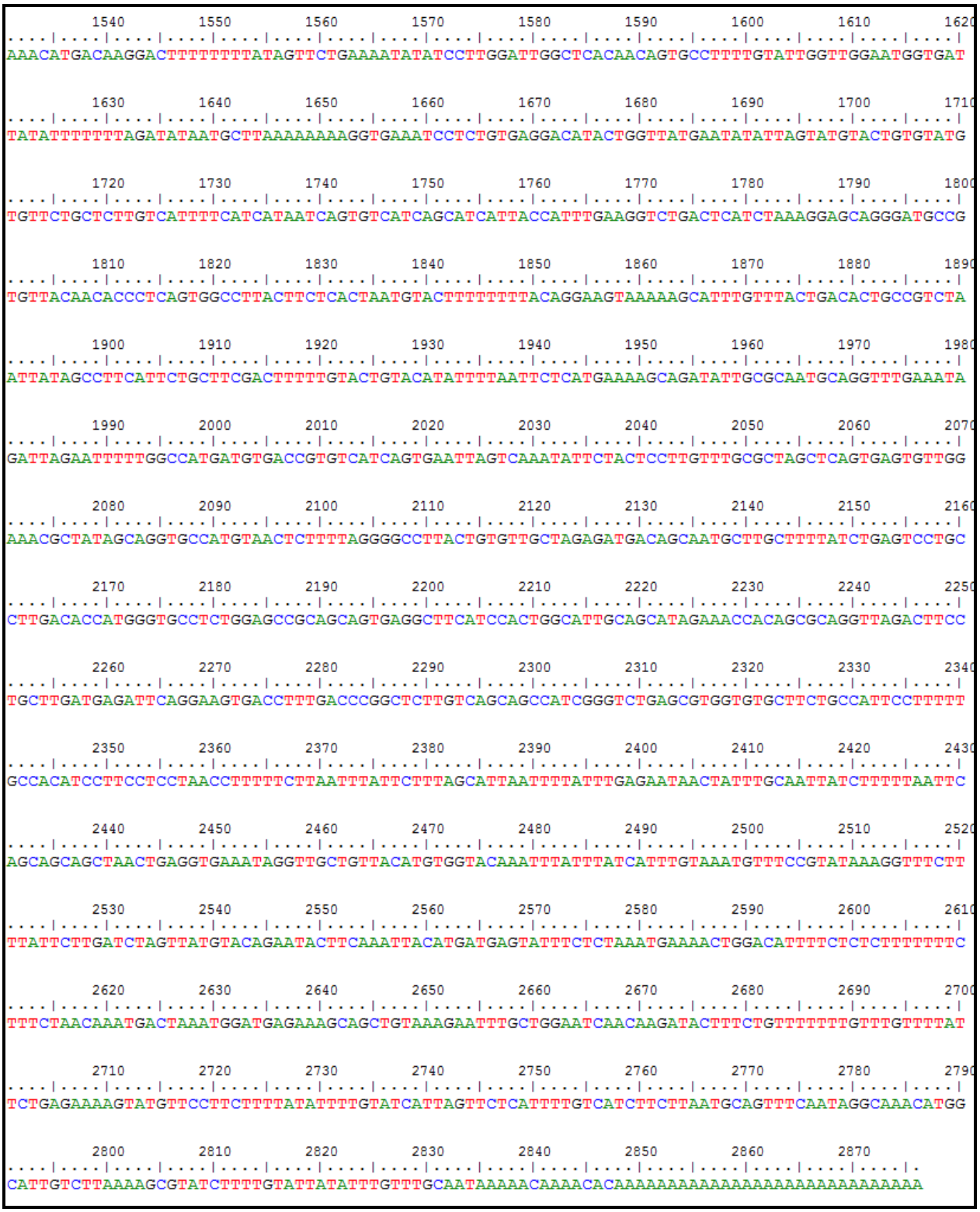

Figure 1 Partial mRNA sequence of *sox9* gene in *H. tsurugae*

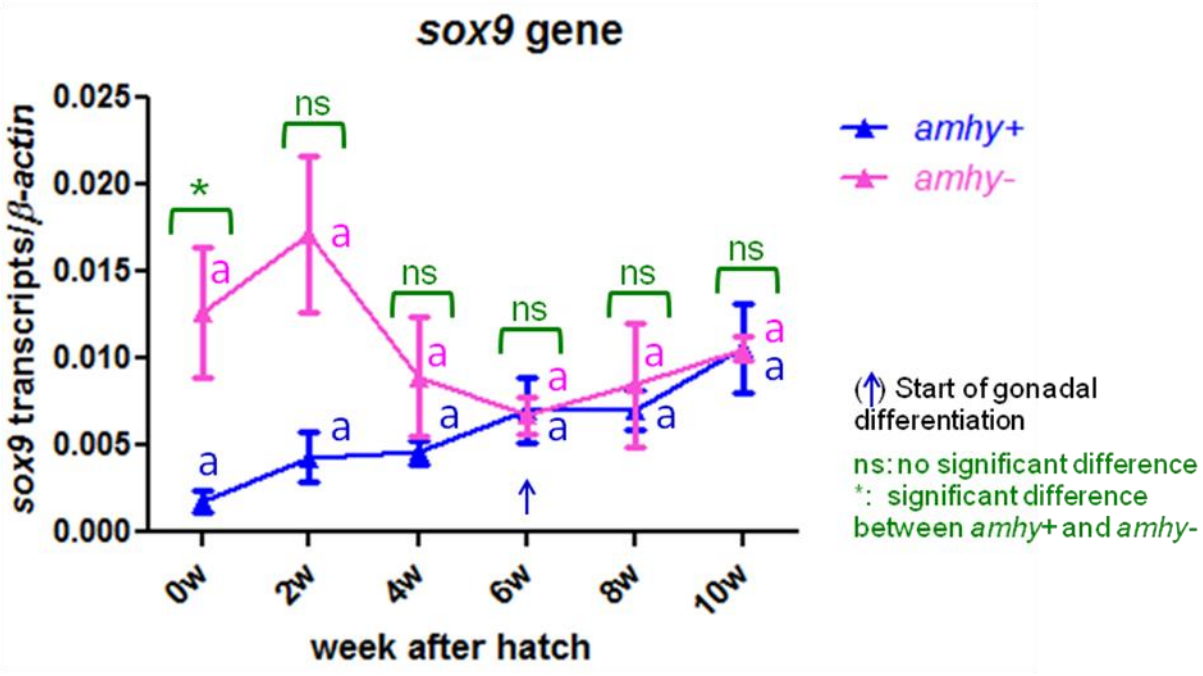

Figure 2 Expression of *sox9* gene in *amhy*+ and *amhy*- individual

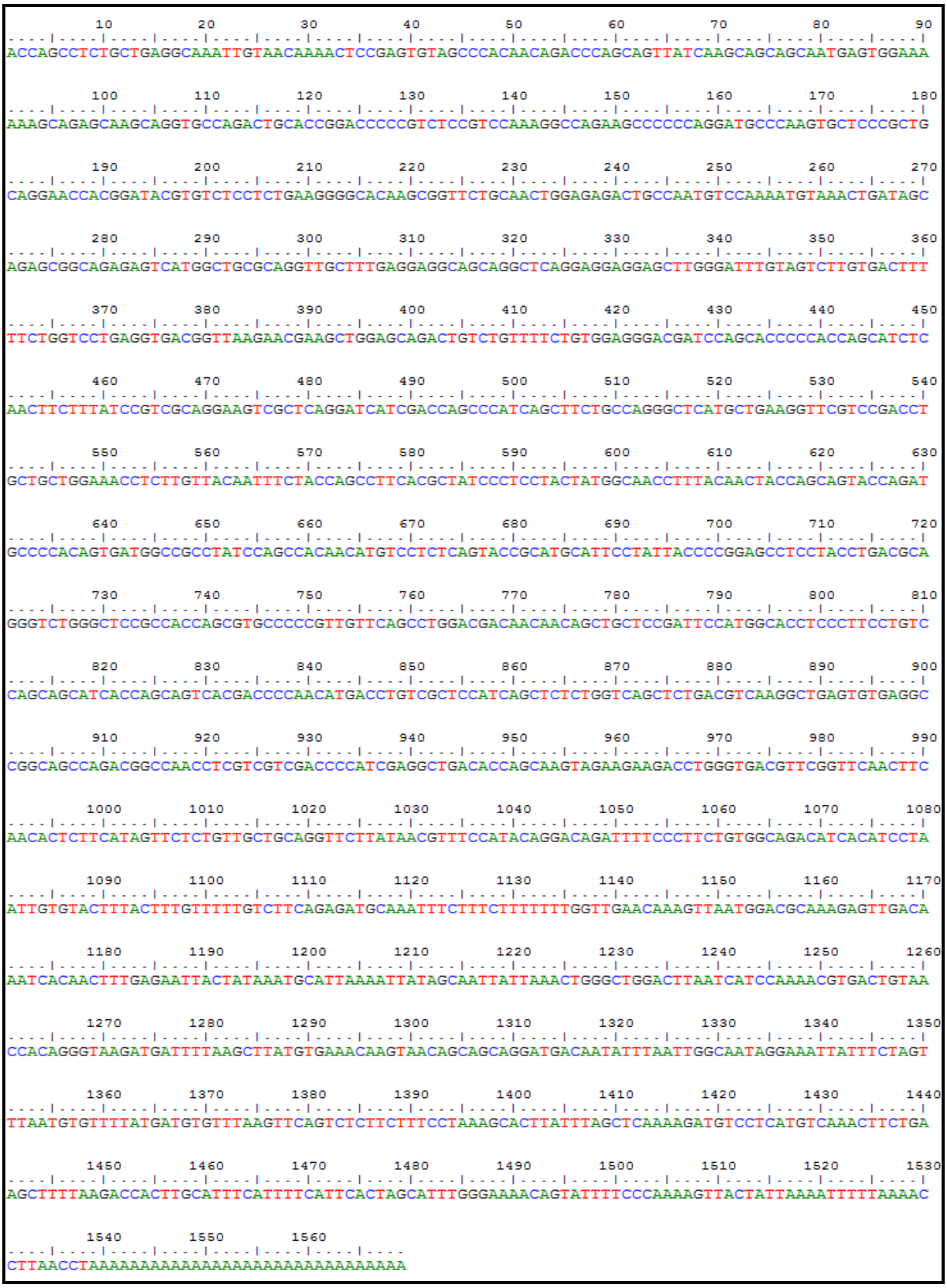

Figure 3 Complete mRNA sequence of *dmrt1* gene in *H. tsurugae*

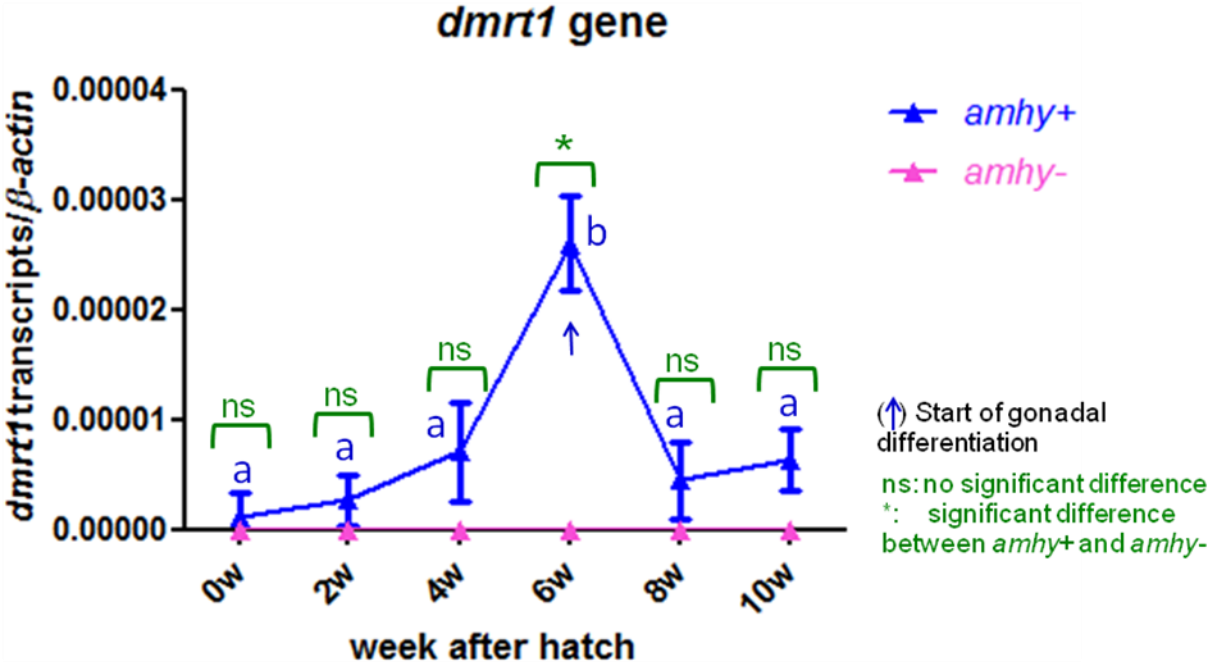

Figure 4 Expression of *dmrt1* gene in *amhy*+ and *amhy*- individual

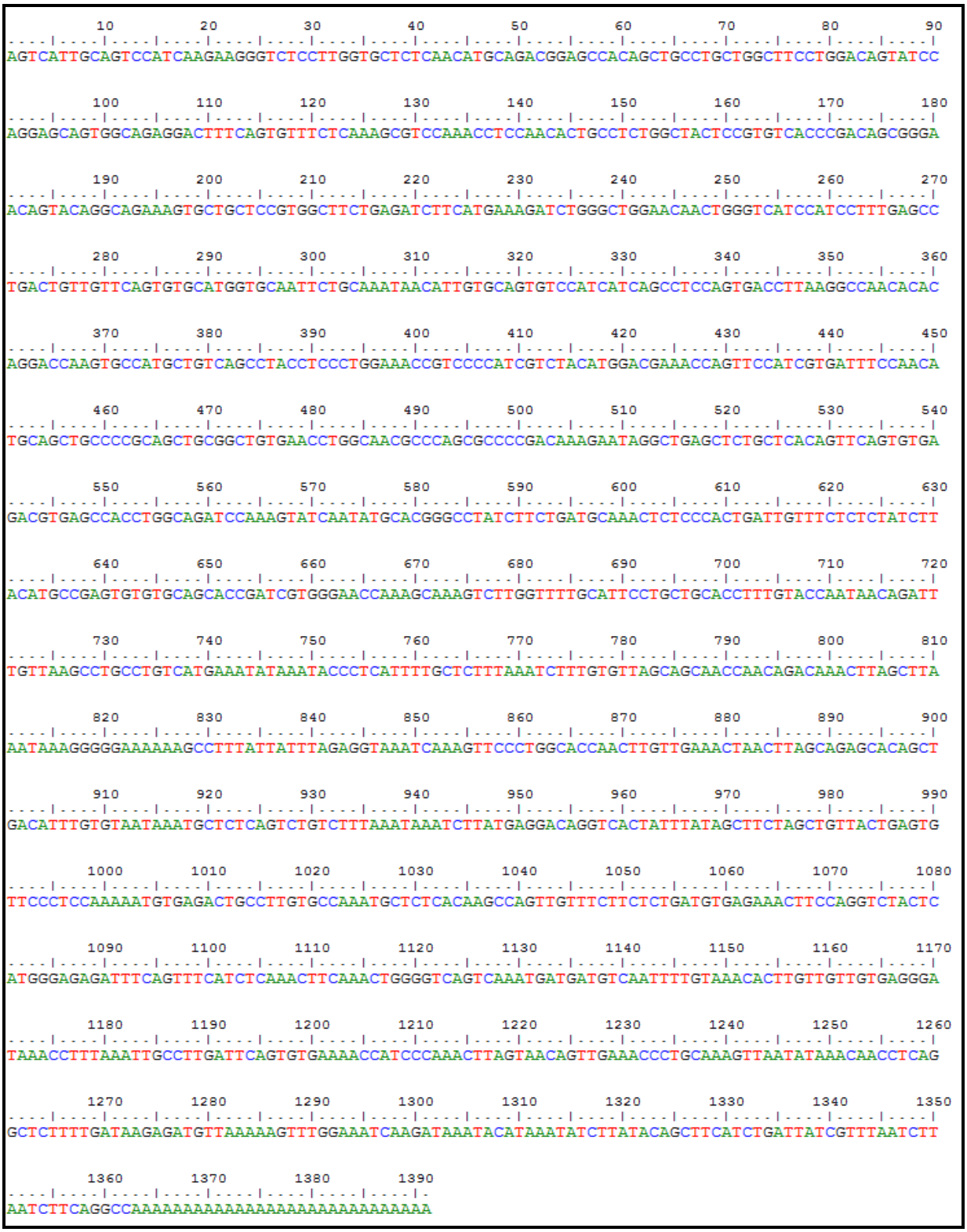

Figure 5 Partial mRNA sequence of *gsdf* gene in *H. tsurugae*

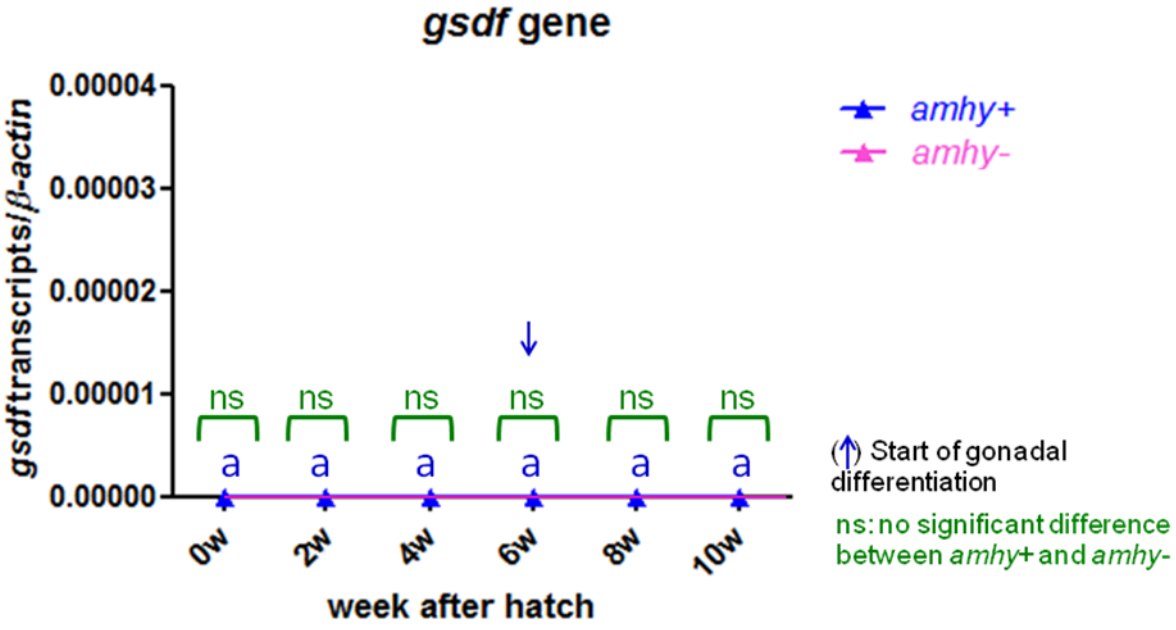

Figure 6 Expression of *gsdf* gene in *amhy*+ and *amhy*- individual

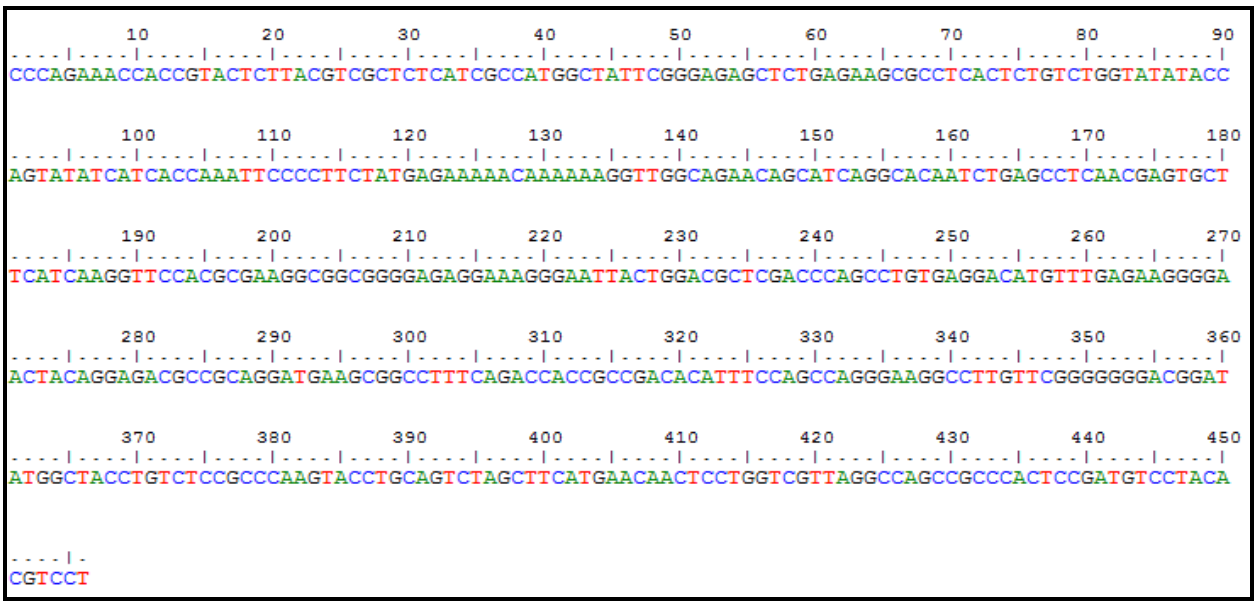

Figure 7 Partial mRNA sequence of *foxl2* gene in *H. tsurugae*

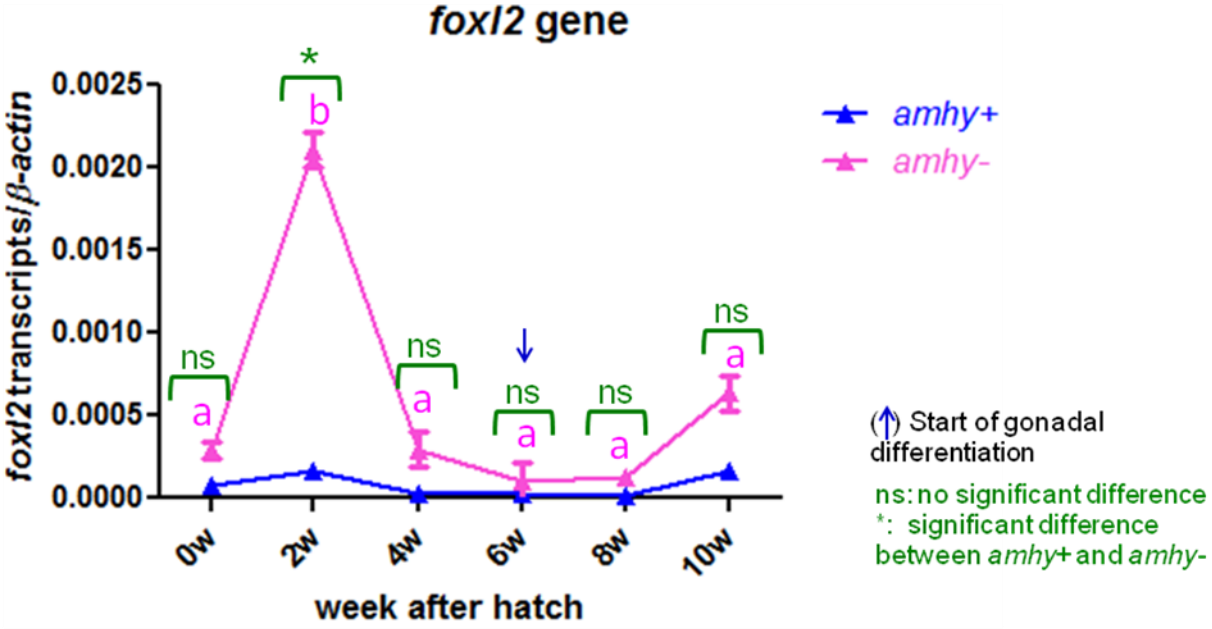

Figure 8 Expression of *foxl2* gene in *amhy*+ and *amhy*- individual

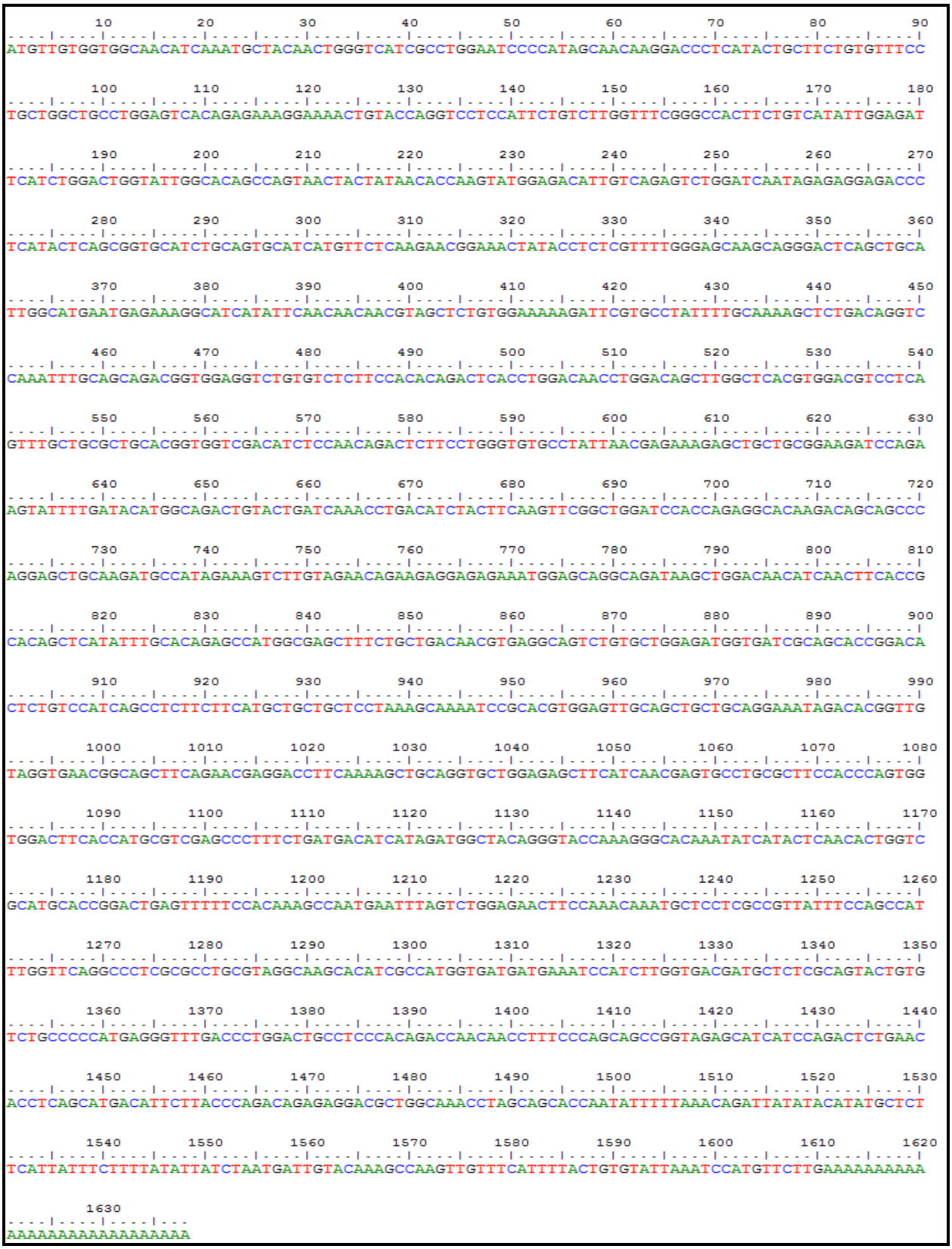

Figure 9 Partial mRNA sequence of *Cyp19a1a* gene in *H.tsurugae*

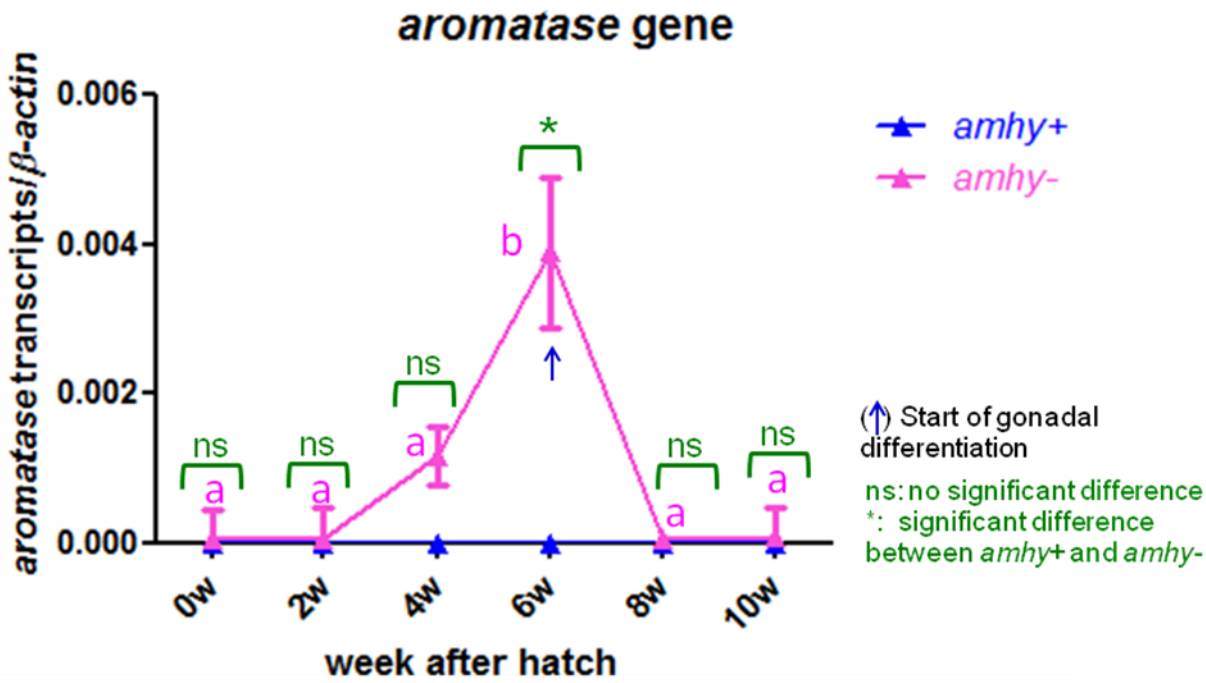

Figure 10 Expression of *aromatase* gene in *amhy*+ and *amhy*- individual

Figure 11 Complete mRNA sequence of *scp3* gene in *H. tsurugae*

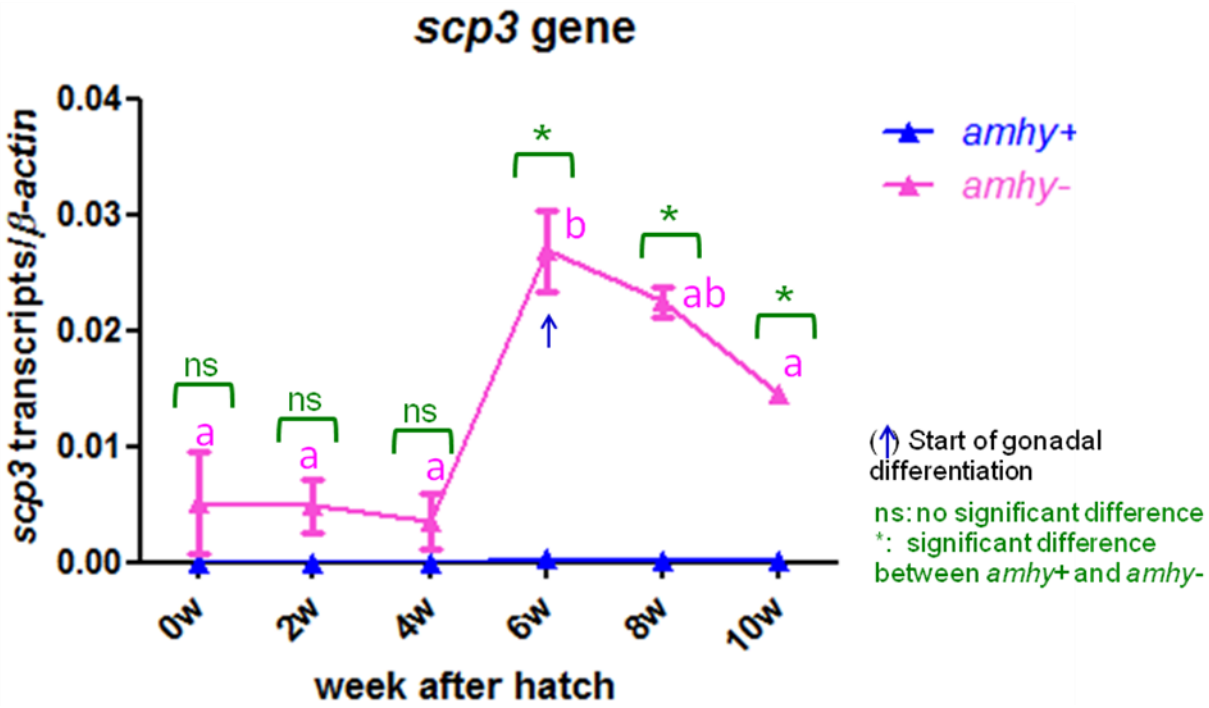

Figure 12 Expression of *scp3* gene in *amhy*+ and *amhy*- individual

General conclusion

From the 1st chapter, the presence of *amhy*, a duplicated Y chromosome-linked *amh* gene, previously known only in Atherinopsidae (New World silversides), is now demonstrated in the cobaltcap silverside *Hypoatherina tsurugae* (Atherinidae). I successfully isolated, cloned and sequenced the *amhy* gene in *H. tsurugae.* The *amha* gene is composed of 2,015 nucleotide bases and seven exons. The TGF-β domain is present in Exon 7 as in other species. The *amhy* gene is composed of 1,838 nucleotide bases and has only 4 exons. Exons 2 and 3 are completely lacking in the *amhy* gene structure. A specific insertion of 195 nucleotide bases is present at the place of exons 2 and 3. The exon 5 sequence is found in genomic sequence but it is not translated. The nucleotide identity between exons of *amha* and *amhy* was more than 80%. The deduced amino acid sequence of Amha (511 aa) and Amhy (340 aa) shared 91% identity. Exons 1, 4, 6 and 7 of *amhy* showed identity to those of *amha* as follows: 71%, 99%, 95% and 81% respectively. Phylogenetic analysis of Amha and Amhy amino acid sequences of *H. tsurugae* and with other species revealed that *H. tsurugae amhy* and *amha* form a clade different from that of *Odontesthes* species *amhy* and *amha*. It signifies they are very far related, although included under same order atheriniformes. In mature domain of both *amha* and *amhy* were compared and found conserved and shared high percentage of homology in both gene. The cysteine knot is also well conserved signifying the signature of TGF-β domain. Though, *amhy* gene lacks three exons still, it may have equivalent potentiality to bind its receptor as like *amha*. The high expression of *amhy* during early larval development, particularly during gonadal sex differentiation, and the high linkage with maleness in captive-reared and wild animals make *amhy* a strong candidate for the sex-determining gene in this species. These results reveal for the first time that *amhy* is

conserved in Old World silversides (Atherinidae) and therefore help shed light on the evolution of genotypic sex determination mechanisms in the order Atheriniformes.

In 2nd chapter, the gene expression profiling in developmental stages showed the fundamental work with a quest of early gonadal differentiation period in *Hypoatherina tsurugae*. The expressions of six potential key sex differentiated genes in *amhy+* and *amhy-* individuals were studied during the early period of gonadal development. All most all genes showed the dimorphic expression in respect to *amhy+* and *amhy-* individuals and an apparent synchronization with *amhy* gene expression but exception to *sox9* and *gsdf* gene. As *sox9* gene is involved in the proliferation of germ cell in both male and female individual during the early developmental stage, therefore it displays the similar type of expression. It is conserved in mammals, birds as well as preserved its structure and function in all teleost fish. Gene *gsdf* express very low in both *amhy+* and *amhy-* individuals. It may be express more in later stage of differentiation period for maintenance of normal function in gonad. The *dmrt1* gene seems to be the only gene whose structure and role are conserved during differentiation and gonad development in males and which has been found throughout vertebrate evolution. It is also concordant with the expression of *dmrt1* gene during the differentiation period of *H. tsurugae*. In females, *foxl2* is one of the earliest markers of ovarian differentiation in vertebrates. *foxl2* directly bind to the promoter region of the cytochrome P450 aromatase (P450arom) gene, resulting in the activation of P450arom transcription. The early expression of *foxl2* in *H.tsurugae* is correlated to aromatase gene; it may be to bind with the promoter of aromatase gene to activate its transcription which essential for the normal function and development of ovary. Thus, analyzing the expression pattern of potential key genes related to sex differentiation give valuable information during the early period of gonadal development in *H.tsurugae*. Future

studies should look in more detail about their relations with as well as their regulation by *amhy* in order to corroborate the status of sex determining gene for *amhy* in *H. tsurugae*.

Future perspective

After successful isolation, cloned and characterization of *amhy* gene, I studied the role of *amhy* gene in early gonadal differentiation period and in relation to other sex related genes. With amalgamation of all results revealed that *amhy* may be a gene of candidate for *H. tsurugae* species. My future plan will be detection of amhy chromosome in the cell by Florescence in situ hybridization (FISH) so that it will be a more supportive data to the *amhy* gene. Next step, it is crucial to know the real function of the amhy gene which can be studied by only the process of gain in function/loss of function. So, I am planning to do the transgenic line of *H. tsurugae* species, so that in female transgenic fish by introducing the *amhy* gene it will develop as male or vice versa. In this way, I can confirm the actual role *amhy* gene during early gonadal developmental period in this species.

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Dilip Kumar Bej – Tokyo, August 31st