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Molecular Cloning and Expression Analysis of FTZ-F1 in the Half-smooth Tongue-sole, *Cynoglossus semilaevis*

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Abstract: To investigate the expression characteristics of sex related gene of FTZ-F1 in the half-smooth tongue-sole (*Cynoglossus semilaevis*), the homologue FTZ-F1 (hsFTZ-F1) full-length cDNA was isolated from the testis by homologous cloning, and the cDNA included the open reading frame and a 66bp 5'-UTR, along with a 1619bp 3'-UTR, encoding a predicted 485 amino acid protein. Sequence, tissue distribution and phylogenic analyses of the FTZ-F1 showed that the hsFTZ-F1 belonged to SF-1/Ad4BP group. The hsFTZ-F1 transcripts were highly abundant in the gonads, kidneys, brain and head-kidneys, but weakly in other tissues. However, the expression level in the brain and head-kidney of female was highly abundant than in the male. The hsFTZ-F1 expression was highly abundant in the embryo than in the larvae, which suggested that the hsFTZ-F1 may be involved in the organogenesis in the tongue sole.

Key words: *Cynoglossus semilaevis*; FTZ-F1; cDNA cloning; Expression

半滑舌鳎 FTZ-F1 cDNA 克隆及表达分析

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摘要：为研究性别相关基因 FTZ-F1 在半滑舌鳎鱼中的表达特征，采用同源克隆策略,从其精巢分离了 3143bp 长的半滑舌鳎 FTZ-F1（hsFTZ-F1）的全长 cDNA，该序列包含 1458 bp 开放阅读框，66 bp 长的 5'末端非编码区（UTR），1 619 bp 长的 3'末端 UTR。mRNA 的组织分布、氨基酸序列和系统发生分析表明：hsFTZ-F1 属于 SF-1/Ad4BP 类群。RT-PCR 分析表明：hsFTZ-F1 mRNA 的分布广泛，几乎在所有组织都有表达，但在性腺、肾脏、脑和头肾组织中表达最强，其他组织表达较弱，雌鱼脑和头肾中的表达量明显高于雄性。胚胎发育过程中表达量都高于孵化后仔鱼的表达量，表明 hsFTZ-F1 可能参与了半滑舌鳎的器官形成过程。

关键词：半滑舌鳎；FTZ-F1；cDNA 克隆；表达
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The fushi tarazu factor-1 (FTZ-F1) is a member of the nuclear receptor superfamily and was originally found as a regulator of the Drosophila homeobox segmentation gene FTZ (Lavorgna et al, 1991). FTZ-F1 homologues have been identified in human, mouse, *Xenopus laevis* and in a number of teleost species (Oba et al, 1996; Lala et al, 1992; Ellinger-Ziegelbauer et al, 1994; Zhang et al, 2006, Chai & Chan, 2000; Watanabe et al, 1999; Von et al, 2002). In mammals these genes have been classified as either SF-1/Ad4BP (steroidogenic factor-1/adrenal 4 binding protein) or LRH/FTF (liver receptor hormone/a-fetoprotein transcription factor) related. Mammalian LRH/FTF receptors are expressed in the pancreas, liver, intestine, and ovary, and are mainly involved in the homeostasis of cholesterol and bile acid (Lu et al, 2001). Mammalian SF-1/Ad4BP was expressed

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in the adrenal cortex, ovary, testis, placenta, adipocyte, and brain (Honda et al, 1993). It has been shown that the SF-1/Ad4BP is a key regulator of the development of the hypothalamic pituitary adrenal and gonadal axis and an essential factor in sex differentiation (Parker & Schimmer, 1997; Hammer & Ingraham, 1999). The mammal SF-1/Ad4BP genes are important regulators of steroid biosynthesis by controlling transcription of many P450 enzymes (Hammer & Ingraham, 1999). They are expressed in steroidogenic tissues and are involved in the embryonic development of adrenals and gonads (Sadovsky et al, 1995). The SF-1/Ad4BP also involved in the regulation of MIS (Mullerian inhibiting substance) transcription and promotion of testis development (Shen et al, 1994; Giuili et al, 1997).

In fish, several FTZ-F1 have been isolated, and some forms of FTZ-F1 homologues could not be put into either group (Von et al, 2001). The teleost FTZ-F1 may be involved in tissue differentiation and may play a role in the sexual maturation. However, the information needs to be greatly expanded in respect to the functional study as well as the diversity of fish species.

The half-smooth tongue-sole, *Cynoglossus semilaevis*, is a newly exploited and commercially important cultured marine flatfish in China, in which females grow 2−4 times faster than males. Regarding the potential role of FTZ-F1 homologues in the gonadal development, it would be of interest to study the FTZ-F1 homologue in the tongue sole. In this report, a homologue FTZ-F1 cDNA was cloned from the testis of the tongue sole, and its spatio-temporal expression patterns was examined.

1 Materials and methods

1.1 Materials

For the cloning and measurement of FTZ-F1 mRNA in the tongue sole, the gonads, liver, spleen, kidneys, brain, heart, muscle, head-kidneys, gills, skin, intestine and eyes were collected from a two-year-old fish. They were snap frozen in liquid nitrogen, and stored at −80℃ until use. In addition, the embryo of germ cell, zygote, 8-cells stage, blastula stage, early of gastrula, middle of gastrula, neurula stage, tail-bud stage, heart beating stage, and the larvae of hatching, 1day after hatching (dah), 12dah, 19dah, and 25dah were also collected. The fish were obtained from the Haiyang 863 High-Tech Experimental Base, Haiyang city, China.

1.2 RNA extraction and cDNA cloning

Total RNA extraction and reverse transcription were carried out as described (Chen et al, 2001). A pair of degenerate primers (P1: 5'-TBCTCACVTGYGAGAGC-TGYAAG-3'; P2: 5'-GMAGCATYTCRATVAGVAGGT-TG-3') were designed according to the conserved sequences of the FTZ-F1 gene in other teleosts and used to amplify a FTZ-F1 cDNA fragment of approximately 1217 bp from the tongue sole testis. PCRs were carried out at 94℃ (30s), 55℃ (30s), and 72℃ (60s) for 35 cycles using a PTC-Peltier thermal cycler. Then, 5'- and 3'-RACE were performed to obtain the 5' and 3' cDNA ends of Sox10 using the SMART RACE Kit (Clontech) according to the manufacturer's instructions. Two gene-specific primers were used for RACE:
P3: 5'-AATTGCCTGCCTGAAGTTCATCGTCCTC-3';
P4: 5'-CTTCCAACCGCATCCCGACATTCAGACA-3'.

The amplified fragments were separated and purified with a QIAEX Ⅱ Gel Extraction Kit (QIAGEN). The purified fragments were then cloned into pMD18-T vector (Takara), propagated in *E. coli* DH5α, and were sequenced using an ABI 377 sequencer.

1.3 Sequence analysis and alignment

The alignment of the amino acid sequence of FTZ-F1 protein was performed using DNAstar. The sequences used for comparison and their GenBank accession numbers were as follows: *Oryzias latipes* (mdFTZ-F1: BAA32394), *Acanthopagrus schlegelii* (sbFTZ-F1b: AAS75792), *A. schlegelii* (sbFTZ-F1a: AAS75791), *Oncorhynchus mykiss* (rtFTZ-F1: AAW83490), *Danio danio* (zebrafish ff1b: AAF43283), *Ictalurus punctatus* (ccNR5A1: AAY45704), *Clarias gariepinus* (acFTZ-F1a: AAG49004), *C. gariepinus* (acFTZ-F1b: AAG49005), *Carassius auratus* (gFTZ-F1: AAM89250), *D. danio* (zebrafish ff1d: AAO59489), *Mus musculus* (mLRH-1: AAA39447), *O. mykiss* (rtLRH-1: BAE71417), *D. danio* (zFF1A: AAK54449), *Rana rugosa* (rrFTZ-F1a: BAA94077), *Gallus gallus* (cFTF/LRH-1: BAA22838), *Homo sapiens* (hSF-1: AAB53105), *H. sapiens* (hFTF: AAD03155), *Trachemys scripta* (tuSF-1: AAD01975), *R. rugosa* (rrSF-1: BAA36789), *M. musculus* (mSF-1: AAB28338), *G. gallus* (cSF-1: BAA22839), *Epinephelus coioides* (grouper FTZ-F1: AAQ72771), *D. danio* (zebrafish ff1c: AAK19303), *Drosophila melanogaster* (dFTZ-F1: P33244), *Oreochromis niloticus* (tSF-1: BAC75890) , *Bos taurus* (bAd4P: BAA02764) and *C. semilaevis* FTZ-F1 (ABQ41307). A phylogenetic tree was constructed with the neighbour-joining method using Mega 3.1.

1.4 RT-PCR analysis of FTZ-F1 expression in the tissues, embryos and larvae

Total RNA was isolated from various tissues, embryos and larvae at different phase. Then, cDNA was synthesized and RT-PCR was employed for analysis of FTZ-F1 expression. Total RNA isolated from tissues was used as the initial template for RT-PCR. The PCR reaction was performed as described above. A pair of gene specific primers (P5: 5'-TCATCGTCCTCTTCAA-CCCCAAT-3'; P6: 5'-GTTCATGTAGCACAGGTAGTC-CT-3') were used to amplify 206 bp FTZ-F1 cDNA fragments by PCR of 94℃ (30 s), 55℃ (30 s) and 72℃ (60 s) for 35 cycles using a PTC-200 Peltier thermal cycler (Fig.1a). A 450 bp β-actin fragment was amplified as an internal control with a pair of β-actin primers (actinN1: 5'-AGGTGATGAAGCCCAGAGCA-3'; actinC1: 5'-GCAGTGGTGGTGAAGGAGTAG-3'). All the PCR products were electrophoresed on 1.2% agarose gel and gels were stained with ethidium bromide to visualize bands.

2 Results

2.1 Isolation, characterization and phylogenetic analysis of FTZ-F1 cDNA

After RT-PCR and subsequent 5' and 3'-RACE, a 3 143 bp FTZ-F1 cDNA was obtained (Fig.1). This clone contains the poly (A) tail. The FTZ-F1 cDNA included the open reading frame and a 66 bp 5'-UTR, along with a 1 619 bp 3'-UTR, encoding a predicted 485 amino acid protein, and including the highly conserved DNA-binding and ligand-binding regions (I, II, and III, FTZ-F1 box, and the activation function-2 (AF-2) hexamer (Fig.2).

The deduced amino acid sequence of the tongue sole FTZ-F1 had 16.1%−71.5% identity with that of the other vertebrate. A higher level of identity was found when the tongue sole FTZ-F1 was compared to the other FTZ-F1 proteins. A higher level of identity was found when the tongue sole FTZ-F1 was compared to the other teleosts FTZ-F1 proteins. The deduced amino acid sequence of the tongue sole FTZ-F1 had 71.5%, 70.7%, 63.3% and 57.2% identity with that of the *Oncorhynchus latipes* FTZ-F1, *O. niloticus* FTZ-F1, *O. mykiss* FTZ-F1 and *A. schlegelii* FTZ-F1, respectively; had 53%, 48.4% and 46.2% with that of the *Gallus gallus* SF-1, *Homo sapiens* SF-1 and *Bos taurus* Ad4BP respectively; had 44.7%, 42.3% and 37.5% with that of *O. mykiss* LRH-1, *G. gallus* LRH-1 and *Mus musculus* LRH-1 respectively. The lowest sequence identity was with the *Drosophila melanogaster* FTZ-F1. The tongue sole FTZ-F1 was 40.8%, 37.5%, 35.5% and 25.3% sequence identity with *Danio danio* ff1b, ff1a, ff1d and ff1c, respectively. The tongue sole FTZ-F1 amino acid sequence and several published FTZ-F1 in various vertebrate species were used to infer phylogenetic relationships. And the phylogenetic tree shows that the tongue sole FTZ-F1 clustered firmly with other teleosts FTZ-F1 (Fig.3).

2.2 Tissues, embryos and larvae expression of FTZ-F1

The FTZ-F1 mRNA expression in different tissues of the tongue sole was analyzed using RT-PCR. It was demonstrated that the FTZ-F1 transcripts were highly abundant in testis, spleen, female brain and female head-kidney, intermediately in ovary and male head-kidney, and weakly in kidneys, liver, heart, muscle, gills, skin, intestine and eyes (Fig.4). And the FTZ-F1 transcripts in female brain and head-kidneys was highly abundant than in male brain and head-kidneys.

The tongue sole FTZ-F1 expression in the embryos and larvae was also analysed using RT-PCR (Fig.5). The FTZ-F1 was expressed from the germ cell to the 25 dah. The FTZ-F1 transcripts were highly abundant during the embryo development. However, the FTZ-F1 decreased during the larvae development.

3 Discussion

We have successfully cloned a homolog FTZ-F1 from the tongue sole testis. Phylogenetic analysis of the tongue sole FTZ-F1 showed that it clustered firmly with other teleosts FTZ-F1. Amino acids alignment analysis showed that the FTZ-F1 is characteristic of nuclear receptor superfamily, with highly conserved regions of Ⅰ, Ⅱ, Ⅲ, FTZ-F1 box and AF-2 hexamer. The tongue sole FTZ-F1 shared 86%−100%, 92%−100% and 83.3%−100% with other vertebrate Region Ⅰ, FTZ-F1 box and AF-2 hexamer. The sequence identity showed that the tongue sole FTZ-F1 was the highest with *Oncorhynchus latipes* FTZ-F1, *O. niloticus* FTZ-F1, *O. mykiss* FTZ-F1 and *Acanthopagrus schlegelii* FTZ-F1, higher with vertebrate SF1/Ad4BP, lower with LRH-1, and the lowest with *Drosophila melanogaster* FTZ-F1.

The tongue sole FTZ-F1 expressed widely in the tissues, with highly abundant in gonad, spleen, brain and head-kidneys, which suggested that the FTZ-F1 belonged to SF-1/Ad4BP (Parker et al, 1997; Hammer et al, 1999). However, the tongue sole FTZ-F1 expressed weakly in the liver, which implied that the FTZ-F1 is some of LRH/FTF (Lu et al, 2001). During embryogenesis of zebrafish, the expression of homologues FTZ-F1 was detected in the pituitary, mandibular arch, pronephric duct, liver, rostral diencephalons, hindbrain, and pancre-

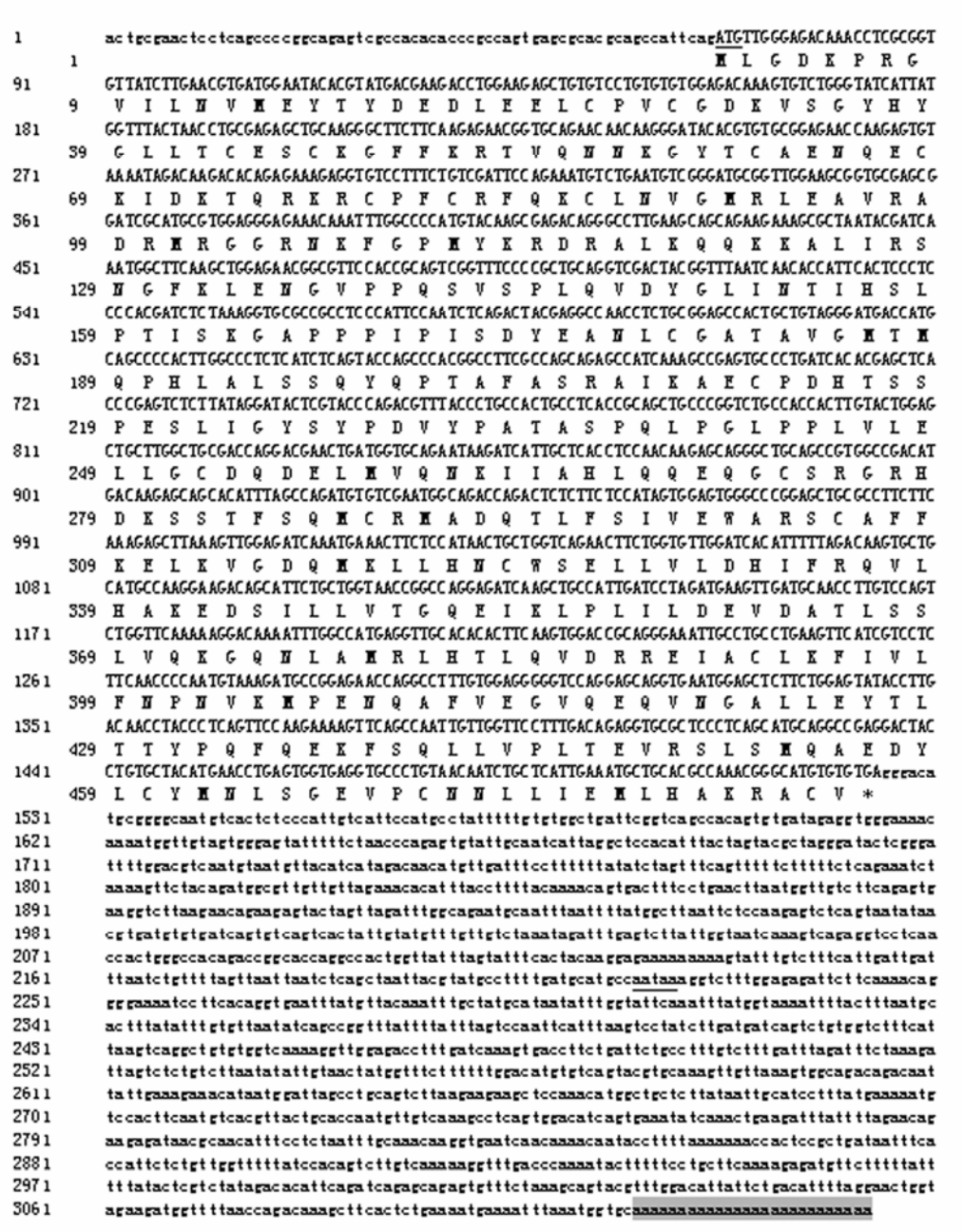

Fig. 1 cDNA nucleotide (GenBank Accession No: EF555726) and predicted amino-acid sequences of the *Cynoglossus semilaevis* FTZ-F1

Nucleotides are indicated above and numbered to the left of each lane (upper row). The deduced amino acid sequence is shown below the nucleotide sequence. Amino acids are numbered to the left of each lane (lower row).The start codon ATG is underlined; the stop codon is indicated by an asterisk; the poly (A) signal is shaded in gray; the lowercase indicated 5' and 3'UTR.

as (Liu et al, 1997; Chai et al, 2000; Von Hofsten et al, 2001), indicating that they may be involved in tissue differentiation. The present results indicated that the expression of the tongue sole FTZ-F1 was detected in all stages examined from zygote to 25 dah of the tongue sole. High levels of expression during embryogenesis as suggested by the gel image indicated that the tongue sole FTZ-F1 might be involved in the organogenesis of the tongue sole embryo.

The orange-spotted grouper *Epinephelus coioides* is a protogynous hermaphroditic fish, and the expression of FTZ-F1 in the gonad also decreased significantly in response to MT treatment (Zhang et al, 2006). The Ad4BP/SF-1 increased in parallel with the onset of the

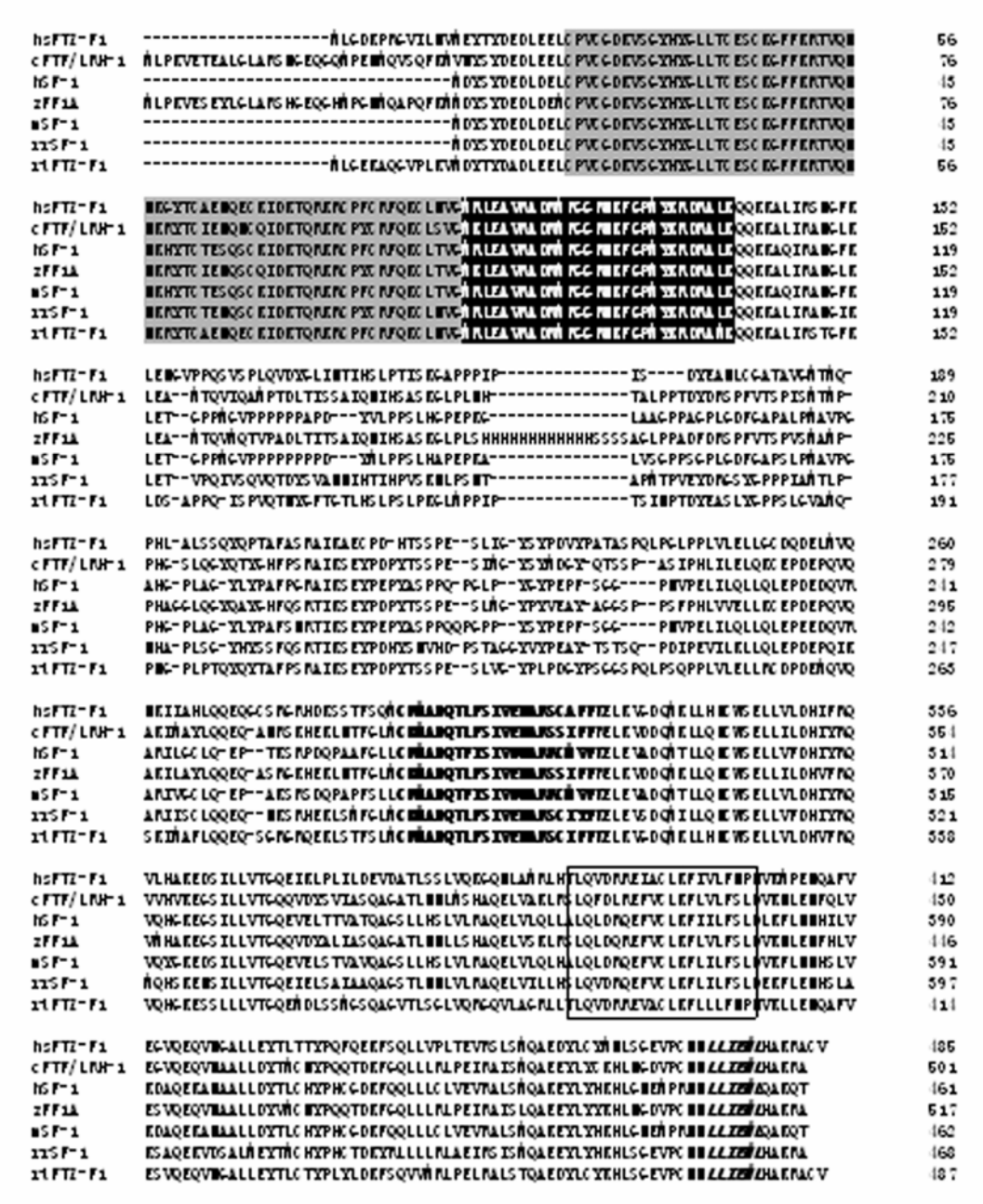

Fig. 2 Amino acid alignment of the *Cynoglossus semilaevis* FTZ-F1 (hsFTZ-F1: ABQ41307), *Gallus gallus* (cFTF/LRH-1: BAA22838), *Homo sapiens* (hSF-1:AAB53105), *Danio danio* (zFF1A: AAK54449), *Mus musculus* (mSF-1: AAB28338), *Rana rugosa* (rrSF-1: BAA36789), *Oncorhynchus mykiss* (rtFTZ-F1: AAW83490)

The highly conserved regions of Ⅰ, Ⅱ, Ⅲ, FTZ-F1 box and AF-2 hexamer are shown in gray box, boldface, boxes, white letters in black boxes and italics, respectively. And gaps used to maximize the alignment are shown by dashes.

female-phase and decreased as female became male in the serial sex changing goby, *Trimma okinawae* (Kobayashi et al, 2005). In *O. latipes* and *O. niloticus*, the aromatase promoter contains potential binding site for steroidogenic factor 1 (SF-1), which is involved in transcriptional regulation of P450 steroidogenic genes (cyp11A and B, steroid hydroxylases; aromatase) and in the formation of the gonads (Tanaka et al, 1995; Yasutoshi et al, 2003; Watanabe et al, 1999; Kuhl et al, 2005). It suggested the FTZ-F1 was involved in the regulation of sex reversal. There is a potential binding site for steroidogenic factor 1 in tongue sole gonad P450 aromatase (GenBank Accession No.: EF421177). However it remained to investigate weather the tongue

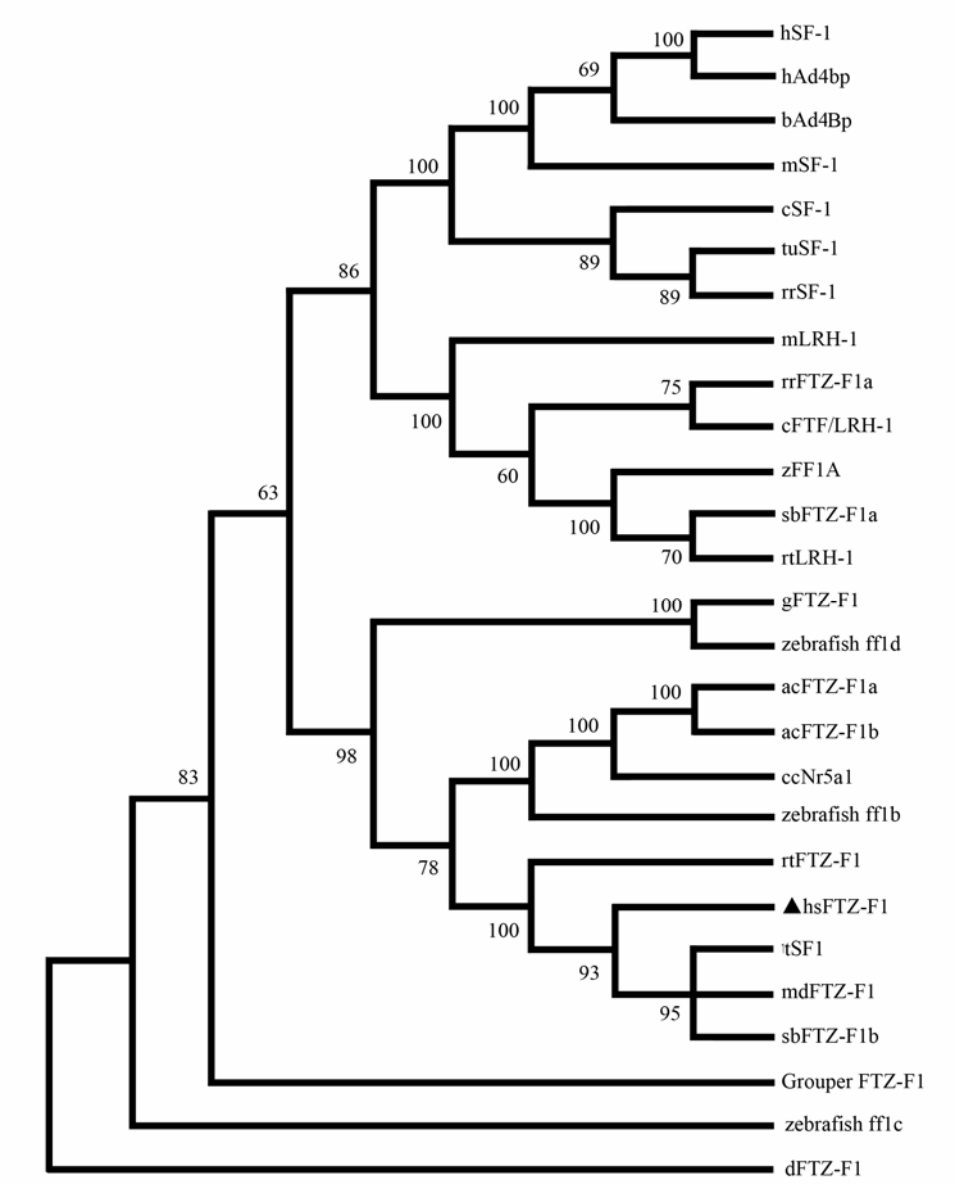

Fig. 3 Neighbour joining tree of the *Cynoglossus semilaevis* FTZ-F1 and FTZ-F1 from other organisms based on amino acid sequence

Distances are used to construct the phylogenetic tree and bootstrap values based on 1000 resampling replicates. The bottom scale refers to percentage divergence (p-distance).

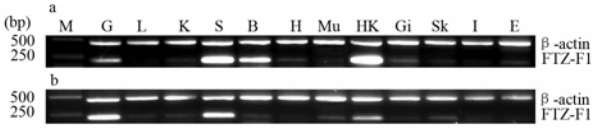

Fig. 4 RT-PCR analysis of FTZ-F1 expression from various tissues of two year old female(a) and male(b) *Cynoglossus semilaevis*

G, gonads; L, liver; K, kidneys; S, spleen; B, brain; H, heart; Mu, muscle; HK, head-kidneys; Gi, gill; Sk, skin; I, intestine; E, eyes and M, marker (DL2000).

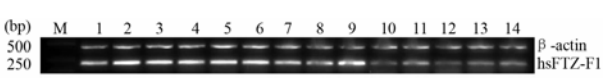

Fig. 5 RT-PCR analysis of the FTZ-F1 expression in the *Cynoglossus semilaevis* embryos and larvae

1. germ cell, 2. zygote, 3. 8-cells stage, 4. blastula stage, 5. early of gastrula, 6. middle of gastrula, 7. neurula stage, 8. tail-bud stage, 9. heart beating stage, 10. hatching, 11. 1day after hatching (dah), 12. 12dah, 13.19 dah,14. 25 dah.

tongue sole FTZ-F1 homologues may play roles similar to those of their mammalian counterparts. However, the information needs to be greatly expanded in respect to the functional study as well as the diversity of fish species.

References:

Chai C, Chan WK. 2000. Developmental expression of a novel Ftz-F1 homologue, *ff1b* (NR5A4), in the zebrafish *Danio rerio*[J]. *Mech Dev*, **91**: 421-426.

Ellinger-Ziegelbauer H, Hihi AK, Laudet V, Keller H, Wahli W, Dreyer C. 1994. FTZ-F1-related orphan receptors in *Xenopus laevis*: transcriptional regulators differentially expressed during early embryogenesis[J]. *Mol Cell Biol*, **14**(4): 2786-2797.

Giuili G, Shen WH, Ingraham HA. 1997. The nuclear receptor SF-1 mediates sexually dimorphic expression of Müllerian inhibiting substance, *in vivo*[J]. *Development*, **124**(9): 1799-1807.

Hammer GD, Ihgraham HA. 1999. Steroidogenic factor-1: Its role in endocrine organ development and differentiation[J]. *Front Neuroendocrinol*, **20**(3): 199-223.

Honda S, Morohashi K, Nomura M, Takeya H, Kitajima M, Omura T. 1993. Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily[J]. *J Biol Chem*, **268**(10): 7494-7502.

Kobayashi Y, Sunobe T, Kobayashi T, Nakamura M, Suzuki N, Nagahama Y. 2005. Molecular cloning and expression of Ad4BP/SF-1 in the serial sex changing gobiid fish, *Trimma okinawae*[J]. *Biochem Biophys Res Commun*, **332**(4): 1073-1080.

Kuhl AJ, Manning S, Brouwer M. 2005. Brain aromatase in Japanese medaka (*Oryzias latipes*): Molecular characterization and role in xenoestrogen-induced sex reversal[J]. *Journal of Steroid Biochemistry & Molecular Biology*, **96**(1): 67-77.

Lala DS, Rice DA, Parker KL. 1992. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is a mouse homolog of fushi tarazu-factor I[J]. *Mol Endocrinol*, **6**(8): 1249-1258.

Lavorgna G, Ueda H, Clos J, Wu C. 1991. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of fushi tarazu[J]. *Science*, **252**(5007): 848-851.

Lu TT, Repa JJ, Mangelsdorf DJ. 2001. Orphan nuclear receptors as eLiXiRs and FiXeRs of sterol metabolism[J]. *J Biol Chem*, **276**(41): 37735-37738.

Oba K, Yanase T, Nomura M, Morohashi K, Takayanagi R, Nawata H. 1996. Structural characterization of human ad4bp (SF-1) gene[J]. *Biochem Biophys Res Commun*, **226**(1): 261-267.

Parker KL, Schimmer BP. 1997. Steroidogenic factor 1: a key determinant of endocrine development and function[J]. *Endocrine Rev*, **18**(3): 361-377.

Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, Simburger K, Milbrandt J. 1995. Mice deficient in the orphan receptor steroidgenic factor 1 lack adrenal glands and gonads but express P450 side-chaincleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids[J]. *Proc Natl Acad Sci*, **92**(24): 10939-10943.

Shen WH, Moore CC, Ikeda Y, Parker KL, Ingraham HA. 1994. Nuclear receptor steroidogenic factor 1 regulates the Müllerian inhibiting substance gene: A link to sex determination cascade[J]. *Cell*, **77**(5): 651-661.

Tanaka M, Fukada S, Matsuyama M, Nagahama Y. 1995. Structure and promoter analysis of the cytochrome P-450 aromatase gene of the teleost fish, medaka (*Oryzias latipes*) [J]. *J Biochem*, **117**: 719-725.

Von Hofsten J, Jones I, Karlsson J, Olsson PE. 2001. Developmental expression patterns of FTZ-F1 homologues in Zebrafish (*Danio rerio*) [J]. *Gen Comp Endocrinol*, **121**: 146-155.

Watanabe M, Tanaka M, Kobayashi D, Yoshiura Y, Oba Y, Nagahama Y. 1999. Medaka (*Oryzias latipes*) FTZ-F1 potentially regulates the transcription of P-450 aromatase in ovarian follicles: cDNA cloning and functional characterization[J]. *Mol Cell Endocrinol*, **149**: 221-228.

Yasutoshi Y, Balasubramanian S, Masatada W, Oba Y, Kobayashi T, Nagahama Y. 2003. Synergistic Expression of Ad4BP/SF-1 and Cytochrome P-450 Aromatase (Ovarian Type) in the Ovary of Nile Tilapia, *Oreochromis niloticus*, During Vitellogenesis Suggests Transcriptional Interaction1[J]. *Biology of Production*, **68**(5): 1545-1553.

Zhang W, Zhang Y, Zhang L, Zhao H, Li X, Huang H, Lin H. 2006. The mRNA expression of P450 aromatase, gonadotropin beta-subunits and FTZ-F1 in the orange-spotted grouper (*Epinephelus coioides*) during 17alpha-methyltestosterone-induced precocious sex change[J]. *Mol Reprod Dev*, **74**(6): 665-673.