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Quantitative mRNA expression of *sox3* and DMRT1 during sex reversal, and expression profiles after GnRHa administration in black porgy, *Acanthopagrus schlegeli*

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ABSTRACT

We cloned full-length *sox3* cDNA from testis of black porgy, *Acanthopagrus schlegeli*. Black porgy *sox3* cDNA consists of 897 base pairs (bp) and encodes a protein of 298 amino acids. We have investigated the expression pattern of *sox3* and DMRT1 mRNA during the sex-reverse process from male to female (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary and ovary). The expression of *sox3* and DMRT1 mRNA was high in mature testis of black porgy during sex-reverse process. In a histological analysis, testicular portion of gonad was degenerated and the ovary portion was increased during sex reversal from male to female, and then oocytes were increased in ovary. Also we examined the expression of *sox3* and DMRT1 mRNA after gonadotropin-releasing hormone analogue (GnRHa) treatment in immature black porgy. The expression of *sox3* and DMRT1 mRNA was increased after GnRHa treatment (*in vivo* and *in vitro* experiment) in immature black porgy. Therefore, we concluded that *sox3* and DMRT1 were involved in the development of testis than ovary in black porgy.

1. Introduction

The *Sry*-related higher mobility group [HMG]-box (*sox*) genes are well known transcriptional regulators involved in the decision of cell fates during developmental processes as well as the development of the central nervous system, differentiation and growth (Pevny and Lovell-Badge, 1997). *Sox* genes were detected in various cells and tissues concerning the regulation of diverse development processes such as the formation of genital gland and organ development (Lefebvre et al., 1998). *Sox* genes were first cloned from mouse, *Mus musculus* and human, *Homo sapiens* and there are twenty *sox* genes in vertebrate to date, and also *sox* isoforms (etc., *sox3*, -9, -10, -11, -19 and -24) were detected in fish (Bowles et al., 2000). Among the *sox* family genes which are conserved HMG-box domain, sex determining region of the Y chromosome (*Sry*) gene was first detected and is located in the Y chromosome and was known as the testis (male)-determining factor in mammals (Graves, 1998). *Sox* genes were cloned based on the HMG-box domain of *Sry* genes (Sinclair et al., 1990). For example, *sox3* obtained in this study was cloned from the HMG-box domain of *Sry* gene, and *sox3* and *Sry* have overlapping DNA-binding specificity (Bergstrom et al., 2000). This feature, combined with the evolutionary relationship of these proteins, led to the hypothesis that *sox3* might be involved in sex-determination similar to *Sry* gene (Graves, 1998). Moreover, *sox3* is important for normal oocyte development and male testis differentiation and gametogenesis in mouse (Weiss et al., 2003). Raverot et al. (2005) found that *sox3* knock-out (KO) mice lead to defects of spermatozoa formation, testis development and maturity. Therefore we supposed the hypothesis that *sox3* might be involved in male-specific development (Graves, 1998; Weiss et al., 2003). Many studies have demonstrated the involvement of other genes of the *sox* family as well as *sox3* in gonadal development (sex differentiation, maturation and/or sex changes). For example, *sox17* in the rice field eel, *Monopterus albus* (Wang et al., 2003) and in the sea bass, *Dicentrarchus labrax* (Navarro-Martín et al., 2009), and *sox11b* in the orange-spotted grouper, *Epinephelus coioides* (Zhang et al., 2008). Through these studies, we found that *sox* genes were involved in gonadal development.

With *sox3*, it is well known that doublesex- and mab-3 related transcription factor (DMRT) plays a role in fish sex differentiation and male-specific differentiation (Fernandino et al., 2006). DMRT encodes a putative transcription factor containing a DNA-binding motif (DM) domain, first identified in the *Drosophila melanogaster* doublesex (dsx) (Erdman and Burtis, 1993) and *Caenorhabditis elegans* mab-3 (Raymond et al., 1998) genes. To date, the various DMRT genes (DMRT1, -2, -3, -4, -5, -6 and -7) were reported in vertebrate including fish (Hong et al., 2007). Among these genes, DMRT1 is only expressed in testis of fish such as rainbow trout, *Oncorhynchus mykiss* (Marchand

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et al., 2000), zebrafish, *Danio rerio* (Gao et al., 2005), Pejerrey, *Odontesthes bonariensis* (Fernandino et al., 2006, 2008), olive flounder, *Paralichthys olivaceus* (Jo et al., 2007), also in testis of vertebrate such as mammalian, birds (Smith et al., 1999; Raymond et al., 1999) and reptilian (Smith et al., 1999). So we concluded that DMRT1 was involved in testicular differentiation.

GnRH stimulates the pituitary to promote the synthesis and excretion of GTH subunits for playing the critical role of sex differentiation in the gonochorism and hermaphrodite fish (Dubois et al., 2002). For example, European sea bass, *D. labrax* developed from immature to male or female between 200 and 250 days after hatching, and GnRH was increased in brain of European sea bass in this point of time (Moles et al., 2007). In addition, in our previous study using black porgy, *Acanthopagrus schlegeli*, GnRH isoforms mRNA expressed high in mature male and female gonad, and pituitary GTH subunit mRNA expression pattern was increased in immature black porgy treated with GnRHa (An et al., 2008b). So we hypothesized that GnRH might affect gonadal development and stimulate the expression of *sox3* and DMRT1 mRNA in gonad of immature black porgy.

Black porgy is a widely distributed marine protandrous hermaphrodite and a candidate for commercial aquaculture in various parts of Asia including Korea. Black porgy is a functional male for the first 2 years of life, and then about 70% of males change sex to female during their third spawning season. So, black porgy is of utility value for the study on sex-reverse mechanism from male to female. To date, despite many studies on sex reverse of black porgy (Chang et al., 1994; Lee et al., 2001; An et al., 2008a,b), investigating the cloning and expression of *sox3* and DMRT1 during sex reverse is lacking for protandrous hermaphrodite fish.

Hence, we cloned the full-length *sox3* cDNA from testis of male black porgy, and investigated the expression of *sox3* and DMRT1 in gonads during the sex-reverse process as part of the reproduction mechanism of black porgy. Also, to understand the role of gonadotropin-releasing hormone, we investigated the expression of *sox3* and DMRT1 mRNA by *in vivo* (GnRHa injection) and *in vitro* (tissue culturing) experiments in immature black porgy.

2. Materials and methods

2.1. Experimental fish

The study was carried out on immature male (51.0 ± 2.3 g, 1-year; yr), mature male (220.0 ± 14.2 g, 2-yr), sex changing male (489.2 ± 11.5 g, 3-yr) and female black porgy (948.5 ± 51.6 g, 4-yr). Sexual maturity was determined via gonadal examination upon excision. Maturity was designated by the presence of ova and sperm. All fish were netted and anesthetized in tricaine methane sulfonate (MS-222, Sigma-Aldrich, USA) prior to tissue collection. Gonad samples ($n = 3$) from black porgy at each gonad maturity stage (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary and ovary) were removed, immediately frozen in liquid nitrogen and stored at −80 °C until the total RNA was extracted for analysis.

2.2. Identification of sox3 cDNA

Primers for *sox3* were designed using highly conserved regions of barramundi perch, *Lates calcarifer sox3* (GenBank accession no. DQ915951) and orange-spotted grouper, *E. coioides sox3* (DQ219298): *sox3* forward primer (5′-GCT GAC TGG AAA CTT CTG AC-3′) and *sox3* reverse primer (5′-ACA TCA TGG GGT ACT GGA G-3′). Total RNA was extracted from the gonads using a TRIzol kit (Gibco/BRL, USA). Reverse transcription (RT) was performed using M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed using a 2× Taq Premix I (Solgent, Korea). PCR was carried out as follows: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 20 s, annealing at 56 °C for 40 s, and extension at 72 °C for 1 min; followed by 7 min at 72 °C for the final extension. The amplified PCR product was separated on 1% agarose gels. The PCR product was purified and ligated into pGEM-T Easy Vector (Promega, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Korea), and plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea). Based on the plasmid DNA, *sox3* cDNA sequence data was analyzed using an ABI DNA Sequencer (Applied Biosystems, USA).

2.3. Sox3 rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the *sox3* RACE reaction, total RNA was extracted from the gonads using a TRIzol kit (Gibco/BRL, USA). Using 2.5 μg of total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo (dT) anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA $T(T)_{18}$-3′) and a CapFishing™ adaptor (Seegene, Korea).

Sox3-specific primers were selected from the PCR product by RT-PCR. For 3′ RACE, the 50-μL PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 mM 3′ RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 mM 3′ RACE *sox3*-specific primer (5′-CCT ACC CTC AGC ATC ACA GCA TGA ACA G-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was performed as follows: initial denaturation at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 1 min; followed by 5 min at 72 °C for the final extension.

For 5′ RACE, the 50-μL PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 mM 5′ RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 mM 5′ RACE *sox3*-specific primer (5′-CAG CGT CGG TCA GAA GTT TCC AGT CAG-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 1 min; followed by 5 min at 72 °C for the final extension. The amplified PCR product was processed by electrophoresis in 1% agarose gels. Transformation and sequencing were performed as described above.

2.4. Phylogenetic analysis

Group B1 *sox* phylogenetic analysis was conducted using other known species of *sox* amino acid sequences aligned using BioEdit software (Hall, 1999). The sequences compared to black porgy *sox3* were as follows: zebrafish *sox1* (zf*sox1*, NM_001002483), Western clawed frog, *Xenopus (Silurana) tropicalis sox1* (wc*sox1*, BC159121), chicken, *Gallus gallus sox1* (ck*sox1*, AB011802), house mouse *sox1* (hs*sox1*, NM_009233), human *sox1* (hm*sox1*, NM_005986), Japanese medaka, *Oryzias latipes sox2* (jm*sox2*, NM_001104764), zebrafish *sox2* (zf*sox2*, NM_213118), Western clawed frog *sox2* (wc*sox2*, NM_213704), chicken *sox2* (ck*sox2*, NM_205188), house mouse *sox2* (hs*sox2*, NM_011443), pig, *Sus scrofa sox2* (pg*sox2*, EU519824), human *sox2* (hm*sox2*, NM_003106), black porgy *sox3* (bp*sox3*, EF605272), barramundi perch *sox3* (bm*sox3*, DQ915951), orange-spotted grouper *sox3* (og*sox3*, DQ219298), Japanese medaka *sox3* (jm*sox3*, AJ245396), zebrafish *sox3* (zf*sox3*, AB117960), goldfish *sox3* (gf*sox3*, EF174418), Wrinkled Frog, *rana rugosa sox3* (wf*sox3*, AB295441), African clawed frog, *Xenopus laevis sox3* (af*sox3*, NM_001090679), zebra finch, *Taeniopygia guttata sox3* (zb*sox3*, DQ206644), chicken *sox3* (ck*sox3*, AB011803), house mouse *sox3* (hs*sox3*, NM_009237) and human *sox3* (hm*sox3*, NM_005634). The phylogenetic tree was constructed using the neighbor-joining method with the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

2.5. Gonad histological analysis

The gonad tissues from each developmental stage (mature testis, testicular and ovarian portions of the sex changing gonad and ovary) for the analysis of gonads during sex change were fixed in Bouin's solution. The samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin. Sections (5 μm thick) were selected and stained with haematoxylin–eosin for observation under a light microscope (Leica DM 100, Germany) and images were captured with a digital camera (Leica DFC 290, Germany).

2.6. In vivo experiment (GnRHa injection)

GnRHa (des Gly^{10}-[$D\text{-}Ala^{6}$] LHRH ethylamide, Sigma, USA) was dissolved and diluted in 0.9% physiological saline. After anesthesia, the immature black porgy (51.0 ± 2.3 g, 1-yr) were injected at a dose of 0.2 μg GnRHa/g body mass (BW) at volume of 1 μL/g BW. After injection, gonads were sampled from five fish at each of the following time periods: 0, 6, 12, 24 and 48 h. All of the fish survived the experimental period.

2.7. In vitro experiment (tissue culturing by treatment GnRHa)

After gonad tissues were removed from ten immature black porgy, the gonad tissues were placed in a 24-well culture plate (SPL Life Sciences, Korea). The gonad tissues were treated with GnRHa 10^{-5} and 10^{-3} M in medium 199 (Invitrogen, USA) according to the manufacturer's instructions, and cultured for 0, 6, 12, 24 and 48 h. Testis was cultured under this condition for 48 h in an incubator at 18 °C, 100% humidity, and 5% CO_2 in air. Following the incubation period, each sample was centrifuged (20 °C, 10,000 *g*, 15 s) and supernatant was removed, and stored in individual micro-centrifuge tubes at −80 °C.

2.8. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of *sox3* and DMRT1 mRNA in total RNA extracted from the gonads. Primers for QPCR were designed with reference to the known sequences of black porgy (EF605272 (*sox3*); AY323953 (DMRT1)) as follows: *sox3* forward primer (5′-AGT GTC AGC GAC CAG GAG CG-3′), *sox3* reverse primer (5′-TTT CCA GTC AGC CCC GAG C-3′), DMRT1 forward primer

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bpsox3   1:MYSMMETEIKTPLPQ--SGSAQGAKNNSVSDQERVKRPMNAFMVWSRGQRRKMAQENTKM  58
bmsox3   1:MYNMMETEIKTPLPQSNSGSAPGAKNNSASDQERVKRPMNAFMVWSRGQRRKMAQENPKM  60
ogsox3   1:MYNMMETELKTPLPQSNSGSAPGAKNNSASDQERVKRPMNAFMVWSRGQRRKMAQENPKM  60
jmsox3   1:MYNMMETEIKTPLPQSNSGSAAGAKNNSANDQERVKRPMNAFMVWSRGQRRKMAQENPKM  60
zfsox3   1:MYNMMETEIKSPIPQSNTGSVTGGKNNSANDQDRVKRPMNAFMVWSRGQRRKMAQENPKM  60
                                           *********

bpsox3  59:HNSEISKRLGADWKLLTDAEKRPFIDEAKRLRAMHMKEHPDYKYRPRRKTKTLLKKDKYS 118
bmsox3  61:HNSEISKRLGADWKLLTDAEKRPFIDEAKRLRAMHMKEHPDYKYRPRRKTKTLLKKDKYS 120
ogsox3  61:HNSEISKRLGADWKLLTDAEKRPFIDEAKRLRAMHMKEHPDYKYRPRRKTKTLLKKDKYS 120
jmsox3  61:HNSEISKRLGADWKLLTDAEKRPFIDEAKRLRAMHMKEHPDYKYRPRRKTKTLLKKDKYS 120
zfsox3  61:HNSEISKRLGADWKLLTDAEKRPFIDEAKRLRAMHMKEHPDYKYRPRRKTKTLLKKDKYS 120

bpsox3 119:LPGGLLAPGANAVNNSVSVGQRMDGYAHMNGWTNSAYSLMQDQLAYPQHHSMNSPQIQQM 178
bmsox3 121:LPGGLLPPGANAVNNSVSVGQRMDGYAHMNGWTNSAYSLMQDQLAYPQHHSMNSPQIQQM 180
ogsox3 121:LPGGLLAPGANAVNNSVSVGQRMDGYAHMNGWTNSAYSLMQDQLAYPQHHSMNSPQIQQM 180
jmsox3 121:LPGGLLAPGANAVNNSVSVGQRMDGYAHMNGWTNSAYSLMQDQLAYPQHPSMNSPQIQQM 180
zfsox3 121:LPGGLLAPGANAVNNAVSVGQRMD-YTHMNGWTNSAYSLMQDQLAYPQHPSMNSPQIQQM 179

bpsox3 179:HRYEMAGLQYPMMSSAQTYMNAASTY-SMSPAYTQQTGSAMGLSSMASVCKTEPSSPPPA 237
bmsox3 181:HRYEMAGLQYPMMSSAQTYMNAASTY-SMSPAYTQQTTSAMGLSSMASVCKTEPSSPPPA 239
ogsox3 181:HRYEMAGLQYPMMSSAQTYMNAASTY-SMSPAYTQQTTSAMGLSSMASVCKTEPSSPPPA 239
jmsox3 181:HRYEMAGLQYPMMSSAQTYMNAASTY-SMSPAYTQQTGSAMGLSSMGSVCKTEPSSPPPA 239
zfsox3 180:HRYDMAGLQYPMMSTAQTYMNAASTYSSMSPAYTQQTSSAMGLGSMASVCKTEPSSPPPA 239

bpsox3 238:ITSHSQRACLGDLRDMISMYLPPGGDSAEHSSLQSSRLHSVHPHYQTAGTAVNGTLPLTH 297
bmsox3 240:ITSHSQRACLGDLRDMISMYLPPGGDSAEHSSLQSSRLHSVHPHYQTAGTGVNGNLPLTH 299
ogsox3 240:ITSHSQRACLGDLRDMISMYLPPGGDSAEHSSLQSSRLHSVHPHYQTAGTGVNGTLPLTH 299
jmsox3 240:ITSHSQRACLGDLRDMISMYLPPGGDSAEHSSLQSSRLHSVHPHYQSAGTGVNGTLPLTH 299
zfsox3 240:ITSHSQRACLGDLRDMISMYLPPGGDSADHSSLQTSRLHSVHPHYQSAGTGVNGTLPLTH 299

bpsox3 298:I 298
bmsox3 300:I 300
ogsox3 300:I 300
jmsox3 300:I 300
zfsox3 300:I 300
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Fig. 1. Comparison of *sox3* amino acid sequence of black porgy, *Acanthopagrus schlegeli sox3*, barramundi perch, *Lates calcarifer sox3*, orange-spotted grouper, *Epinephelus coioides sox3*, Japanese medaka, *Oryzias latipes sox3*, zebrafish, *Danio rerio sox3* optimally aligned to match identical residues, indicated by a shaded box. The sequences were taken from the GenBank/EMBL/DDBJ sequence database. The GenBank accession numbers for the *sox3* sequences used for alignment are as follows: black porgy (bp*sox3*), barramundi perch (bm*sox3*), orange-spotted grouper (og*sox3*), Japanese medaka (jm*sox3*) and zebrafish (zf*sox3*). The HMG-box domain is boxed and an asterisk indicates specificity amino acids in HMG-box domain.

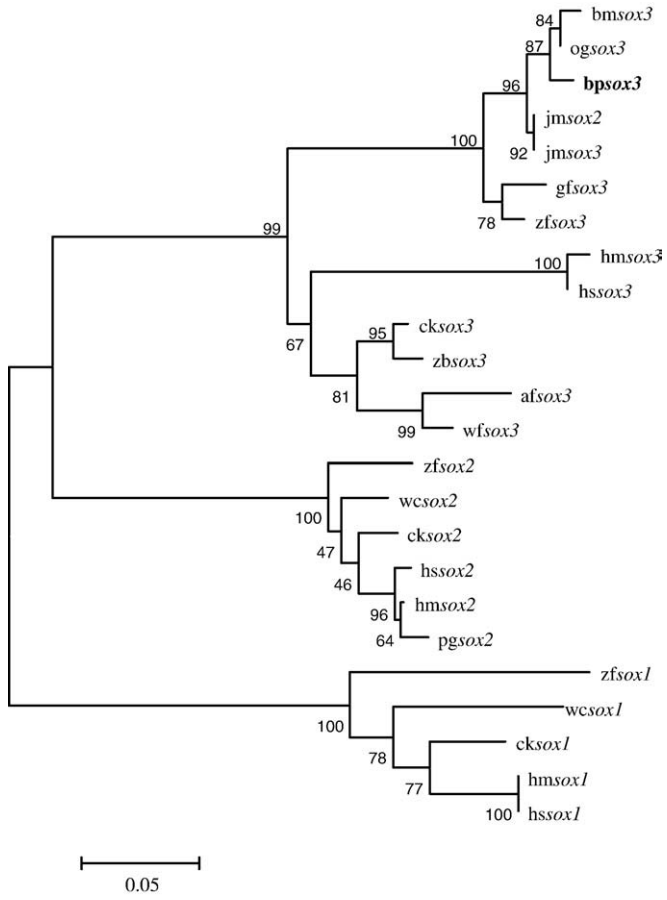

Fig. 2. Phylogenetic tree based on an amino acid alignment for *sox3* in teleost fish, reptile, bird and mammals. Bootstrap values (%) are indicated (1000 replicates). The score between two protein sequences, which is a measure of the relative phylogenetic relationship between the two proteins, is represented by the horizontal distance in this tree, i.e. the shorter the distance, the more related they are. Genbank accession number of the sequences are: zebrafish *sox1* (zf*sox1*), Western clawed frog *sox1* (wc*sox1*), chicken *sox1* (ck*sox1*), house mouse *sox1* (hs*sox1*), human *sox1* (hm*sox1*), Japanese medaka *sox2* (jm*sox2*), zebrafish *sox2* (zf*sox2*), Western clawed frog *sox2* (wc*sox2*), chicken *sox2* (ck*sox2*), house mouse *sox2* (hs*sox2*), pig *sox2* (pg*sox2*), human *sox2* (hm*sox2*), black porgy *sox3* (bp*sox3*), barramundi perch *sox3* (bm*sox3*), orange-spotted grouper *sox3* (og*sox3*), Japanese medaka *sox3* (jm*sox3*), zebrafish *sox3* (zf*sox3*), goldfish *sox3* (gf*sox3*), Wrinkled frog *sox3* (wf*sox3*), African clawed frog *sox3* (af*sox3*), zebra finch *sox3* (zb*sox3*), chicken *sox3* (ck*sox3*), house mouse *sox3* (hs*sox3*) and human *sox3* (hm*sox3*).

(5′-CCT CCT ATT ACA ACT TCT ACC-3′), DMRT1 reverse primer (5′-GAC AGG CGG CCA TCA CC-3′), β-actin forward primer (5′-GCA AGA GAG GTA TCC TGA CC-3′) and β-actin reverse primer (5′-CTC AGC TCG TTG TAG AAG G-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad, USA) according to the manufacturer's instructions. QPCR was performed as follows: denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s. As an internal control, experiments were duplicated with β-actin, and all data were expressed as change with respect to corresponding β-actin calculated threshold cycle (CT) levels. The amplified PCR product was processed by electrophoresis in 1% agarose gels. Transformation and sequencing were performed as described above, and then we confirmed the *sox3* sequences.

2.9. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One-way ANOVA followed by *post hoc* Duncan's multiple range test was used to compare the differences to control in the data ($P<0.05$).

3. Results

3.1. Identification of full-length sox3 cDNA

A single PCR product of the expected size (352 base pairs [bp]) was obtained by RT-PCR. A PCR-based cloning strategy (RT-PCR followed by 3′ and 5′ RACE) was used to clone full-length cDNA encoding *sox3*. The full-length *sox3* cDNA contained 897 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 298 amino acids (EF605272). The amino acid sequence of black porgy *sox3* was compared to those deduced from the cDNA of other teleost species. The amino acid similarities were as follows: 96% with barramundi perch *sox3* (DQ915951), 96% with orange-spotted grouper *sox3* (DQ219298), 96% with Japanese medaka *sox3* (AJ245396), and 91% with zebrafish (AY316135). Black porgy *sox3* has a HMG-box domain including 9 amino acids (RPMNAFMVW) that indicated HMG-box domain (Fig. 1).

3.2. Phylogenetic analysis

The phylogenetic tree obtained by clustal analysis of the sequences described below was shown in Fig. 2. The black porgy *sox3* was most closely related to barramundi perch *sox3* and orange-spotted grouper *sox3*.

3.3. Expression of sox3 and DMRT1 mRNA during sex reversal

The expression changes of *sox3* and DMRT1 mRNA during sex reversal were shown in Fig. 3. The expression of *sox3* mRNA was significantly high in mature testis and testicular portion of mostly testis ($P=0.001$), and the DMRT1 mRNA was the highest in mature testis ($P=0.015$) during sex reversal.

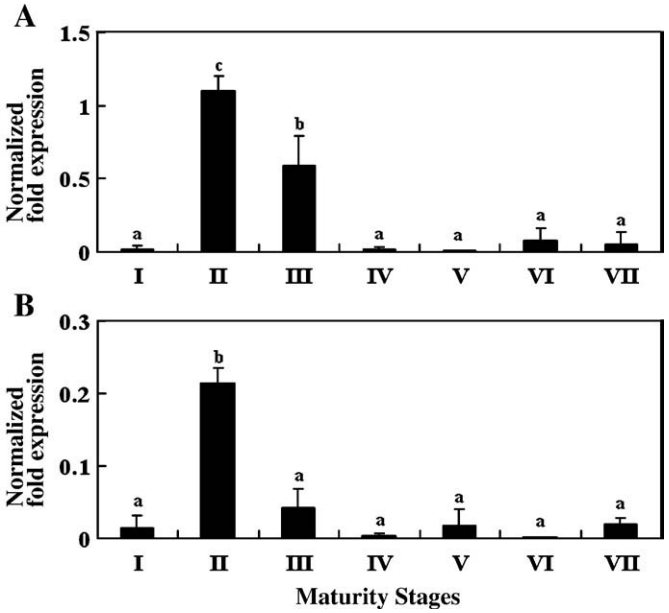

Fig. 3. Expression of *sox3* (A) and DMRT1 (B) mRNA in gonads of black porgy during sex reverse by quantitative real-time PCR. 2.5 μg of total RNA prepared from the gonads was reverse-transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expressions with respect to β-actin levels for the same sample. Maturity stages were divided into seven stages during the sex-reverse process from male to female (I, immature testis; II, mature testis; III, testicular portion of mostly testis; IV, ovarian portion of mostly testis; V, testicular portion of mostly ovary; VI, ovarian portion of mostly ovary; VII, ovary). Values with different superscripts indicate significant difference from control in the same time of sampling ($P<0.05$). All values are means ± SD ($n=3$).

3.4. Histological analysis

The histological examination of gonad during sex reversal was shown in Fig. 4. Testicular tissue was dominant in the mature testis (Fig. 4A), and then the testicular portion of the gonad was degenerated (Fig. 4B) and the ovarian portion of the gonad was increased during sex reversal (Fig. 4C), and oocytes were increased in diameter in ovary (Fig. 4D).

3.5. Quantification of sox3 and DMRT1 mRNA by GnRHa injection (in vivo)

The expression changes of *sox3* and DMRT1 mRNA by GnRHa injection were shown in Fig. 5. Gonad was injected by GnRHa, the expression of *sox3* mRNA was significantly increased at 24 h ($P=0.001$, Fig. 5A), and the expression of DMRT1 mRNA was significantly increased at 12 h and peaked at 24 h and then decreased ($P=0.005$, Fig. 5B).

3.6. Quantification of sox3 and DMRT1 mRNA by GnRHa treatment (in vitro)

The expression changes of *sox3* and DMRT1 mRNA by gonad tissues culturing were shown in Fig. 6. At the 10^{-5} M GnRHa group, the expression of *sox3* mRNA was increased at 12 h and peaked at 24 h ($P=0.015$, about 3 times than control) and then decreased, and the DMRT1 mRNA was increased at 6 h and peaked at 24 h ($P=0.02$, about 3.3 times than control) and then decreased. At the 10^{-3} M GnRHa group, the expression of *sox3* mRNA peaked at 24 h ($P=0.001$, about 5.1 times than control) and DMRT1 mRNA increased at 6 h and peaked at 24 h ($P=0.001$, about 6.3 times than control) and then decreased.

4. Discussion

Sox family in vertebrates characterizes very well the conservation of the DNA-binding domain, the HMG-box domain, and is classified into ten groups according to amino acid identity (Bowles et al., 2000). In addition, Bowles et al. (2000) named a *sox* gene which has the specific amino acids (RPMNAFMVW) in the HMG-box domain, and *sox* gene from black porgy in this study also belongs to the *sox* family as this gene has the specific amino acids (RPMNAFMVW, residues 37–45, Fig. 1). We conducted a phylogenetic analysis using amino acids of black porgy *sox*, it is placed in the B1 group (B1: *sox1*, *-2*, *-3* and *-19*; B2: *sox14* and *-21*) of the *sox* B group. Therefore considering the amino acids homology and phylogenetic analysis, we named the protein as *sox3* (Fig. 2).

The black porgy *sox3* expressed very high in testis and testicular portion of mostly testis during sex reversal (Fig. 3A). The previous

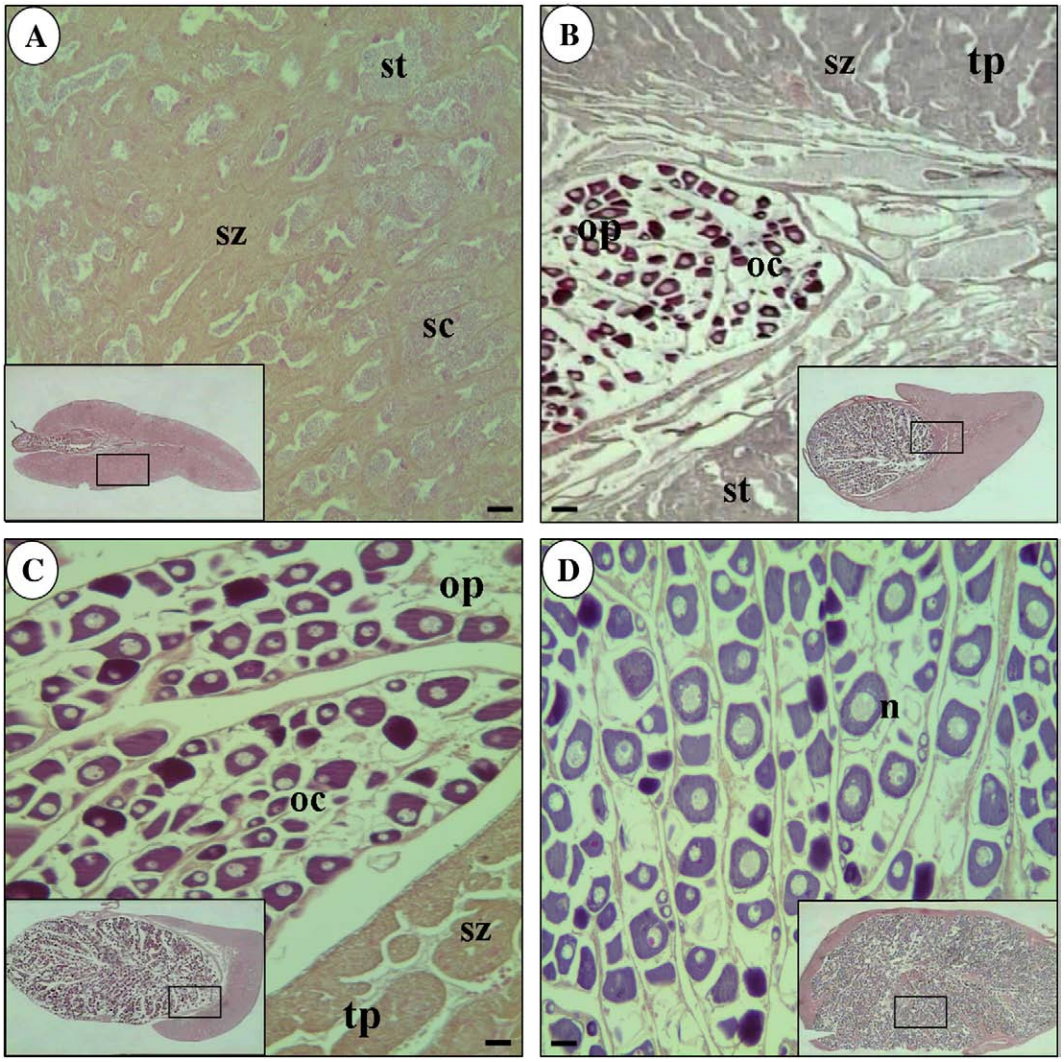

Fig. 4. Photomicrograph of gonad maturity stages during the sex change of black porgy. A: mature testis, B: mostly testicular gonad (III and IV in Fig. 1), C: mostly ovarian gonad (V and VI in Fig. 1), D: ovary. The insets in the photomicrograph were the whole gonad photograph. tp: testis part, st: spermatids, sz: spermatozoa, sc: spermatocytes, op: ovary part, oc: oocytes, n: nucleus. Scale bar = 100 μm.

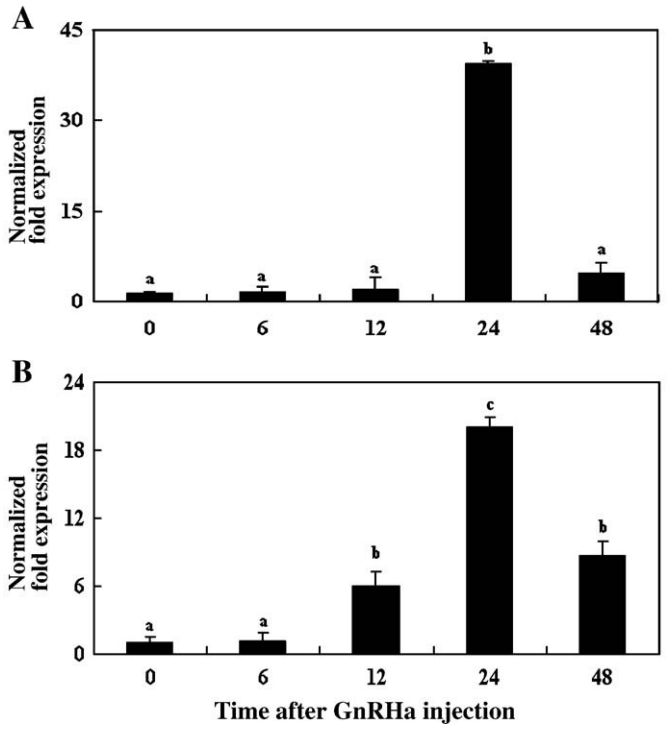

Fig. 5. Expression of *sox3* (A) and DMRT1 (B) mRNA in gonads of black porgy by GnRHa injection (*in vivo*). 2.5 μg of total RNA prepared from the gonads was reverse-transcribed and amplified using gene-specific primers by quantitative real-time PCR. Results are expressed as normalized fold expressions with respect to β-actin levels for the same sample (0, 6, 12, 24 and 48 h). Values with different letters indicate significant difference from control in the same time of sampling ($P<0.05$). All values are means ± SD ($n=5$).

studies examined *sox3* as related to testicular development (Graves, 1998; Bergstrom et al., 2000), for example, *sox3* increased through feeding with food containing methyltestosterone in female red-spotted grouper (Yao et al., 2007), and increased in the spermatogonia and Sertoli cell constituted seminiferous tubules of mice testis (Raverot et al., 2005). These reports corresponded with the present results. Also, the testis weight of male *sox3*-KO mice was reduced significantly and seminiferous tubules were irregular in shape and small (Weiss et al., 2003; Rizzoti et al., 2004). Despite many studies on the expression *sox3* mRNA in testicular growth and maturation was not performed in fish as compared to mammalian, considering the results of the previous studies (Weiss et al., 2003; Rizzoti et al., 2004; Raverot et al., 2005; Yao et al., 2007) and this study, we suggested the fact that *sox3* was involved in testicular growth and maturation.

Also, the expression of DMRT1 mRNA was high in testis of mature black porgy similar to that of *sox3* (Fig. 3B). In fish, DMRT1 mRNA was highly expressed during testicular differentiation in rainbow trout (Marchand et al., 2000) and the expression of DMRT1 mRNA was high during ovary degeneration and spermatocyte was increased in the protogynous honeycomb grouper (Alam et al., 2008). Through the result of previous studies that DMRT1 was only expressed in testis of flounder (Jo et al., 2007) and mice (Raymond et al., 1998), it is concluded that DMRT1 is the transcription factor which is involved in testicular differentiation and development (Nanda et al., 1999; Fernandino et al., 2006, 2008). Recently, Wang et al. (2006) reported that DMRT1 mRNA was increased as repressing cytochrome P450 aromatase mRNA and promoted the synthesis of androgen, and in the event, regulated the testicular development. Therefore, we suggest that DMRT1 plays an important role in testicular maturation in black porgy.

Also, we observed the histological analysis of gonad during sex reversal. Spermatogonia, spermatocyte, and spermatids were present in mature testis, and then these cells were degenerated and oocytes were increased during sex reversal from male to female. Through the QPCR results the expression of *sox3* and DMRT1 mRNA was increased in mature testis, and *sox3* and DMRT1 played essential roles in testicular development of black porgy. In addition to that, Guo et al. (2005) reported that DMRT1 was expressed in developing germ cells, including spermatogonia, spermatocytes, and spermatids in testis by hematoxylin–eosin staining and *in situ* hybridization in zebrafish.

We investigated the expression changes of *sox3* and DMRT1 mRNA by GnRHa to figure out the effect of GnRH in immature black porgy gonad. The results of *in vivo* experiment by intraperitoneally injected GnRHa and *in vitro* experiment by treated GnRHa at gonad tissue culturing showed that both *sox3* and DMRT1 mRNA expression were increased in immature black porgy gonad (Figs. 5 and 6). The importance of GnRH has been documented during spermatogenesis in juveniles and early maturing striped bass (Holland et al., 2002). Dubois et al. (2001) reported that GnRH could be the biotic factor causing testis development observed in immature male catfish. Despite the study of *sox3* and DMRT1 mRNA expression by GnRH treatment being almost one, the study of Collignon et al. (1996) and Koopman et al. (1991) reported that *sox3* is critically concerned in initial gonad development in mouse and Marchand et al. (2000) reported DMRT1 is concerned in initial testis development in rainbow trout, we can suggest that these genes are concerned in testicular development. Hence, the expression of these genes by treated GnRHa in this study is a first, but after considering other previous studies, increasing *sox3* and DMRT1 by treated GnRHa, activated hypothalamus-pituitary-gonad axis by GnRHa which is concerned in vertebrate gonad differentiation and development, we suppose the gonad *sox3* and DMRT1 mRNA expression increased to develop the testis.

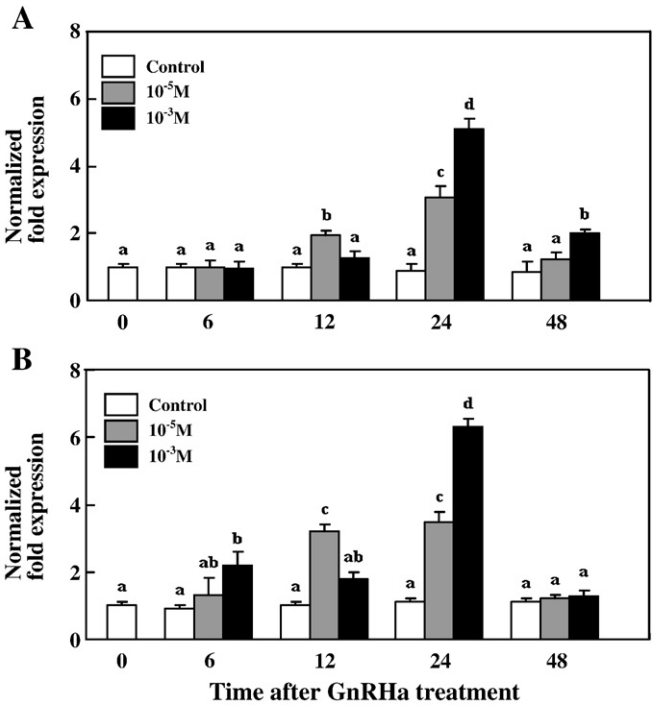

Fig. 6. The expression of *sox3* (A) and DMRT1 (B) mRNA in gonads of black porgy by GnRHa treatment (*in vitro*). The black porgy gonads incubated for 0, 6, 12, 24 and 48 h with media alone, 10^{-5} M and 10^{-3} M GnRHa. 2.5 μg of total RNA prepared from the gonads was reverse-transcribed and amplified using gene-specific primers by quantitative PCR. Results are expressed as normalized fold expressions with respect to β-actin levels for the same sample. Values with different letters indicate significant difference from control in the same time of sampling ($P<0.05$). All values are means ± SD ($n=5$).

In conclusion, *sox3* and DMRT1 play a critical role in male gonadal maturation, and additional studies will be necessary about the relation of *sox3* and DMRT1 as the factor of testicular development.

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