

Research Article

Molecular Characterization, Phylogeny, and Expression Profiles of *SoxE* Subfamily in *Scophthalmus maximus*

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SOXE transcription factors, including SOX8, SOX9, and SOX10, regulate several developmental events, such as sex determination, chondrogenesis, and neurogenesis. This study systematically identified six *SoxE* subfamily genes from the turbot (*Scophthalmus maximus*) genome and transcriptome, including *SmSox8a*, *SmSox8b*, *SmSox9a*, *SmSox9b*, *SmSox10a*, and *SmSox10b*. The duplicates of three SOXE members revealed that *SoxE* subfamily genes in the turbot underwent significant expansion. Relatively conserved exon-intron structures and intron insertions were detected using genomic structural analysis. Besides, phylogenetic analysis supported the previous classification of the *SoxE* subfamily. Results of the expression profile revealed that turbot *SoxE* subfamily genes may be involved in different activities, such as neurogenesis and gonad development. These findings would assist in the understanding of fish *SoxE* gene subfamily activities and evolution.

1. Introduction

The *Sox* family encodes various transcription factors (TFs) in the animal kingdom that regulate diverse biological processes [1]. The *Sox* TF family is characterized by the presence of a sry-related high-mobility group (HMG). On the basis of the structural homology of the HMG domain together with partial regions outside the HMG-box, the *Sox* family is divided into 11 subfamilies (A–K) [2, 3]. More than 80% homology in HMG-box sequences is observed for different *Sox* genes in a subfamily; moreover, they exhibit similar biochemical properties and biological functions [4, 5]. Studies on the structure and function of *SoxE* subgroups were the most extensive when compared with other *Sox* subgroups [6, 7].

In mammals and other higher vertebrates, the *SoxE* family is composed of three members, namely, *Sox8*, *Sox9*, and *Sox10*. For example, three *SoxE* genes were discovered in

Homo sapiens, *Mus musculus*, and *Gallus domesticus* [7–9]. Although fish have a low evolutionary status among vertebrates, they are the most widely distributed and account for nearly half of the existing vertebrate species. Because teleost fish underwent teleost-specific whole-genome duplication (3R-WGD), a greater number of *SoxE* genes are present in fish compared with other vertebrates. For example (as shown in Table 1), channel catfish (*Ictalurus punctatus*) has 4 putative *SoxE* genes, zebrafish (*Danio rerio*) has 5, tongue sole (*Cynoglossus semilaevis*) has 6, Japanese flounder (*Paralichthys olivaceus*) has 6, pufferfish (*Tetraodon fluvialis*) has 6, Nile tilapia (*Oreochromis niloticus*) has 6, and common carp (*Cyprinus carpio*) has 10 [3, 10–14]. Because of its special phylogenetic status, the study of *SoxE* evolution and function in fish is more attractive. *SoxE* modulates different bioprocesses in vertebrates, such as nervous system development [7, 15], skeletogenesis [16], and sex determination and differentiation [17, 18]. In fish, *Sox9* is the

TABLE 1: SoxE copy numbers across *Scophthalmus maximus* and other vertebrates.

Subfamily classification	Soxgenes	<i>Homo sapiens</i>	<i>Mus musculus</i>	<i>Gallus domesticus</i>	<i>Ictalurus punctatus</i>	<i>Danio rerio</i>	<i>Paralichthys olivaceus</i>	<i>Cynoglossus semilaevis</i>	<i>Tetraodon fluviatilis</i>	<i>Oreochromis niloticus</i>	<i>Cyprinus carpio</i>
SoxE subgroup	Sox8	1	1	1	1	2	2	2	2	2	4
	Sox9	1	1	1	2	2	2	2	2	2	4
	Sox10	1	1	1	1	1	2	2	2	2	2
	Total	3	3	3	4	5	6	6	6	6	10

most studied *SoxE* that exerts a crucial effect on sex determination and differentiation in *P. olivaceus* and medaka (*Oryzias latipes*) [19, 20]. In spotted sea bass (*Lateolabrax maculatus*), *Sox8b*, *Sox9b*, and *Sox10* are upregulated in the brain, indicating that *SoxE* is a key regulator in central nervous system (CNS) development [21].

Turbot (*Scophthalmus maximus*) is a valuable commercial farming fish in the Chinese aquaculture industry, especially in Northern China [22–25]. The genome sequence of *S. maximus* has been published (GenBank Accession: PRJNA821077). The systemic characterization of the *SoxE* subfamily has been completed in certain fish species but not in *S. maximus*. This study comprehensively identified genes, analyzed sequence structures, and evaluated the evolutionary characteristics for the systemic analysis of the turbot *SoxE* subfamily. Moreover, this work analyzed gene expression profiles to investigate possible *SoxE* activities in adult tissues. Our findings would contribute to a better understanding of *SoxE*-related biological activities in turbot and in other teleost species.

2. Materials and Methods

2.1. Animals and Sample Collection. Turbot individuals (age: 2 years, mean length: 38 ± 3.62 cm, and mean weight 1.82 ± 0.21 kg) were obtained from an aquatic product market in Lianyungang (Jiangsu, China). The turbot individuals were anesthetized using MS-222, followed by the collection of tissues, such as the brain, gill, liver, heart, spleen, stomach, kidney, muscle, intestines, and gonad (ovary or testis). The collected tissues were frozen immediately in liquid nitrogen and stored at -86°C until RNA purification. All animal-based experiments were approved by the Animal Research and Ethics Committee of Jiangsu Ocean University, and the detailed experimental operations were consistent with previous studies [26, 27].

2.2. Identification of *SoxE* in *S. maximus*. The genes were identified on the basis of the conserved HMG-box in the *SoxE* genes of zebrafish (*Danio rerio*). The sequences were obtained from Ensembl (https://asia.ensembl.org/Danio_rerio/Info/Index) and compared against the tblastn of BLAST in NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), with an E-value threshold level of $1e^{-6}$. All gene candidates were analyzed to determine the presence of the core motif RPMNAFMVW, which verified that the gene was *Sox* [2].

2.3. Sequence Analysis and Structure Construction of *SoxE*. We obtained DNA, mRNA, and protein sequences of the *S. maximus SoxE* subfamily by using NCBI (<https://www.ncbi.nlm.nih.gov>). Moreover, the exon-intron structures were acquired using reference genome-related annotation files. On the basis of the principle of equivalence, introns, exons, HMG-boxes, and open-reading frames were further identified, followed by mapping of the *SoxE* subfamily gene structure. Subsequently, the EXPASY compute pI/MW approach (<https://www.expasy.org/tools/>) was used to measure the isoelectric point (pI), molecular weight (MW), and other attribute

values of every *SoxE* protein. SMART (https://smart.embl.de/smart/show_motifs.pl) was adopted to predict conserved HMG sequences in *SoxE* proteins, while DNAMAN 8.0 was used to perform multiple alignments. WebLogo (<https://weblogo.berkeley.edu/logo.cgi>) was used to present six multiple sequence alignments of *S. maximus*.

2.4. Phylogenetic Analyses of *SoxE*. A total of 59 full-length sequences of *SoxE* proteins were downloaded from NCBI and Ensembl. The sequences of different organisms, such as *M. musculus*, *H. sapiens*, *G. domesticus*, *X. laevis*, *O. Latipes*, *D. rerio*, *I. punctatus*, *P. olivaceus*, *C. semilaevis*, *T. rubripes*, *O. niloticus*, and *C. carpio*, were downloaded. SMART was used to identify and retrieve HMG domains for phylogenetic analysis. ClustalW was used to align HMG-box sequences, followed by phylogenetic tree construction by using the neighbor-joining (NJ) approach. For this, the Poisson model from MEGA 7.0, with 1000 bootstrapping replicates, was used.

2.5. RNA Isolation and Real-Time Quantitative Reverse Transcription PCR. Total RNA was extracted from the tissues by using TRIzol (Invitrogen, CA, USA), and first-strand cDNA was synthesized using MMLV and oligo (dT)18 reverse transcriptase (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Specific primers (Table 2) were designed using Oligo 7.0. Primer specificities were examined through alignment against *S. maximus* transcriptomes (unpublished data) by using BLASTN, with the E-value being $1e^{-8}$ and β -actin being the endogenous control gene. Figure S1 displays the melting curves of different genes. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using the Light Cycler 480 Real-time PCR System (Roche Diagnostics, Mannheim, Germany) in triplicate. The reaction volume was $20\ \mu\text{L}$ and consisted of $10\ \mu\text{L}$ TB Green Premix EX Taq Mix (Takara, Japan), $1.6\ \mu\text{L}$ cDNA templates, $8\ \mu\text{L}$ ddH₂O, and $0.2\ \mu\text{L}$ respective primers. PCR conditions were 30 s at 94°C ; 10 s at 94°C ; 30 s at 60°C ; and 30 s at 72°C for 35 cycles. The $2^{-\Delta\Delta\text{Ct}}$ approach was used to determine *SoxE* expression, which was consistent with the previous method of calculating gene expression [28, 29]. SPSS 19.0 was employed for statistical analysis through an independent sample *t*-test. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. *SoxE* Identified in the *S. maximus* Genome. Six *SoxE* genes were identified in the *S. maximus* genome by using Ensembl and NCBI (Table 3). The cDNA length in *S. maximus* ranged from 2211 to 4592 bp, while the corresponding gene-encoded proteins were 464–499 aa in length. In addition, the predicted MWs were 50.88–54.42 kDa, and the pIs were 6.14–7.17. The *S. maximus SoxE* number is similar to those of *P. olivaceus*, *C. semilaevis*, *T. fluviatilis*, and *O. niloticus* (Table 1). In these teleost mentioned above, members of the *SoxE* subfamily all evolved into two orthologs in their genome (including *Sox8a*, *Sox8b*, *Sox9a*, *Sox9b*, *Sox10a*, and *Sox10b*). The reason for this

TABLE 2: Primers for qRT-PCR.

Gene name	Primer sequence (5'-3')	Tm (°C)	Amplicon length (bp)
<i>β-actin</i>	F: TGAACCCCAAGCCAACAGG R: AGAGGCATACAGGGACAGCAC	60	107
<i>Sox8a</i>	F: CCCGCCACAGACCTCAGATA R: AAGACGACGAGGAGGAGGTG	60	147
<i>Sox8b</i>	F: CGCAGCTTCTTTCTCCAGCA R: TAGGGCCGTCTGGATGAGTG	60	129
<i>Sox9a</i>	F: CTGCCGTTTTCTGTCCACTTC R: TCTTGTTCCCTCCGTCATCTTC	60	204
<i>Sox9b</i>	F: CCGGGAGAACACTCAGGTCA R: CAGGTCAGCTTTGCTGGAGG	60	87
<i>Sox10a</i>	F: ACACAGAGCAGGCATTCATACTTGG R: GAATCCGTGAGTTCCCGAGTTTCC	60	87
<i>Sox10b</i>	F: ATCAGAGAAGCGGTCAGTCAG R: GTCCTTCTTGTGCTGTTTCCT	60	288

TABLE 3: Basic information on the turbot *SoxE* subfamily.

Gene	Intron number	cDNA length (bp)	Protein length (aa)	HMG position	pI	Molecular weight (Da)	Accession number
<i>SmSox8a</i>	2	2211	481	96–168	7.17	52349.86	XM.035613581
<i>SmSox8b</i>	2	3294	464	100–172	6.48	50880.02	XM.035614518
<i>SmSox9a</i>	2	3246	479	104–176	6.2	52589.18	XM.035613389
<i>SmSox9b</i>	2	3470	499	107–179	6.14	54416.22	XM.035615649
<i>SmSox10a</i>	3	4592	496	106–178	6.45	52785.02	XM.035614967
<i>SmSox10b</i>	3	4305	498	104–176	6.37	52493.78	XM.035637005.2

phenomenon may be that these bony fish have undergone whole-genome duplication (3R-WGD events) during evolution, resulting in a significant expansion of members of the *SoxE* subfamily [30–32]. A similar phenomenon of double copies of members of the *SoxE* subfamily has also been seen in other teleost, such as the large yellow croaker and spinyhead croaker [33, 34]. However, *Cyprinus carpio* has more *SoxE* (including *CySox8a*, *CySox8b*, *CySox8c*, *CySox8d*, *CySox9a*, *CySox9b*, *CySox9c*, *CySox9d*, *CySox10a*, and *CySox10b*) compared with *S. maximus* and other aforementioned fish, implying that *C. carpio* underwent additional genome duplication events during evolution (4R-WGD events) [14].

3.2. Genomic Structure and Sequence Alignment of Turbot *SoxE*. The genomic structure of *S. maximus SoxE* was investigated and constructed using an online analysis tool (<https://gsds.gao-lab.org>). Relatively conserved exon-intron structures were observed in a *SoxE* subgroup [10]. On the basis of the structural distribution characteristics of introns and exons, turbot *SoxE* can be divided into two categories, those consisting of two and three introns, respectively. Specifically, *SmSox8a*, *SmSox8b*, *SmSox9a*, and *SmSox9b* have two introns, whereas *SmSox10a* and *SmSox10b* have three introns (Figure 1(a)). Intron insertion occurred in the HMG domain of all *SmSoxE* genes. The presence of introns in HMG boxes has also been observed in *SoxE* of other fish, such as *Lateolabrax maculatus* (*LmSox8a*, *LmSox8b*, and *LmSox10*), *Danio rerio* (*DrSox10*), and *Paralichthys olivaceus* (*PoSox8a*, *PoSox8b*, *PoSox9a*, *PoSox9b*, *PoSox10a*, and

PoSox10b) [10, 13, 21]. Because of the genetic diversity among species, only the insertion state of flounder is completely consistent with that of turbot; however, only some *SoxE* in other fishes exhibit this phenomenon. We further aligned the six HMG domains and explored the corresponding intron positions in the domains (Figure 2). The logo plots of the HMG-box domain revealed relatively conserved genes. Moreover, all HMG domains comprised a core motif of RPMNAFMVW, which recognized and bound to cis-regulatory elements in relevant target genes' promoters [12, 35] located at residues 5 to 13 (Figures 2(a) and 2(b)). The insertion locations of introns in the HMG domain of all *SmSoxE* genes were identical (Figure 2(a)). Therefore, the intron positions are evolutionarily conserved in the *SoxE* subgroup among species, a result consistent with those of previous studies [3, 13, 21].

3.3. Phylogenetic Analysis of Turbot *SoxE*. To identify six *SoxE* members and their clade, we used MEGA 7.0 to construct a nonrooted phylogenetic tree with 59 full-length sequences of *SoxE* proteins in twelve species. Turbot *SoxE* were clustered with corresponding counterparts, and three clades, namely *Sox8*, *Sox9*, and *Sox10*, were identified, conforming to prior classification results (Figure 3). Interestingly, most *SoxE*, including *Sox9a*, *Sox9b*, *Sox10a*, and *Sox10b*, can be clustered into their respective branches, indicating that *SoxE* of different species may have a common evolutionary origin. However, phylogenetic trees have some anomalies. For example, most *Sox8a* and *Sox8b* are clustered into different branches, which are close to *Sox10* and *Sox9* in the evolutionary tree structure

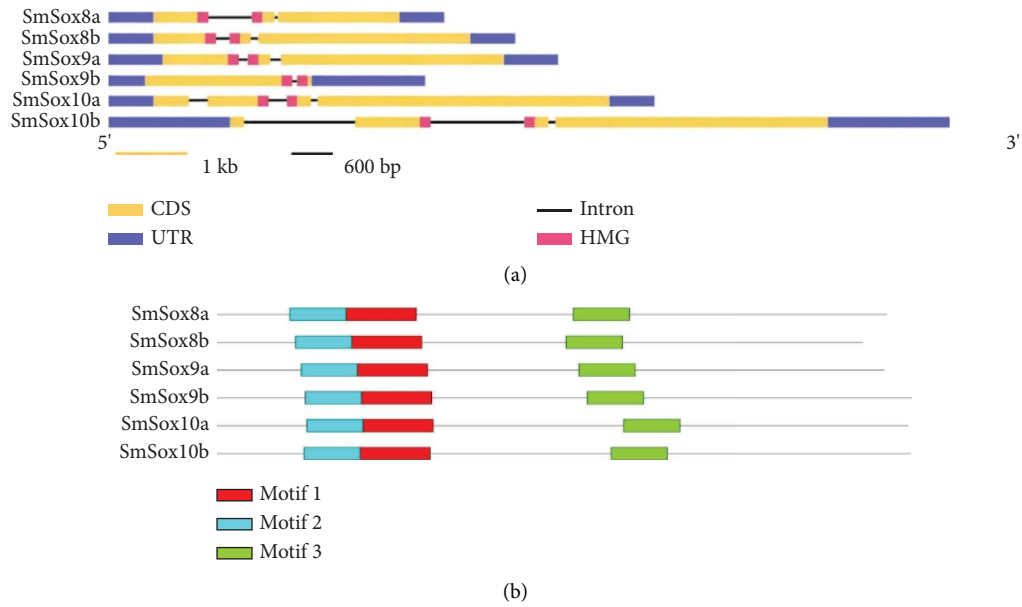


FIGURE 1: Exon-intron structures in *SoxE* together with motif analysis in *Scophthalmus maximus*. (a) Exon-intron structures in *SoxE* from *Scophthalmus maximus*. Yellow boxes, black horizontal lines, and blue boxes indicate the exons, introns, and noncoding regions, respectively. The fuchsia box indicates the HMG-box domain. (b) Motif structures in *SoxE* from *Scophthalmus maximus*. MEME was employed to identify conserved motifs. Different colors indicate different motifs.

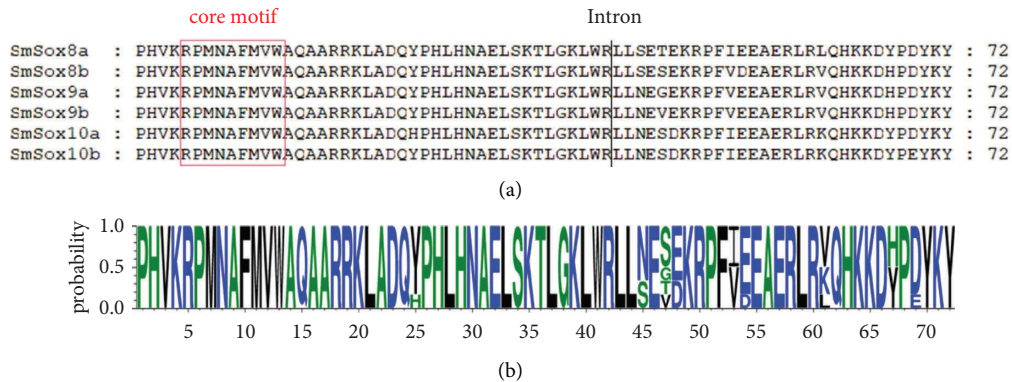


FIGURE 2: HMG-box domain sequences in *SoxE* from *Scophthalmus maximus*. (a) HMG-box domain sequence multiple alignments. Amino acids in red boxes indicate the core motif. The black long-line positions indicate the intron positions. (b) Sequence logo diagrams indicate sequence patterns and relative frequencies at diverse positions in the HMG-box domain. Horizontal axis numbers represent the numbers of HMG-box domain residue positions.

branches. Similar results were also reported in the closely related species of Japanese flounder (*P. japonicus*) and tongue sole (*C. semilaevis*) [13, 36]. This may be attributable to the third WGD event, leading to all members of the *SoxE* subfamily having two parallel homologous genes. The *Sox8a* and *Sox8b* of flounder fish are more likely to act as substitutes for *Sox10* and *Sox9*, respectively, thereby supplementing evolutionary adaptation and innovation.

3.4. Gene Patterns for Turbot *SoxE* in Different Tissues. *SoxE* TFs are involved in different physiological and biochemical events through the activation or inhibition of specific targets, depending on tissue and development

[6, 21]. This study analyzed gene patterns for six turbot *SoxE* genes in 11 adult tissues. Expression patterns were observed for all turbot *SoxE* genes (Figures 4(a)–4(f)). Results showed that turbot *Sox9a* and *Sox9b* had higher levels in the gill, while the other *SoxE* genes had very low or negligible expression levels in the liver and gill. Notably, most *SoxE* genes expressed relatively higher in brain and intestine, and *Sox8b* as well as *Sox10b* expressed highest in brain compared with their expression in other tissues. In addition, expression of *Sox8a* in muscle, *Sox9a* in gill, *Sox9b* in stomach and *Sox10a* in testis were all relatively higher in their own expression profiles. We also observed the weak expression levels of all *SoxE* genes in the heart, spleen, and kidney, indicating the role of *SoxE* genes in these organs' development needed

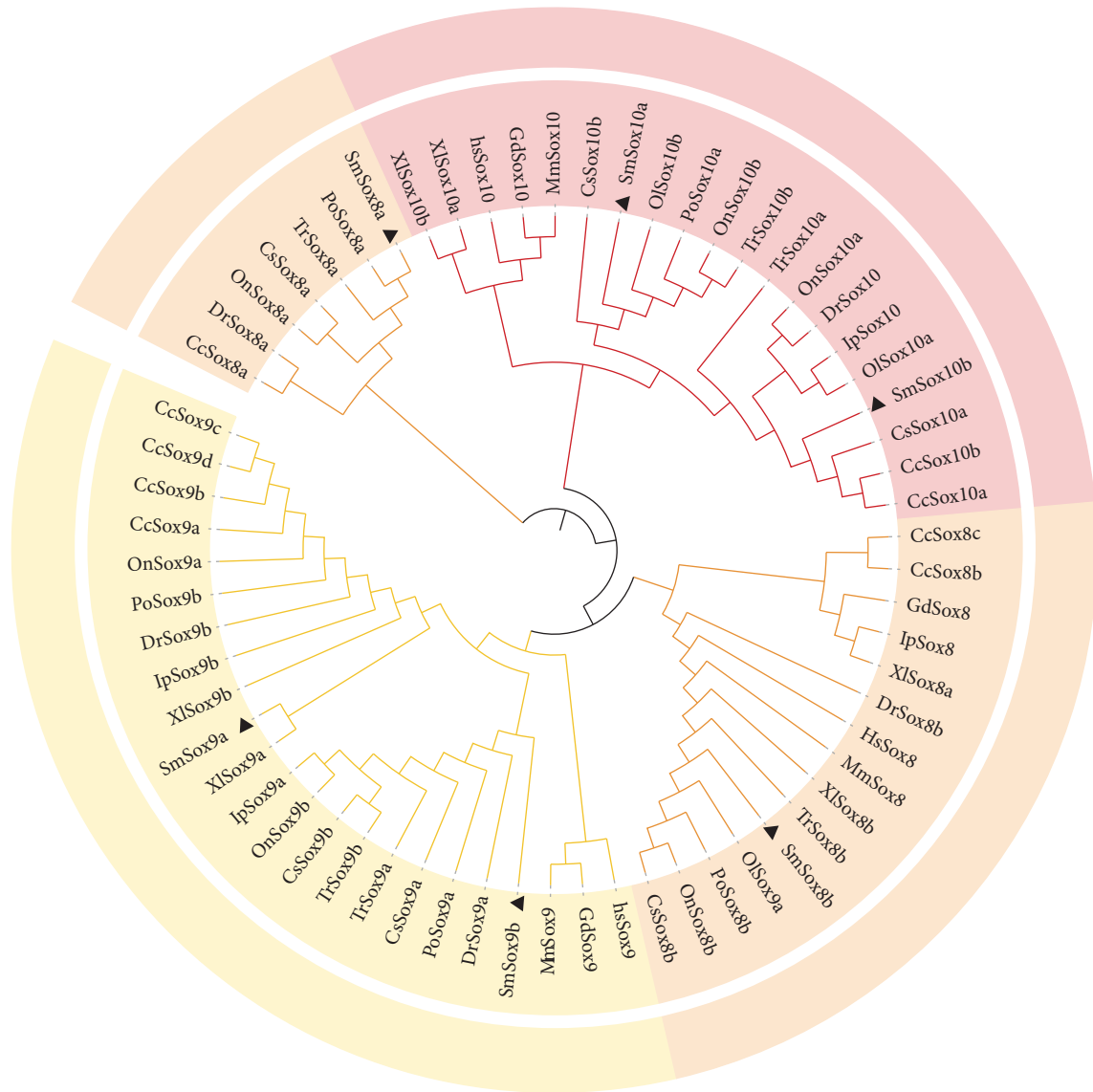


FIGURE 3: Phylogenetic tree for *SoxE*. The neighbor-joining phylogenetic tree was established using amino acid sequences of *SoxE* in vertebrates by using MEGA 7.0 with 1000 bootstraps. *SoxE* genes from *Scophthalmus maximus* are marked using a black triangle, whereas colored arcs indicate diverse subfamilies. Orange bars surrounding the phylogenetic tree indicate the amino acid sequences of *Sox*. Hm: *Homo sapiens*, Ol: *Oryzias latipes*, Mm: *Mus musculus*, Gd: *Gallus domesticus*, Xl: *Xenopus laevis*, Dr: *Danio rerio*, Ip: *Ictalurus punctatus*, Cs: *Cynoglossus semilaevis*, Po: *Paralichthys olivaceus*, On: *Oreochromis niloticus*, Tr: *Takifugu rubripes*, and Cc: *Cyprinus carpio*.

further investigation. Generally, *Sox8*, *Sox9*, and *Sox10*, the TFs belonging to the subgroup E *Sox* protein family, exert crucial effects on numerous nervous system developmental processes in vertebrates. These TFs participate in the original neural crest occurrence and ensure pluripotency maintenance and survival in migratory neural crest stem cells. Moreover, they are crucial regulatory factors for glial norms in the CNS and peripheral NS [7, 37]. Therefore, several *SoxE* genes are simultaneously expressed in an organ, suggesting their coordinating activity in performing a function. Such activity is possibly essential for neurogenesis or for maintaining bioprocesses in the turbot brain.

In vertebrates, all three *SoxE* proteins (*SOX8*, *SOX9*, and *SOX10*) from diverse species exhibit high similarity in HMG nonbox structural conservation and box homology, with

95% similarity in amino acid sequence. This may be the reason behind the conserved function of *SoxE* among species [6, 7]. For example, *SoxE* proteins play multiple roles in the specification and differentiation of mammalian sex. Among the *SoxE* proteins, *Sox9* is the most important and conserved sex-determining protein. After sex determination, a complicated positive feedback pathway exists among *Sox8*, *Sox9*, and *Sox10*; the pathway is necessary to maintain spermatogenesis and fertility in males [38]. *SoxE* paralogs *Sox8*, *Sox9*, and *Sox10* have been isolated from many fish, and the research on gonad function has also made some progress. In adult Japanese flounder, *Sox8b*, *Sox9a*, *Sox9b*, and *Sox10a* are expressed in the testis and also in the ovary to a certain extent [13]. In adult spotted sea bass, *Sox8a*, *Sox8b*, *Sox9b*, and *Sox10* are highly expressed in the testis and ovaries,

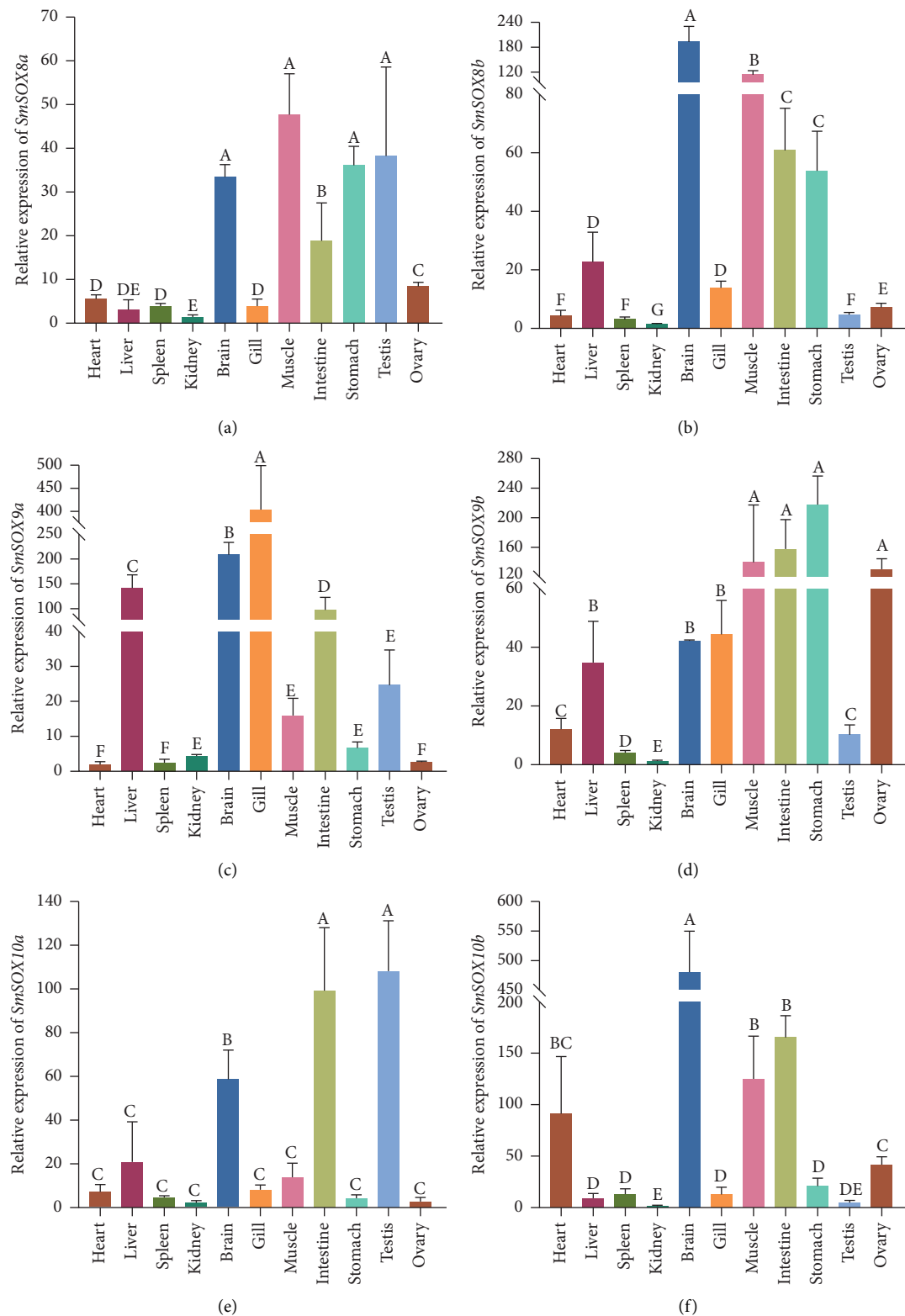


FIGURE 4: Spatial expression patterns for six *SoxE* genes in 11 tissues. The expression of one gene was determined on the basis of its minimum expression level across diverse tissues. Vertical bars indicate the mean $\pm$ S.E. P values were determined using the SPSS 20.0 through an independent sample t -test. Different letters represent statistical significance ($P < 0.05$) or the common letters across diverse groups represent nonsignificance.

whereas *Sox9a* is almost not expressed in both testis and ovaries [14]. According to our results, *Sox8a*, *Sox9a*, and *Sox10a* are upregulated in testis. The *Sox9b* upregulation in the ovary indicated its critical regulatory effect on testis and ovary development. The role of *SoxE* in turbot sex determination and development requires further exploration in gonads at different development stages and should be verified using gene knockout or RNAi. In addition, almost all *SoxE* genes were expressed at low levels in the spleen and kidney, indicating that these genes may not participate in spleen or kidney development and function maintenance, a result consistent with those of previous reports [3, 13, 14].

4. Conclusion

The present work identified six *SoxE* genes in *S. maximus*. Gene expression patterns in adult tissues provide crucial data regarding turbot *SoxE* activities, and the data should be verified using technologies such as gene knockout and RNAi. These findings would assist in the understanding of fish *SoxE* subfamily activity and evolution.

Data Availability

All data included in this study are available upon request upon contact with the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1: Melt curves of all genes used in this study. (A) *Sox8a*, (B) *Sox8b*, (C) *Sox9a*, (D) *Sox9b*, (E) *Sox10a*, and (F) *Sox10b*. (Supplementary Materials)

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