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Molecular cloning and mRNA expression pattern of *Sox9* during sex reversal in orange-spotted grouper (*Epinephelus coioides*)

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ABSTRACT

Sox9 is a key gene in male sex determination and gonad development. To study its potential function in the female-to-male sex reversal in orange-spotted grouper, we conducted the following studies. The *Sox9* gene was cloned and full-length sequence of *Sox9* mRNA was determined using the rapid amplification of cDNA ends method. The *Sox9* mRNA consists of 3277 bp in size with a 328 bp 5′ untranslated region and a 1511 bp 3′ untranslated region. The 1437 bp opening reading frame encodes a 479 amino acid protein. The *Sox9* gene contains 3 exons and 2 introns; the beginning and end of both introns conform to the "GT-AG" rule. RT-PCR analysis indicated that *Sox9* mRNA was expressed in brain, kidney, heart, liver, muscle, stomach, intestine, spleen, testis and ovary in adult orange-spotted grouper. Artificial sex reversal was successfully performed by implanting a medicinal strip containing 17α-methyltestosterone into the groupers. By real-time PCR, we found that *Sox9* was weakly expressed in the ovary-stage gonads (before treatment). Once the sex reversal began (1 week after treatment), *Sox9* mRNA expression level significantly increased. However, at 2 weeks after treatment, *Sox9* mRNA expression level significantly decreased to a level that was only slightly higher than that before treatment. *Sox9* mRNA expression increased again at 4 weeks after treatment, and at 6 and 8 weeks, it was maintained at a high level close to that at 1 week after treatment. The results suggest that *Sox9* may be one of the important factors initiating and maintaining the masculinization of orange-spotted grouper during sex reversal.

1. Introduction

Sox (Sry-related high mobility group box) is a gene family encoding a set of transcription factors such as the mammalian male-determining factor SRY which contain the high mobility group (HMG) box. The HMG box is a DNA binding domain of approximately 79 amino acids, which helps SRY and SOX bind to the target DNA. By convention, HMG domains of SOX proteins are at least 50% identical to the HMG domain of SRY. To date, more than 30 members of the *Sox* family have been identified, and they are divided into several subfamilies according to their different structures and functions. *Sox9*, a prominent member of the *Sox* family, was first cloned from an autosomal sex reversal patient accompanied by campomelic dysplasia (Wagner, et al., 1994). *Sox9*, belonging to the *SoxE* subfamily together with *Sox8* and *Sox10* according to Bowles' classification (Bowles, et al., 2000), has been shown to be related to many developmental events and diseases, such as notochord maintenance (Barrionuevo, et al., 2006), prostate development (Thomsen, et al., 2008), and campomelic dysplasia (Lecointre, et al., 2009). The role of *Sox9* in sex determination and gonadal development in vertebrates is a particular focus of study now for many researchers. In mammals and birds, dimorphic *Sox9* expression during the process of sex determination and gonadal development suggest that it may play an important role in these two processes. In mice, *Sox9* expression in the urogenital ridge was limited to a faint diffuse band on the lateral side of the genital ridge in both sexes at 10.5 day post-coitum (dpc); at 11.5 dpc, *Sox9* expression differed strikingly between males and females, with strong expression in male, but not in female genital ridges. In chickens, *Sox9* expression was first detected at 6.5 and 7.5 days of incubation in the male genital ridge, but not detected in the female genital ridge (Kent, et al., 1996). Moreover, gain-and-loss function studies further revealed the role of *Sox9* in sex determination and gonad development in mammals. Using a "yeast artificial chromosome (YAC) knock-in" approach, Vidal et al. (2001) constructed a transgenic mouse model in which *Sox9* expression was controlled by the regulatory region of the Wilms' tumor suppressor gene *Wt1*. *Wt1* was expressed at embryonic day 10.5 (E10.5) similar to *Sox9* in the urogenital region, but its expression was similar between sexes while *Sox9* expression was sexually dimorphic. While *Sox9* was expressed in the urogenital region in both male (XY) and female (XX) type mice in this model, all XX

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transgenic animals (21/21) were phenotypically male and had normal mounting behavior (Vidal, et al., 2001). However, in *Sox9* knock-out XY mouse, not only the formation of testis was suppressed, the female-specific markers such as *Bmp2* and follistatin were also activated (Chaboissier, et al., 2004). Additionally, several male-specific cellular events, such as glycogenesis, coelomic epithelium proliferation, mesonephric migration and vasculogenesis were induced in XY gonads following the onset of *Sox9* expression (Kanai, et al., 2005). All these studies suggest that *Sox9* is an upstream key gene in male determination and testis formation.

Fish have a more complicated mechanism of sex determination and differentiation than mammals, and it is currently a popular animal model in this area of research. Many members of the *Sox* gene family have been studied as potential candidate genes that may drive gonadal sex determination and differentiation in fish. Navarro-Martín et al. (2009) summarized that several *Sox* genes, including Sox5/9a/9b/11/17/23/24, had been found to possess gonadal expression in fish. However, their exact role in sex determination and gonadal differentiation is generally unknown. Nakamoto et al. (2005) studied *Sox9a2* expression in several stages during gonad development in medaka (*Oryzias latipes*). At 3–4 days after fertilization (daf), *Sox9a2* was strongly expressed at similar levels in somatic cells surrounding gonadal primordial germ cells (PGCs), but was not detected in the somatic cells around PGCs that migrated to the gonadal region in both males and females; at 7.5 daf, *Sox9a2* expression was also similar between males and females; at 10 days after hatching (dah), sexually dimorphic expression of *Sox9a2* appeared; at 30 dah, *Sox9a2* expression could only be detected in developing testes but not in developing ovaries (Nakamoto, et al., 2005). Kobayashi's study in Nile Tilapia (*Oreochromis niloticus*) showed that *Sox9a* expression was similar between XX and XY gonads before 25 dah. Thereafter, *Sox9a* expression could only be detected in testes but not in ovaries (Kobayashi, et al., 2008). These results imply that *Sox9* might be important for the formation and development of the testis in fish.

Orange-spotted grouper (*Epinephelus coioides*) is a coral fish of high commercial value, and farming of this pricey fish has been the focus of fin fish mariculture in Southeast Asia and especially in China. Grouper is also a good and popular model for studying sex differentiation and sex reversal because of its protogynous hermaphrodites. As mentioned above, *Sox9* is important in male determination and testis formation in the early development in vertebrates. To understand whether *Sox9* also plays a role in the female-to-male sex reversal, we cloned its mRNA and gene, and quantified its mRNA expression in gonads during induced sex reversal using the orange-spotted grouper model.

2. Materials and methods

2.1. Animals, artificial sex reversal and sample collection

The body weights of the groupers used in the experiments generally ranged from 2.2–2.9 kg, although several were lighter than 2 kg. The artificial sex reversal was performed by implanting medicinal strips containing 17α-methyltestosterone (17α-MT) into the fish as previously described (Zhang, et al., 2007). The implantation procedure was performed on May 16, 2007. Four groupers without implants were taken as controls on that day. Groups of 4 fish were taken at 1, 2, 4, 6, and 8 week(s) after implantation. At each collection time, the fish were decapitated after anesthesia on ice, and the tissue samples were dissected immediately followed by flash-freezing in liquid nitrogen, and finally stored at −80 °C for RNA extraction. Part of the gonads from each fish was fixed in Bouin's solution for histological examination. All animal experiments were conducted in accordance with the guidelines and approval of the Animal Research and Ethics Committees of Chinese Academic of Science.

2.2. Paraffin sectioning and histological analysis

The gonad samples were dehydrated by a series of graded ethanol solutions (70–100%) after fixing, cleared in xylene and embedded in paraffin. Sections were cut (7 μm) and stained with hematoxylin and eosin (H&E).

2.3. Isolation of DNA and RNA and synthesis of cDNA 1st strand

The traditional phenol-chloroform method was used to isolate the tissue DNA. The total RNA was isolated using TRIzol regent (Invitrogen, U.S.A) according to the users' manual. The reverse transcription reaction was performed in a 20 μl volume containing 2 μg of the treated RNA using ReverTra Ace (TaKaRa, Japan) with 5 pmol oligo d (T)-20 primer.. The reaction mixture was incubated 10 min at 30 °C, 30 min at 42 °C, after which the enzymes were inactivated for 5 min at 95 °C.

2.4. 3′ and 5′ RACE and cloning of the Sox9 genomic DNA

The 3′ and 5′ RACE was performed using the GeneRacer Kit (Invitrogen, U.S.A) as described by the manufacturer. The gene-specific primers 3′GSP-1 and 3′GSP-2 for 3′RACE were designed according to part of the sequence of red-spotted grouper (*Epinephelus akaara*) *Sox9* mRNA (GeneBank accession number: AY676309). The RNA used as template for RACE was the RNA pooled from different tissues. After obtaining the 3′ end sequence, the two gene-specific primers for 5′RACE were designed. The primer pair *Sox9*–S1 and *Sox9*–A1 was designed according to the results of the 3′ and 5′ RACE to amplify the *Sox9* genomic DNA. The amplification reaction was performed in a volume of 50 μl containing 0.5 μg genomic DNA as template, 1 μl each primer (100 mM), 1 U Taq polymerase (MBI, Fermentas), 1 μl dNTP (10 mM each), 5 μl 10× buffer for Taq polymerase (MBI, Fermentas). Each PCR cycle included denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min. Thirty-six cycles were performed, followed by a final extension at 72 °C for 5 min. All PCR products of the expected size were directly sub-cloned into the pMD18-T vector (Takara, Japan), and sequenced using an ABI 377 autosequencer.

2.5. Sequence and phylogenetic analyses

Multiple alignments of amino acid sequences were performed using the online tool in EBI (http://www.ebi.ac.uk/Tools/clustalw2/). Phylogenetic trees were generated using MEGA 4 (Tamura, et al., 2007). The data were re-sampled by 1000 bootstrap replicates to determine the confidence indices within the phylogenetic tree.

2.6. RT-PCR

To detect the *Sox9* mRNA expression in different tissues in the adult orange-spotted grouper, cDNA sample of brain, kidney, heart, spleen, stomach, muscle, intestine and liver were used as templates for RT-PCR. The PCR reaction was performed in a volume of 50 μl containing 1 μl cDNA as template, 1 μl each forward primer *Sox9*–S2 and reverse primer *Sox9*–A2 (100 mM), 1 U Taq polymerase (MBI, Fermentas), 1 μl dNTP (10 mM each), 10× buffer for Taq polymerase (MBI, Fermentas). Each PCR cycle included denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. Thirty-two cycles were performed, followed by a final extension at 72 °C for 5 min.

2.7. Real-time PCR

To quantify the *Sox9* mRNA expression level during sex reversal, cDNA samples of gonads in different stages ($n=4$ for each stage)

were used for real-time PCR, and 3 repeats were performed for each sample. Here, we used the standard curve method for relative quantification (Larionov, et al., 2005). The amplification reaction in a volume of 20 μl contained 1 μl cDNA as template, 0.5 μl each forward and reverse primer (100 mM), 10 μl SYBR Green Mix (TOYOBO, Japan) and corresponding volume of water. The primers for *Sox9* used here were the same as the primers used for RT-PCR (*Sox9*–S2 and *Sox9*–A2), and primers for *β-actin* were *β-actin*-S and *β-actin*-A. The reaction conditions for the real-time PCR were: pre-denaturation at 94 °C for 1 min; denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 40 s; 40 cycles were performed. The reaction was performed on the ABI7000 Sequence Detection System (Applied Biosystems, U.S.A). The real-time PCR data were analyzed using the SPSS 15.0 software, and the mean comparisons were performed using the Turkey HSD method ($P<0.05$).

3. Results

3.1. Cloning of orange-spotted grouper Sox9 mRNA

By assembling 3′ and 5′ RACE results, we obtained the full-length sequence of the orange-spotted grouper *Sox9* mRNA (GeneBank accession number: GQ232762). The orange-spotted grouper *Sox9* mRNA is 3277 bp, comprising of a 328 bp 5′ untranslated region and 1511 bp 3′ untranslated region, including a polyadenylation signal (AATAAA) and a poly (A) tail. The 1437 bp open reading frame encodes a 479 amino acid protein. The HMG box of the orange-spotted grouper SOX9 protein is located at 103–173 aa, and it is identical to the HMG box of SOX9 in pejerrey (*Odontesthes hatchery*), mangrove rivulus (*Kryptolebias marmoratus*), zebrafish (*Danio rerio*), and green spotted puffer (*Tetraodon nigroviridis*). Using the Clustal W2 software, we aligned the deduced orange-spotted grouper full-length SOX9 protein sequence with that of other species. The results showed that, in teleost, the homology was 77% to zebrafish SOX9a (AAG09814), 72% to medaka SOX9a (AAX62154), 93% to torafugu SOX9a (AAQ18507), 78% to threespine stickleback SOX9a (AAQ62978); 62% to zebrafish SOX9b (AAG09815), 90% to medaka SOX9b (AAX62154), 74% to torafugu SOX9b (AAQ18508) 92% to threespine stickleback SOX9b (AAQ62979); 93% and 75% to SOX 9a1 and SOX9a2 in rice field eel; in higher vertebrates, the homology was 76% to Africa clawed frog SOX9 (NP_001084276), 73% to chicken SOX9 (AAB09663), 76% to mouse SOX9 (NP_035578) and 75% to human SOX9 (NP_000337) (Fig. 1).

3.2. Gene structure of orange-spotted grouper Sox9

The orange-spotted grouper *Sox9* gene is composed of 2 introns and 3 exons (GeneBank accession number: GU143809). The ends of both intron 1 (381 bp) and intron 2 (304 bp) correspond to the "GT-AG" rule.

3.3. Tissue distribution of Sox9 mRNA in adult orange-spotted grouper

Analysis of the tissue distribution pattern by RT-PCR revealed that *Sox9* was ubiquitously expressed in tissues such as brain, kidney, heart, liver, spleen, muscle, stomach, intestine, testis and ovary. The expression was relatively high in stomach and testis, while it was low in the kidney and heart (Fig. 2).

3.4. Expression of Sox9 mRNA during sex reversal

The histologic sections (Fig. 3A–F) of gonads in different stages showed that the groupers underwent obvious sex reversal. In the groupers without implantation of the medicinal strip (also represented as "week 0"), the gonads were typically ovaries filled with oocytes in different stages (Fig. 3A). The *Sox9* mRNA expression was low at this time (Fig. 4). At 1 week after treatment, many spermatocytes appeared in the gonads; meanwhile, there were also few degenerating oocytes (Fig. 3B), and the *Sox9* mRNA expression increased remarkably compared to the expression at week 0 (Fig. 4). At 2 weeks after treatment, the oocytes could not be clearly detected, and many spermatocytes and spermatids were found in the gonads (Fig. 3C); however, the *Sox9* mRNA expression was significantly lower than the expression at 1 week after treatment, falling to a similar level as that at week 0 (Fig. 4). The gonads at 4 and 6 weeks after treatment were similar. At this time, the gonads became slack, and were filled with spermatocytes in different developmental stages and spermatids (Fig. 3D and E). The *Sox9* mRNA expression increased again at 4 weeks after treatment, and at 6 weeks after treatment, the expression reached a similar expression level as that at 1 week after treatment (Fig. 4). At 8 weeks after treatment, the gonads became compact again (Fig. 3F), but the *Sox9* mRNA expression was kept at a high level similar to the levels at 1 and 6 week (s) after treatment (Fig. 4).

4. Discussion

In the present study, we cloned the *Sox9* mRNA and gene in orange-spotted grouper. The *Sox9* mRNA is 3277 bp encoding a 479 aa protein, and the *Sox9* gene contains 2 introns and 3 exons. The SOX9 protein contains 479 aa, and the HMG box is located at 103–173 aa. This 70 aa HMG box is identical with that of pejerrey, mangrove rivulus, zebrafish, and green spotted puffer. Phylogenetic analysis based on full-length SOX9 protein of many species revealed that, in teleost, SOX9 could be grouped into two main clusters (Fig. 5). One cluster included the orange-spotted grouper SOX9, threespine stickleback SOX9b, torafugu SOX9a, rice field eel SOX9a1, medaka SOX9b and zebrfish SOX9b, and the other cluster included zebrfish SOX9a, torafugu SOX9b, threespine stickleback SOX9a, medaka SOX9a and rice field SOX9a2. The results of the phylogenetic analysis would seem confusing because there are proteins named "SOX9a" and "SOX9b" in each cluster. At present, there is not a recognized terminology for naming *Sox9* genes and proteins. Different numbers of *Sox9* subtypes exist in different species. Searching "*Sox9*" in NCBI, only one type of *Sox9* can be found in higher vertebrates such as human, mouse and chicken, while two types can be found in lower vertebrates such as teleost. On the other hand there is not a recognized principle to classify the *Sox9* subtypes, and the two types of *Sox9* in teleost may be due to the fish special genome duplication (FSGD) (Meyer and Van de Peer, 2005; Volff, 2005). We presume that there were also two types of *Sox9* in grouper, but only one type was cloned in this study, and we cannot classify it as a *Sox9a* or *Sox9b* subtype based on the phylogenetic analysis. Therefore, we only named the cloned gene "orange-spotted grouper *Sox9*", although we recognize that there may potentially be another Sox9 gene in grouper (Table 1).

In our study, *Sox9* was ubiquitously expressed in tissues such as brain, kidney, heart, liver, spleen, muscle, stomach, intestine, testis and ovary in adult grouper. The *Sox9* expressions in various adult tissues have been reported, but the conclusions of the studies are not consistent even in the same species. Here, we focused on its expression pattern in gonads. Klüver, et al. (2005) showed that *Sox9a* and *Sox9b* were both expressed in medaka testis and ovary. However, Yokoi et al. (2002) and Nakamoto et al. (2005) who also studied medaka gave inconsistent results. Zhou et al. cloned two *Sox9* genes in rice field eel (*Monopterus albus*) named *Sox9a1* and *Sox9a2*, which were both expressed in testis and ovary (Zhou, et al., 2003). There were also two *Sox9* genes cloned in yellow catfish (*Pelteobagrus fulvidraco*), and these were also named *Sox9a1* and *Sox9a2*; the *Sox9a1* was expressed in both ovary and testis, and *Sox9a2* was expressed in ovary but not in testis (Yu et al., 2005) Table 2 summarizes the results mentioned above to illustrate that the two *Sox9* subtypes may exert distinct functions in gonads but detailed mechanisms are still unknown.

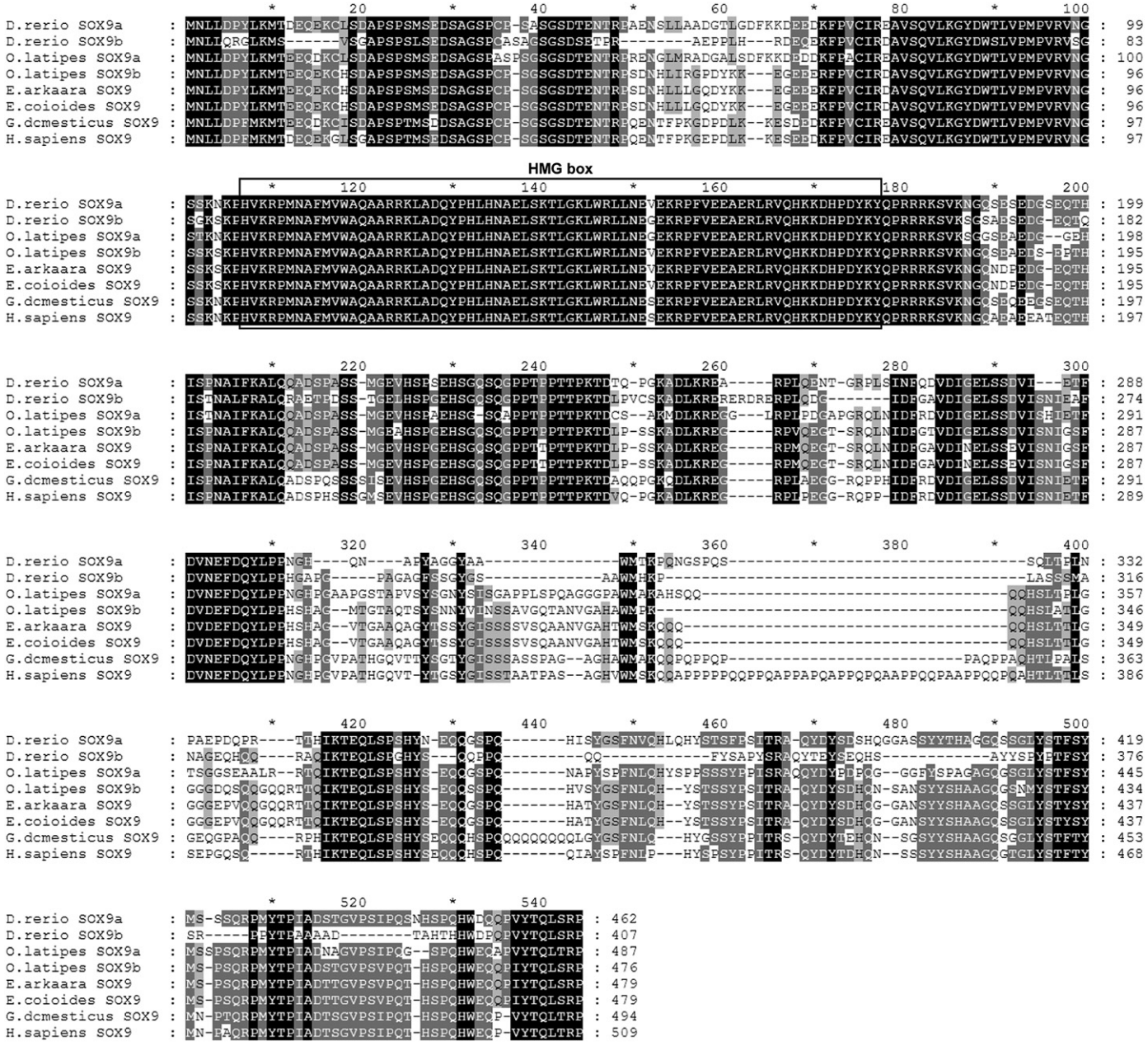

Fig. 1. Amino acid sequences comparison of orange-spotted grouper SOX9 with other orthologs. Sequences in black background indicate the conservative sequence. The black box indicates the HMG box domain.

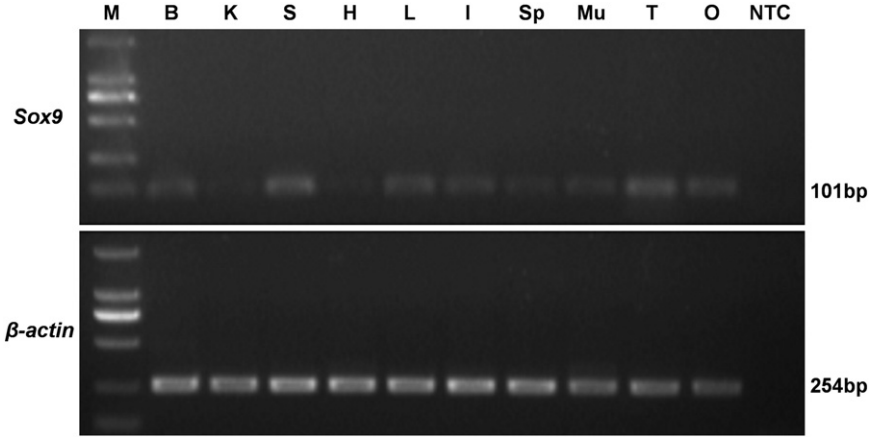

Fig. 2. Tissue distribution of *Sox9* mRNA expression. B. brain, K. kidney, S. stomach, H. heart, L. liver, I. intestine, Sp. spleen, Mu. muscle, T. testis, O. ovary, NTC. no template control.

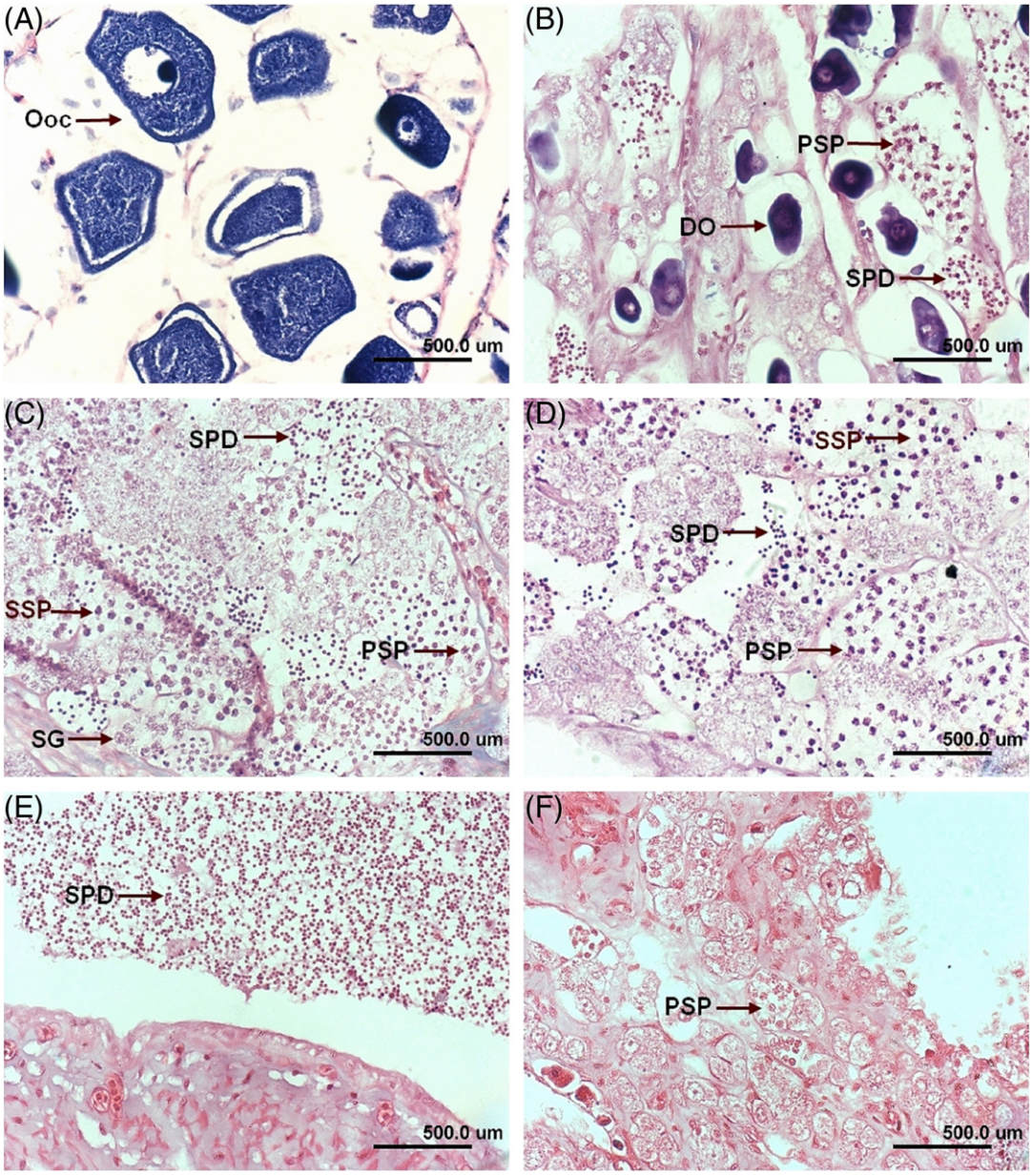

Fig. 3. Images of the gonads of orange-spotted grouper in different stages of sex reversal. A: untreated B–F: 1, 2, 4, 6 and 8 weeks after treatment. Ooc: Oocyte, DO: degenerating oocyte, SP: spermatocyte, SG: spermatogonia. The scale bar represents 500 μm.

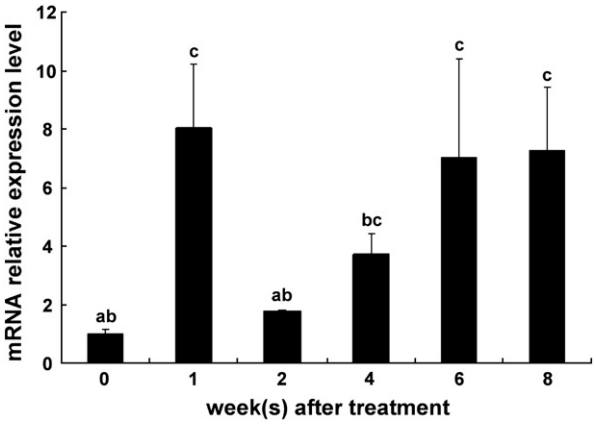

Fig. 4. Change of relative Sox9 mRNA expression during sex reversal. Bars with the same letter indicate no significantly difference from one another ($P<0.05$). Data are given as mean ± S.E.M.

At present, there are many studies focused on sex determination and gonad development in early development, but few on the sex reversal of adult fish. Kobayashi et al. (2008) performed both male-to-female and female-to-male sex reversal in Nile tilapia *(Oreochromis niloticus)* fry by hormone treatment, and studied the *Sox9a* expression pattern before and after the sex reversal. Their results suggested that *Sox9a* was not affected by hormone treatment, and like the normal fry, the sexual dimorphic expression of *Sox9a* was seen 25 days after hatching. Unfortunately, the details of this finding were not shown in their report (Kobayashi, et al., 2008). However, our study showed that the *Sox9* mRNA expression obviously changed after hormone treatment in adult groupers. Our observations of the *Sox9* mRNA expression pattern before and after sex reversal was also not consistent with that of Kobayashi et al. (2008), which may be caused by the different responses of exogenous hormone to the immature gonad of tilapia fry used in their study and the mature gonads of grouper used in our study.

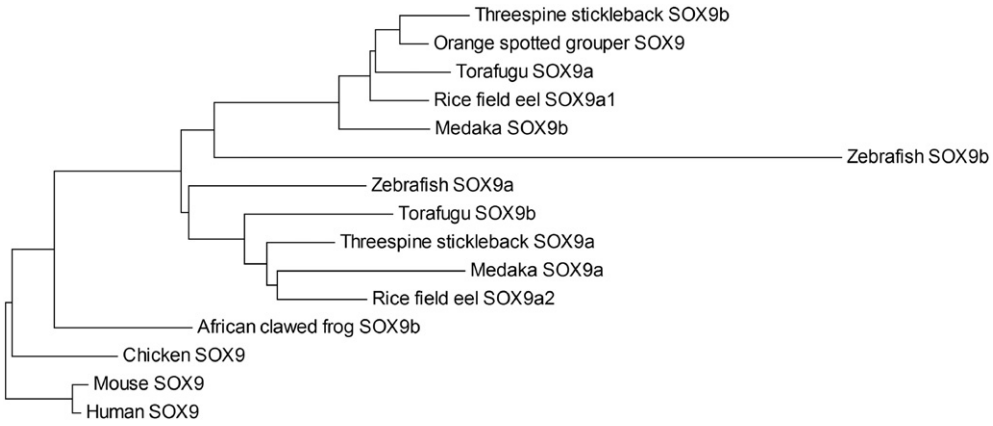

Fig. 5. A neighbor-joining tree based on multiple SOX9 protein sequences, structured by MEGA4.

The sudden increase of *Sox9* mRNA expression at the beginning of sex reversal (1 week after treatment) suggested that *Sox9* was quite likely involved in the initiation of masculinization in grouper. *Sox9* is thought to be the marker gene of Sertoli cells (Hemendinger, et al., 2002). Sertoli cells can produce the anti-Müllerian hormone (AMH), inhibin, activin, androgen binding protein and glial cell line-derived neurotrophic factor (GDNF), and play an important role in spermatogenesis (Boukari, et al., 2009). The increase of *Sox9* mRNA expression may be caused by the re-differentiation and proliferation of Sertoli cells or the enhancement of *Sox9* transcription in Sertoli cells. Therefore, Sertoli cells may play an important role in the sex reversal in grouper.

At 2 weeks after treatment, *Sox9* mRNA expression decreased sharply compared to the expression level at 1 week after treatment. This decline may be due to the negative feedback regulation of SOX9 protein to *Sox9* transcription. The *Sox9* transcription would be repressed when the amount of intracellular SOX9 protein reaches a certain level (Polanco and Koopman, 2007). However, this hypothesis is still needs to be confirmed by further experiments. At the beginning of sex reversal, high levels of SOX9 protein accumulate in the gonad because of the increase of *Sox9* mRNA expression. As a result, the *Sox9* mRNA expression decreases by the negative feedback regulation. Finally, the gonads permanently become testes, and the *Sox9* mRNA expression is maintained at a relatively high level.

Sox9 is involved in many signaling pathways underlying the sex determination and gonad development in both embryo and adult. In mammals, the fate of the primordial gonad is thought to be controlled by the antagonistic regulation between the *Wnt4* and *Fgf9* signal pathways, and the balance between the two pathways is mainly effected by the expression of *Sox9* (Kim, et al., 2006). Recently, *FOXL2* was thought to be involved in the somatic reprogramming of adult ovaries to testes by influencing *Sox9* expression (Uhlenhaut et al., 2009). Our study suggested that the occurrence of sex reversal may be also due to the change of a *Sox9*-related pathway in the gonads in adult grouper. Therefore, discovering this pathway may be the key to understanding the mystery of sex reversal in grouper.

Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)	Purpose
3′GSP-1	CCAAATGCCATATTCAAGG	3′ RACE first round
3′GSP-2	AGCGAGGTCATCTCCAACA	3′ RACE second round
5′GSP-1	CCTTGAATATGGCATTTGG	5′RACE first round
5′GSP-2	TGCCCGTTCTTGACAGAT	5′RACE second round
Sox9–S1	TACTTCAACGATTCAACTT	To amplify the *Sox9* gene
Sox9–A1	GTGTCATGGAGGCACAAA	To amplify the *Sox9* gene
Sox9–S2	GGAAGTCAGAACTACGCTAT	RT-PCR and real-time PCR
Sox9–A2	CTGGGTACTGCCTTGAT	RT-PCR and real-time PCR
β-actin-S	CCCATCTACGAGGGCTAT	RT-PCR and real-time PCR
β-actin-A	ATGTCACGCACGATTTCC	RT-PCR and real-time PCR

Table 2
Sox9 expression in ovaries and testes in different species.

Species	Gene	Testicle	Ovary	Detection methods	Reference
Rainbow trout	*Sox9*	+	−	Northern	Takamatsu et al., 1997
Zebrafish	*Sox9a*	+	−	RT-PCR	Chiang, et al., 2001
	Sox9b	−	+		
Medaka	*Sox9*	*	+	Northern, in situ	Yokoi et al., 2002
Rice field eel	*Sox9a1*	+	+	RT-PCR	Zhou, et al. 2003
	Sox9a2	+	+		
Medaka	*Sox9a*	+	+	RT-PCR, in situ	Klüver et al., 2005
	Sox9b	+	+		
Medaka	*Sox9a2*	+	−	RT-PCR	Nakamoto, et al., 2005
Yellow catfish	*Sox9a1*	+	+		Yu et al., 2005
	Sox9a2	−	+		

*Expression could only be detected by RT-PCR, could not be detected by Northern and in situ.

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