Comparative Biochemistry and Physiology, Part B 177–178 (2014) 56–64

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Molecular characterization and functional divergence of two *Gadd45g* homologs in sex determination in half-smooth tongue sole (*Cynoglossus semilaevis*)

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ARTICLE INFO

Article history:
Received 26 January 2014
Received in revised form 1 May 2014
Accepted 1 September 2014
Available online 8 September 2014

Keywords:
Cynoglossus semilaevis
Gadd45g
Sex differentiation

ABSTRACT

The growth arrest and DNA-damage-inducible protein 45 gamma (Gadd45g) is known to play a major role in embryonic development and sex determination. In this study, two *Gadd45g* genes were isolated from half-smooth tongue sole (*Cynoglossus semilaevis*). Using chromosomal fluorescence in situ hybridization (FISH), *Gadd45g1* and *Gadd45g*2 were located on the W and Z chromosomes, respectively. The full-length cDNA sequences of *Gadd45g1* (1270 bp) and *Gadd45g2* (1181 bp) were predicted to contain a 480-bp coding sequence that could encode a protein of 159 amino acids residues. A phylogenetic tree showed that the predicted Gadd45g1 and Gadd45g2 amino acid sequences clustered closely in one branch. It is proposed that *Gadd45g1* and *Gadd45g2* are paralogous genes derived from the divergence of the sex chromosome. Ka/Ks ratios indicated that *Gadd45g1* and *Gadd45g2* may have undergone a high number of mutations and have a divergence time of only about 68,000 years, although *Gadd45g* homologs are highly conserved. The qRT-PCR demonstrated that *Gadd45g1* and *Gadd45g2* were highly expressed in ovary, and negligibly expressed in testis of male and neo-male. During development of the ovary (from 80 to 150 days), the expression levels of both genes reached high levels. *Gadd45g1* was also highly expressed at 50 days, the stage just before gonad differentiation in *C. semilaevis*. All these findings imply functional divergence of the two *Gadd45g* homologs; *Gadd45g1* may be necessary for sex differentiation in the early stage of gonad development, and then *Gadd45g1* and *Gadd45g2* maintain ovary development and the female character of half-smooth tongue sole.

1. Introduction

Growth arrest and DNA-damage-inducible protein 45 (*Gadd45*) genes encode a small family of multifunctional stress response proteins (Fornace et al., 1988) that mediate diverse cellular processes, including DNA repair, apoptosis, cell cycle arrest, senescence (Hollander and Fornace, 2002; Liebermann et al., 2011), and DNA demethylation (Sheikh et al., 2000; Zhan, 2005; Barreto et al., 2007; Niehrs and Schäfer, 2012). Gadd45 family members Gadd45a, Gadd45b, and Gadd45g appear to have overlapping but non-identical functions and binding partners, and are induced by different stimuli (Liebermann and Hoffman, 2008).

Recently, many studies have focused on the important role of Gadd45g in embryonic development and sex determination. *Gadd45g* was found to be expressed in primary neuron precursors and was reported to promote differentiation by regulation of cell cycle exit in medaka fish (*Oryzias latipes*) and the African clawed frog (*Xenopus laevis*) (de la Calle-Mustienes et al., 2002; Candal et al., 2004). *Gadd45g* genes were also found to be involved in vertebrate neurogenesis (Kaufmann et al., 2011). However, previous studies in mice (*Mus musculus*) found that *Gadd45g* genes were dispensable for embryonic development, because adult mutant mice were viable and did not present obvious malformations (Hollander et al., 1999; Hoffmeyer et al., 2001; Gupta et al., 2006). In-depth studies of *Gadd45g*-mutant mice (Hoffmeyer et al., 2001; Cai et al., 2006; Bouma et al., 2007; Bogani et al., 2009; Warr and Greenfield, 2012) revealed a specific role for *Gadd45g* in mammalian sex determination. It was determined that Gadd45g, but not Gadd45a or Gadd45b, was necessary for activation of the male sex-determining pathway in mice (Johnen et al., 2012) and *Gadd45g*-mutant mice showed complete male-to-female sex reversal. A signaling cascade required for sex-determining region Y (*Sry*) transactivation was also found (Gierl et al., 2012; Warr et al., 2012).

The half-smooth tongue sole (*Cynoglossus semilaevis*) is an increasingly important marine flatfish of potentially great aquaculture value

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http://dx.doi.org/10.1016/j.cbpb.2014.09.001
1096-4959/

in China (Liu et al., 2005). It exhibits prominent sexually dimorphic growth (Ji et al., 2011) in which females grow two to three times faster than males. As a result, female half-smooth tongue sole are favored for cultivation; however, female-to-male sex reversal in this species (Ji et al., 2010) can reduce the proportion of phenotypic females, causing huge financial losses. As a result, the physiological mechanism of sex reversal in *C. semilaevis* is of great interest and still needs to be clarified.

Here, we cloned two *Gadd45g* homologous genes (*Gadd45g1* and *Gadd45g2*) in *C. semilaevis*, and analyzed the physical location, sequence characteristics, evolution, and expression patterns of these two genes. The aim of this study was to reveal the physiological functions and relationship between *Gadd45g1* and *Gadd45g2* to determine their effect on sex determination in *C. semilaevis*.

2. Materials and Methods

2.1. Fish and sampling

The half-smooth tongue soles (*C. semilaevis*) used in the experiments were obtained from Huanghai Aquaculture Ltd. (Haiyang, Shandong Province, China). One-year-old sexually mature fish (three individuals of each gender) were randomly sampled. Spleen, skin, heart, liver, gill, kidney, whole brain, intestine, ovary or testis, muscle, and blood tissues (11 tissues in all), were collected and snap-frozen in liquid nitrogen, and then stored at −80 °C. Additionally, gonads at different developmental stages were collected from larvae from the same family as the sexually mature fishes and stored at −80 °C. The gonads were collected from three individuals of each gender at 20, 35, 50, 65, 80, 95, 120, and 150 days after hatching. To determine the genetic sex of the fish in our study, their fins were collected and stored in 100% ethanol for DNA extraction.

2.2. RNA extraction and genetic sex identification

Total RNA was extracted from the frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was conducted using a PrimeScript™ RT regent Kit with gDNA Eraser (TAKARA, Dalian, China).

Genomic DNA was extracted from fins using a phenol-chloroform protocol (Chen et al., 2007). Female-specific PCR primers, CseF382F and CseF382R (Hu and Chen, 2013), were used to amplify the DNA for genetic sex identification. The PCR products were separated on 1% agarose gels and samples with a 291-bp band were identified as genetic females, while samples with no band were identified as genetic males. The gonad histology was carried out as described (Chen et al., 2008).

Neo-males were identified as individuals that were genetic females, but phenotypic males.

2.3. Primer design and fluorescence in situ hybridization (FISH)

To validate the genome sequences in the extracted RNA and to determine the physical location of *Gadd45g1* and *Gadd45g2* in the *C. semilaevis* genome, two pairs of updated primers (Table 1) were designed based on the *Gadd45g* sequence from the whole-genome sequence results (Chen et al., 2014). All the primers used in our study (Table 1) were designed by Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA).

The positive clone corresponding to the target gene was screened using updated primers from the *C. semilaevis* BAC library (Shao et al., 2010) to enlarge cultivation. Plasmids were extracted from the positive clone using a Mini Plasmid Kit (Biomed, Beijing, China), and then used to prepare FISH probes using DIG-Nick Translation Mix (Roche, Basel, Switzerland). Preparation of the chromosomes of the mature male and female *C. semilaevis* was finished as described previously (Xie et al., 2012).

Table 1
Primers used in this study.

Group	Primers name	Sequences (5′ to 3′)
Sex identification primers	CseF382F	ATTCACTGACCCCTGAGAGC
	CseF382R	TGGCACCATCATTGTAAAACTA
Updated primers	Gadd45g1-A	GCTTTGGGAGAGTGATGCTG
	Gadd45g1-S	CGCCAAGAAGCAGGACTACC
	Gadd45g2-A	CTGGAAGGGTGATGCTGGGC
	Gadd45g2-S	CACGGACAAGACAACACGATGGA
RACE primers	Gadd45g1-3′R	GCGAGGAGAGCCGCAGCGTGTA
	Gadd45g1-3′N	GTGCCCAGCATCACTCTCCCAA
	Gadd45g1-5′R	GCAGTGCGGGTCCTTGGGCTCG
	Gadd45g1-5′N	GCACCCTGTCACTGTTTTCCGTC
	Gadd45g2-3′R	CGCCAAGAATCGGGACTACCTGACG
	Gadd45g2-3′N	AATGTTGACCCAGACAGCGTG
	Gadd45g2-5′R	TCCAACCGTCAGGTAGTCCCGATTC
	Gadd45g2-5′N	TTTTCCATCGTGTTGTCTTGTCC
	UPM-long	CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT
	UPM-short	CTAATACGACTCACTATAGGG
	NUP	AAGCAGTGGTATCAACGCAGAGT
qRT-PCR primers	Gadd45g1-qrt-a	ACACTGTTGCAGGGGAGGAT
	Gadd45g1-qrt-s	TGGTGAAAAGAAAGTCAAGGAGC
	Gadd45g2-qrt-a	GGGTGAAGTGGATCTGGAGC
	Gadd45g2-qrt-s	AAGAATCGGGACTACCTGACG
	Actin-a	GAGTAGCCACGCTCTGTC
	Actin-s	GCTGTGCTGTCCCTGTA

The FISH probes were applied to the chromosome slide and hybridized with target DNA, then incubated with Goat Anti-DIG antibody (Roche) and stained stepwise with secondary fluorescein isothiocyanate (FITC)-labeled donkey anti-sheep IgG antibodies (Roche). After air-drying in the dark, the slides were counterstained with fluorescence quenching agent with propidium iodide (PI) (Sigma-Aldrich, Santa Clara, CA, USA) and covered with a cover slip. The slides were examined under a fluorescence microscope (Nikon Eclipse 80i) equipped with an ×60 oil immersion lens and photographed using a Nikon camera (Nikon Digital Sight Ds-Fi2).

2.4. RACE

Two pairs of RACE-specific primers were designed for 5′- and 3′-RACE, based on the genomic DNA sequences that were identified from the whole-genome sequencing results (Chen et al., 2014). To obtain the full-length sequences of *Gadd45g1* and *Gadd45g2* genes, 5′- and 3′-RACE were performed using a SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Here, to illustrate the method, the full-length cloning of one of the genes (*Gadd45g1*) is described in detail. Gadd45g1-5′R/3′R primers and universal primer 10*UPM were used for touchdown PCR under the following conditions: denaturation at 94 °C for 5 min, followed by 14 cycles (94 °C for 30 s, first cycle 72 °C for 1 min and subsequently the temperature was reduced by −0.5 °C per cycle, and 72 °C for 1 min), 30 cycles (94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min), and then at 72 °C for 10 min. The same method was used for *Gadd45g2*. The 5′- and 3′-RACE product were diluted 100 times with RNase-free ddH_2O and used as template for a nested PCR reaction using Gadd45g1-5′N/3′N primers and nested universal primer NUP under the following conditions: 94 °C for 5 min, followed by 30 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min), and 72 °C for 10 min.PCR reactions were performed using a Veriti™ 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR products were separated on 1.5% agarose gel, purified with Zymo Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA), ligated into a pMD-18 T vector (TaKaRa) and propagated in the *E. coli* Top10 (Tiangen, Beijing, China). Three positive clones were selected for sequencing. The same method was used for the other gene, *Gadd45g2*.

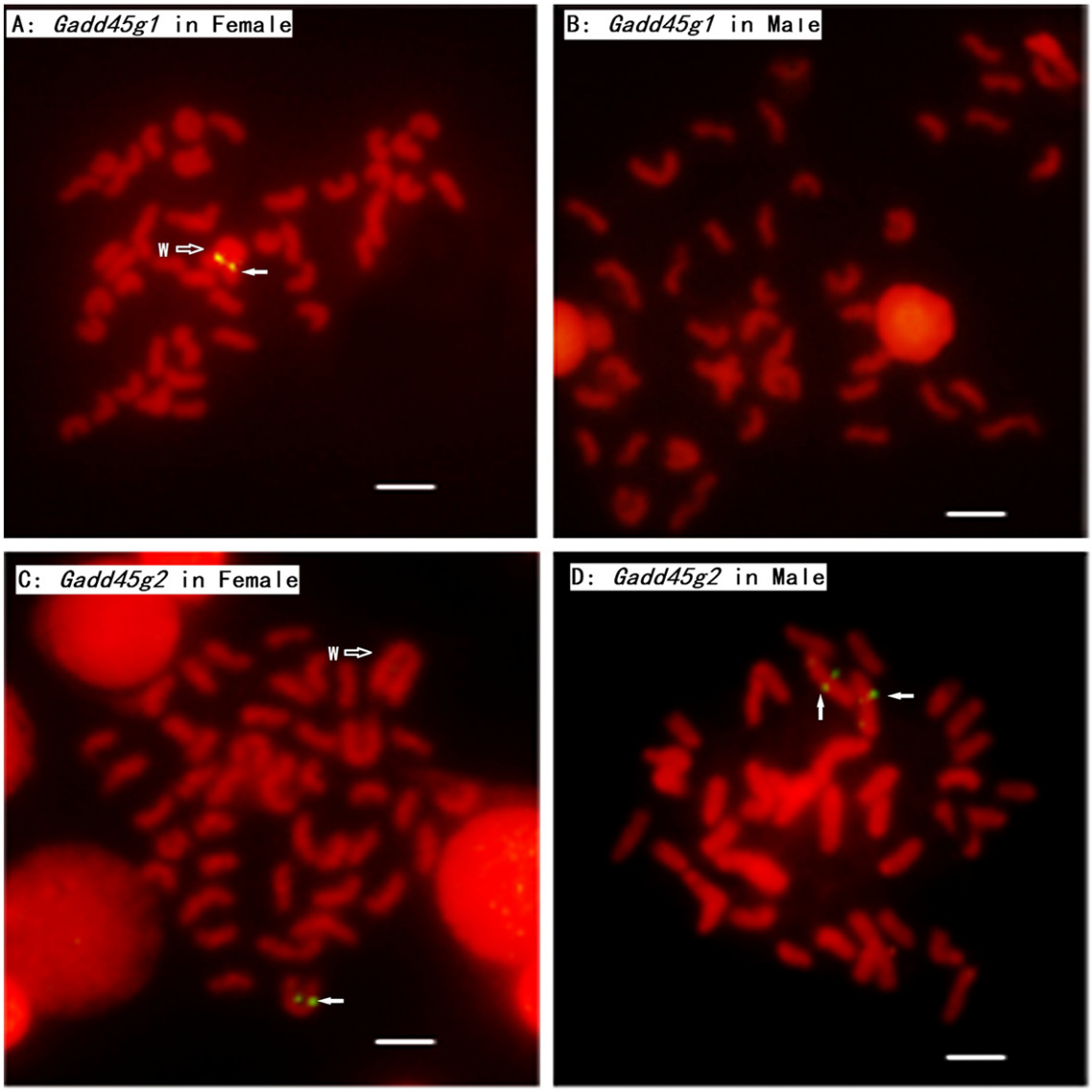

Fig. 1. The result of FISH of *Gadd45g1* (A: *Gadd45g1* in female; B: *Gadd45g1* in male) and *Gadd45g2* (C: *Gadd45g2* in female; D: *Gadd45g2* in male). W indicates the W chromosome; the solid arrows indicate the hybridization signals. Scale bars, 5 μm.

2.5. Sequence and phylogenetic analysis

Sequence data were spliced and analyzed using the DNAStar_LaserGene 7.0 software (DNAStar, Madison, WI, USA). The physico-chemical parameters of the predicted protein sequence were computed using ProtParam (http://www.expasy.org/tools/protparam/). The NCBI basic BLASTp program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search the NCBI protein nr database.

A phylogenetic analysis of genes homologous to *Gadd45g* was conducted using MEGA 5.0 software (Tamura et al., 2011). The phylogenetic tree was constructed using a neighbor-joining method (Saitou and Nei, 1987) based on the Poisson-corrected distances. Tree topological stability was evaluated by 1000 bootstrap re-samplings. The coding sequences (CDSs) of *Gadd45g1* and *Gadd45g2* were used in KaKs_Calculator2.0 (Wang et al., 2010) to estimate the evolution rates.

2.6. Quantitative real-time PCR (qRT-PCR)

The expression of *Gadd45g1* and *Gadd45g2* mRNA in the different tissues and at the different stages of gonad development were detected independently in triplicate. The qRT-PCR primers (Table 1) were designed based on the predicted gene-specific regions of *Gadd45g1* and *Gadd45g2*. The reactions were conducted on a 7500 ABI Real-time PCR system (Applied Biosystems). Amplifications were performed in a 20 μL final volume containing 0.3 μL cDNA template, 10 μL SYBR® Premix Ex TaqTM (Takara), 0.4 μL ROX Reference Dye II, 0.4 μL of each of the gene primers, and 8.5 μL ddH_2O. PCR amplifications were performed in triplicate, using the following conditions: an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 34 s at 60 °C. A dissociation stage was performed after thermo-cycling to determine target specificity. The expression of *β-actin* (Table 1) was used as negative control.

3. Results

3.1. Identification and FISH mapping of Gadd45g gene

We successfully determined the physical location of *Gadd45g1* and *Gadd45g2* in the chromosomes of half-smooth tongue sole using FISH. The two genes were detected, respectively, in the Bam002C14 and Hind029A24 clone of BAC library (Shao et al., 2010). Hybridization

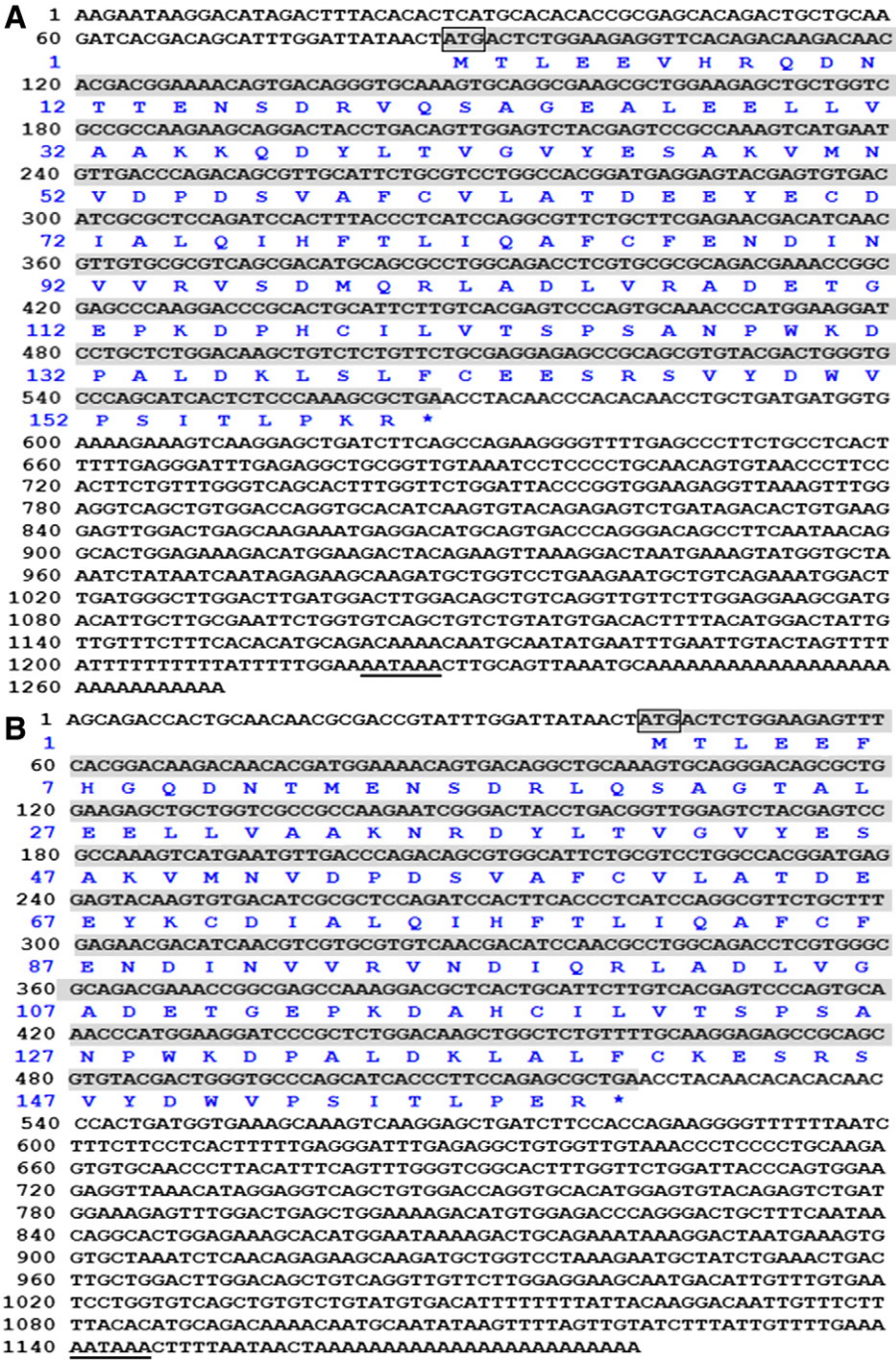

Fig. 2. The structure of the half-smooth tongue sole *Gadd45g1* (A) and *Gadd45g2* (B) gene and the deduced amino acid sequence. The deduced amino acid residues are represented as single-letter abbreviations and numbered from the initiating methionine. The tailing signal peptides are underlined; the translation initiation sites are boxed. The stop codon is marked by an asterisk. The sequence was submitted to GenBank under accession number KF937802 (*Gadd45g1*) and KF937803 (*Gadd45g2*).

signals for *Gadd45g1* and *Gadd45g2* were found on the W and Z chromosomes, respectively (Fig. 1). There are two hybridization signals on one W chromosome in female and no signal in male when *Gadd45g1* was used as the probe. There are two hybridization signals on each of two Z chromosomes of male, while hybridization signals could only be found on one Z chromosome of female when *Gadd45g2* was used as probe.

3.2. Sequence characteristics of two Gadd45g genes

The two full-length *Gadd45g* gene sequences that were successfully cloned by RACE technology have been deposited in the GenBank database under accession no. KF937802 (*Gadd45g1*) and KF937803 (*Gadd45g2*) and the nucleotide base sequence of the two genes are displayed in Fig. 2.

The full-length cDNA of *Gadd45g1* was 1270-bp-long and consisted of an 86-bp 5′ untranslated region (5′UTR), a 704-bp 3′UTR, and a 480-bp CDS. The 3′UTR contained a single typical polyadenylation signal (AATAAA) between nucleotides 1221 and 1226, just 15 bp upstream from the poly(A) tail. The predicted amino acid (AA) sequence of *Gadd45g1* was 159 residues long and had an estimated molecular weight (Mw) of 17.9 kDa and theoretical isoelectric point (p*I*) of 4.38. The full-length cDNA of *Gadd45g2* was 1181 bp long and consisted of

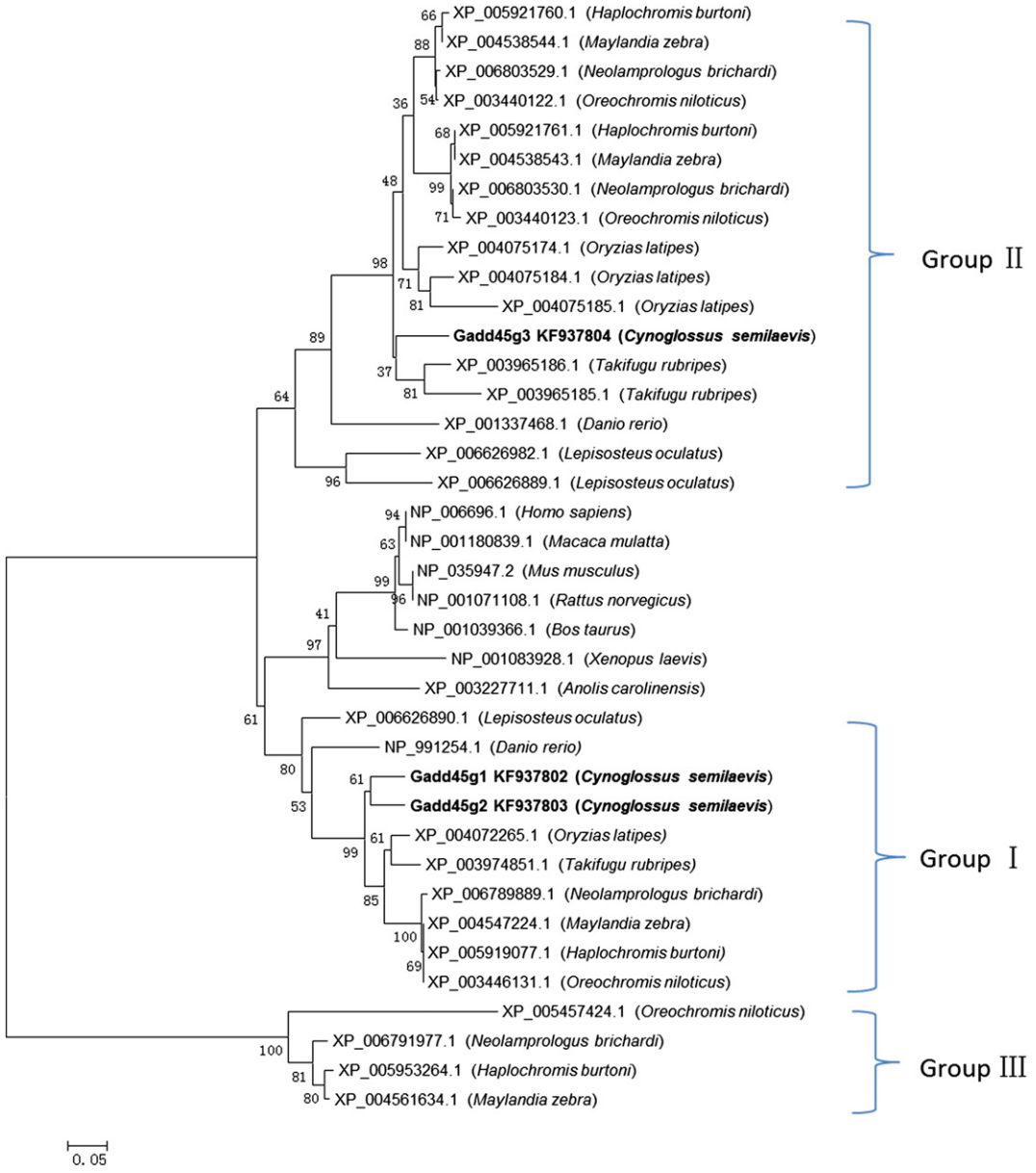

Fig. 3. The phylogenetic tree of Gadd45g based on neighbor-joining (NJ) method. The bootstrap confidence values shown at the nodes of the tree are based on a 1000-bootstrap procedure, and the branch length scale in terms of genetic distance is indicated above the tree. The teleost Gadd45g AA sequences clustered into three groups named I, II, and III.

a 41-bp 5′UTR, a 480-bp CDS, and a 660-bp 3′UTR. A polyadenylation signal (AATAAA) was found in the 3′UTR. The predicted AA sequence of *Gadd45g2* was 159 AA residues long and had an estimated Mw of 17.8 kDa and theoretical p*I* of 4.37.

Alignment of the predicted AA sequences of the *Gadd45g1* and *Gadd45g2* genes by BLASTp searches against the AA sequences from other species showed that the *C. semilaevis* sequences shared a high degree of identity with the sequences from many other fish. For example, the Gadd45g1 AA sequence shared 89% identity with *Oreochromis niloticus* (XP_003446131.1), 88% with *Neolamprologus brichardi* (XP_006789889.1), 87% with *Takifugu rubripes* (XP_003974851.1), 87% with *Oryzias latipes* (XP_004072265.1), and 77% with *Danio rerio* (NP_991254.1). The Gadd45g2 AA sequence shared 89% identity with *O. niloticus* (XP_003446131.1), 87% with *T. rubripes* (XP_003974851.1), and 79% with *D. rerio* (NP_991254.1).

A phylogenetic tree (Fig. 3) was generated and showed that all the teleost Gadd45g sequences clustered into three groups, and the amphibian and mammalian Gadd45g sequences clustered into another group. There are at least two copies of *Gadd45g* gene in teleost. One copy including the Gadd45g1 and Gadd45g2 AA sequences of *C. semilaevis* formed group I. The other copies divided into another two groups (groups II and III). Multiple alignment of the teleost proteins in group I and the amphibian and mammalian Gadd45g sequences (Fig. 4) shows several conserved domains among the members of this family.

3.3. Ka/Ks analysis

The ratio of the number of nonsynonymous substitutions per nonsynonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks) (Laurence, 2002) between any two CDSs in the *Gadd45g* genes from *C. semilaevis, O. niloticus*, *D. rerio*, *M. musculus,* or *H. sapiens* (group I sequences) was calculated using the YN method (Yang and Nielsen, 2000). From the table of the Ka/Ks ratios (Table 2), we found that all the Ka/Ks ratios between sequences in this group were <0.3. The Ka/Ks ratio between *Gadd45g1* and *Gadd45g2* was maximal at 0.288647 ($P = 0.00141284$) and the divergence time was 0.0676534 Mya (million years ago); all the other Ka/Ks ratios between

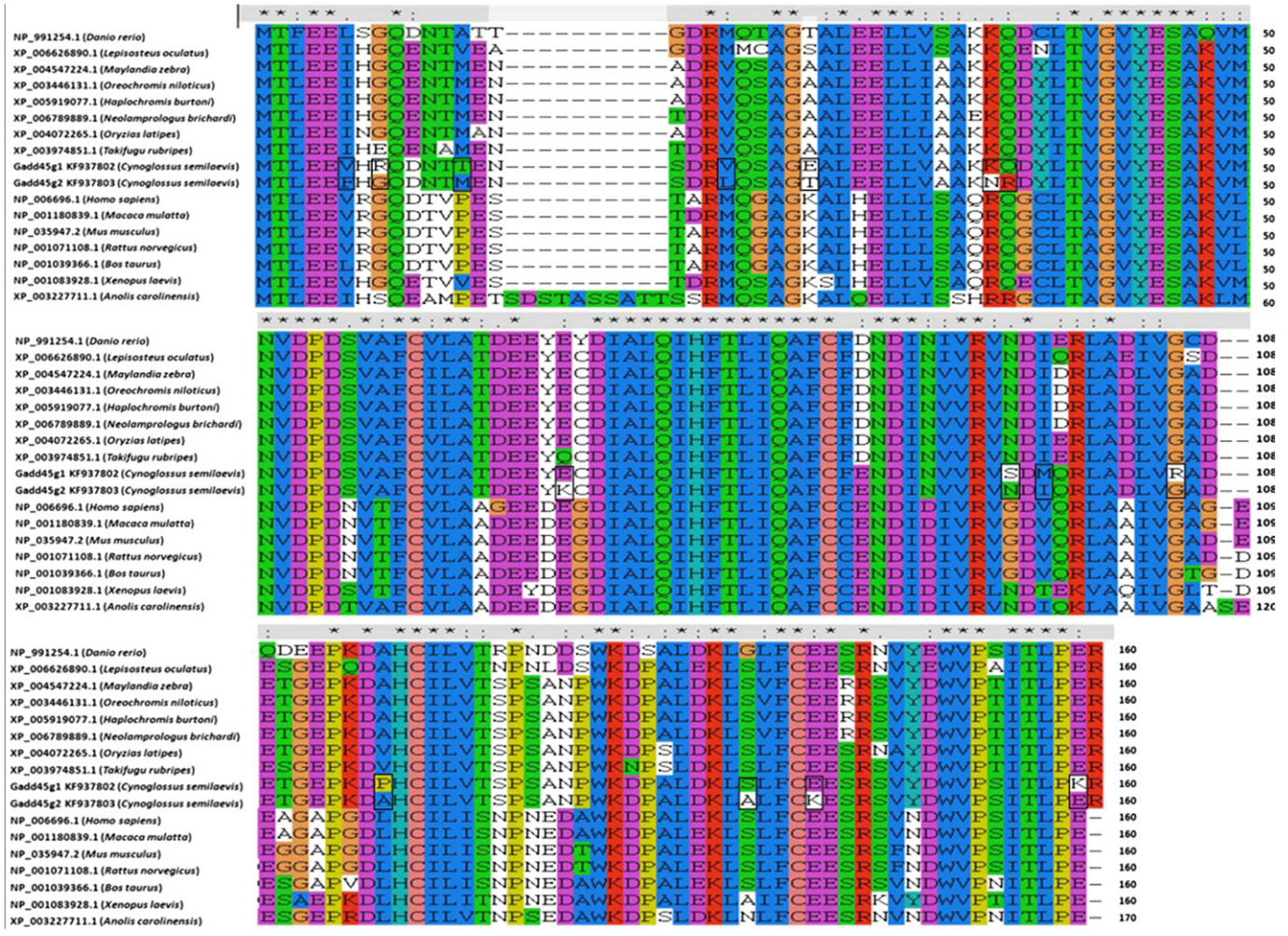

Fig. 4. Multiple alignment of amino acid sequences of different members (all teleost Gadd45g in group I and the amphibian and mammalian Gadd45g sequences). The asterisk indicated residues sharing 100% homology. The different residues between Gadd45g1 and Gadd45g2 were boxed.

two genes were 0.0123411–0.110078 ($P = 1.12233E-14–4.00574E-37$).

3.4. Expression analysis of the two Gadd45g genes by qRT-PCR

When the expression levels of the two Gadd45g homologs in the gonads of female, male, and neo-male of half-smooth tongue sole were compared (Fig. 5), we found that the expression levels in the neo-male and male gonads were significantly lower than in the gonads of the female ($P < 0.05$).

The expression levels of the two *Gadd45g* mRNAs in the tissues of the female and male adults are shown in Fig. 6. In the adult male, no *Gadd45g1* expression was detected in any of the tissues tested, while in the adult female, *Gadd45g1* mRNA was found to be highly expressed

Table 2
The Ka/Ks ratio of *Gadd45g* homologous genes.

Sequence	Method	Ka	Ks	Ka/Ks	*P* (Fisher)	Divergence-Time
Gadd45g1/Gadd45g2	YN	0.04446	0.1534	0.2898	0.001460	0.06810
Gadd45g1/O. niloticus	YN	0.06239	0.6035	0.1034	1.122E-14	0.1838
Gadd45g2/O. niloticus	YN	0.05835	0.5653	0.1032	2.861E-15	0.1840
Gadd45g1/D. rerio	YN	0.1332	1.829	0.07281	3.464E-31	0.5419
Gadd45g2/D. rerio	YN	0.1230	1.351	0.09105	3.254E-28	0.4380
Gadd45g1/M. musculus	YN	0.1664	2.125	0.07831	1.784E-27	0.5995
Gadd45g2/M. musculus	YN	0.1842	1.673	0.1101	5.996E-23	0.5240
Gadd45g1/H. sapiens	YN	0.1669	2.337	0.07142	3.253E-23	0.5927
Gadd45g2/H. sapiens	YN	0.1839	2.472	0.07439	1.149E-21	0.6469
O. niloticus/M. musculus	YN	0.2055	2.156	0.09528	1.152E-26	0.6972
O. niloticus/D. rerio	YN	0.1322	1.723	0.07676	1.788E-23	0.5546
O. niloticus/H. sapiens	YN	0.2083	3.540	0.05884	4.314E-30	1.001
M. musculus/D. rerio	YN	0.20262	3.578	0.05663	4.006E-37	1.048
M. musculus/H. sapiens	YN	0.01358	1.100	0.01234	3.888E-33	0.2530
D. rerio/H. sapiens	YN	0.2045	3.550	0.05761	6.521E-34	1.012

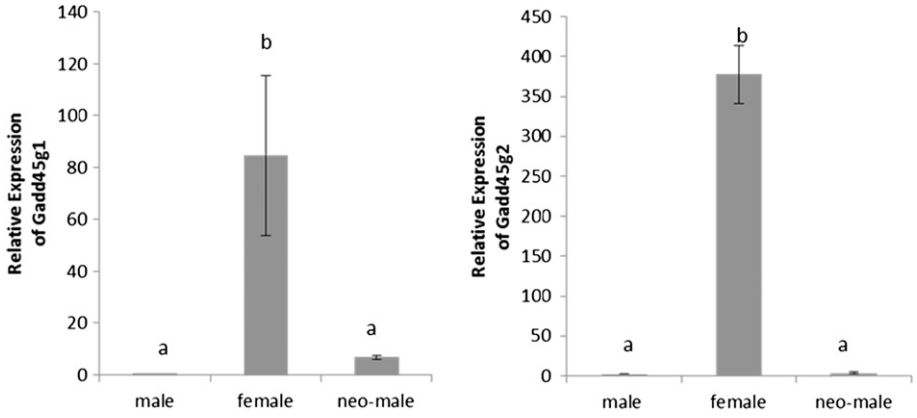

Fig. 5. Relative expression of *Gadd45g1* and *Gadd45g2* gene in male, female, and neo-male gonads of half-smooth tongue sole *C. semilaevis*. The data were analyzed using one-way ANOVA followed by Duncan comparison tests using SPSS 18.0. Bars represent triplicate means ± S.E. from three separate individuals ($n = 3$). Bars with different letters differed at $p < 0.05$.

in liver and ovary, less highly expressed in brain and gill, and negligibly expressed in intestine, kidney, and other tissues. Conversely, the expression level of *Gadd45g2* mRNA was extremely high in ovary, but low in all the other tissues of female and male tissues tested.

The expression patterns of the *Gadd45g1* and *Gadd45g2* genes in the gonads at different development stages are shown in Fig. 7. In contrast with undetectable levels that were found in all stages of testis development, *Gadd45g1* mRNA was detected at all stages of ovary development. In the ovary, the *Gadd45g1* expression levels increased till they reached a peak at 50 days, dropped to a valley at 80 days, and then rose rapidly again. Very low or no significant differences were found in the expression levels of *Gadd45g2* before 80 days in the gonads of both males and females. After 80 days, the expression levels of *Gadd45g2* increased significantly in the ovaries of 95- and 150-day-old fry.

4. Discussion

The *Gadd45g* genes have been cloned in many amphibian and mammalian species, but in fish, it has only been cloned in medaka (Candal et al., 2004). In the present study, we cloned, for the first time, the full-length cDNA sequences of two *Gadd45g* genes named *Gadd45g1* (1270 bp) and *Gadd45g2* (1181 bp) in half-smooth tongue sole, *C. semilaevis*. *Gadd45g1* and *Gadd45g2* were mapped onto W and Z chromosomes, respectively. In the whole-genome sequences of *C. semilaevis,* more than one copy of *Gadd45g* has been found on autosomes of *C. semilaevis*. Indeed, a third *Gadd45g* homolog named *Gadd45g3* was detected in *C. semilaevis*, sequenced, and mapped to an autosome in our study. We intend to carry out further experiments to study these *Gadd45g* genes.

Although only one type of mammalian *Gadd45g* genes has been found, in teleosts, there is more than one copy of *Gadd45g* genes resulting from the fish-specific genome duplication (FSGD or 3R) (Hoegg and Meyer, 2005). The availability of whole-genome sequences for a variety of fish species will provide the sequence data required to

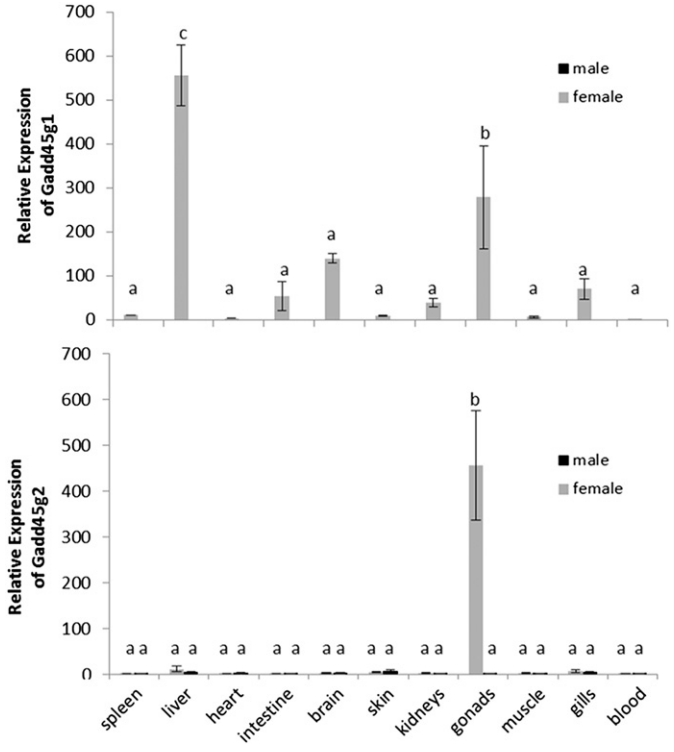

Fig. 6. Relative expression of two *Gadd45g* genes in various organizations from mature female and male of half-smooth tongue sole *C. semilaevis*. Bars represent triplicate means ± S.E. from three separate individuals ($n = 3$). Bars with different letters differed at $p < 0.05$.

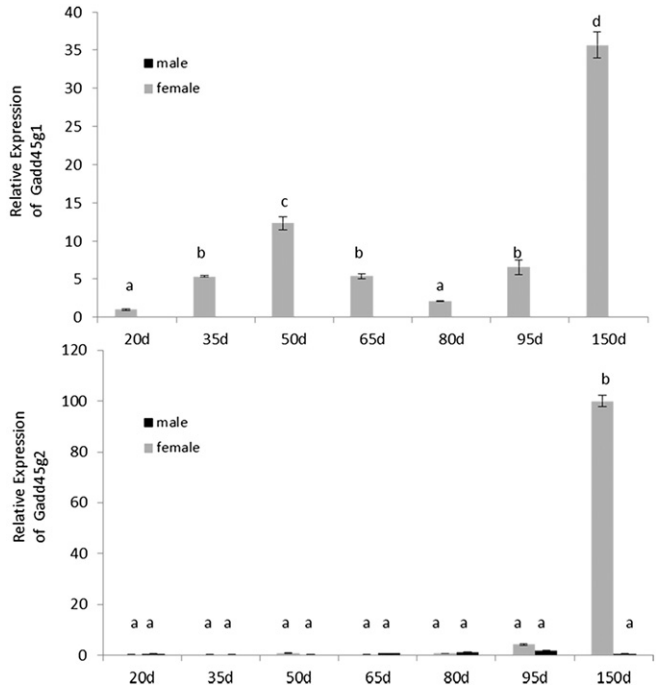

Fig. 7. Relative expression of *Gadd45g1* and *Gadd45g2* gene in each gonad development stages of half-smooth tongue sole *C. semilaevis*. Bars represent triplicate means ± S.E. from three separate individuals ($n = 3$). Bars with different letters differed at $p < 0.05$.

detect new copies of the *Gadd45g* gene. For example, in the NCBI database, there are four different Gadd45g gene sequences from *O. latipes*.

Phylogenetic analysis revealed the phylogenetic relationship of the Gadd45g AA sequences from different species. The translated Gadd45g1 and Gadd45g2 sequences formed a group (group I) with one copy of fish Gadd45g sequences, which were then grouped with the mammalian Gadd45g sequences, suggesting that the Gadd45g1 and Gadd45g2 sequences in half-smooth tongue sole were orthologous to mammalian Gadd45g, while the Gadd45g3 in half-smooth tongue sole was produced by whole genome duplication. *Gadd45g1* and *Gadd45g2* may work as paralogous genes that were created by the divergence of the sex chromosomes.

The Ka/Ks ratio can be used to identify the type of sequence evolution that might have occurred (Laurence, 2002). Comparing the Ka/Ks ratios reported for homologous *Gadd45g* genes in several species, Ka was much smaller than Ks and all Ka/Ks ratios were <0.3. This finding indicates that the *Gadd45g* genes may have been evolutionarily conserved by purification selection and may still retain their original function. Peculiarly, *Gadd45g1* and *Gadd45g2* in *C. semilaevis* exhibited the highest Ka/Ks ratio among the gene pairs tested. This result might imply that a great mutation had occurred in the *Gadd45g1* and *Gadd45g2* genes that had caused them to evolve in different functional directions, although the divergence time for these genes in relatively recent (about 68,000 years ago) compared with the divergence times for orthologous genes among different species.

The qRT-PCR results revealed that the *Gadd45g* genes may have a special function in female sex determination in *C. semilaevis.* After the role of Gadd45g in mammalian male sex determination was identified, it attracted considerable attention. In mouse, the expression levels of *Gadd45g* were found to be similar in both ovary and testis (Small et al., 2005). However, in *Gadd45g*-mutant mice, normal *Sry* gene expression is inactivated and the mice show complete male-to-female sex reversal (Gierl et al., 2012). In mammals, Gadd45g was reported to work as a major developmental regulator, which activated normal expression of *Sry* gene, the sex determination gene (Hiramatsu et al., 2009). We propose that, contrary to the function of the *Gadd45g* gene in mammals, the two *Gadd45g* paralogous genes in *C. semilaevis* may be involved directly in sex determination and differentiation, and the sex reversal of *C. semilaevis* may be triggered by the inhibition of *Gadd45g1* and *Gadd45g2* expressions in female. In normal *C. semilaevis* population, the skewed sex ration toward phenotypic male was a result of a natural female-to-male sex reversal phenomenon. The expression patterns of *Gadd45g1* and *Gadd45g2* were sexually dimorphic; namely, *Gadd45g1* and *Gadd45g2* genes were found to be specifically and highly expressed in the ovary, while they were negligibly expressed in the testis of male and neo-male. This result may indicate that sex reversal in *C. semilaevis* is accompanied by changes in the expression levels of the two *Gadd45g* mRNA transcriptions.

In mouse, *Gadd45g* was found to be highly expressed in skeletal muscle, kidney, and liver, but low in heart, brain, spleen, lung, and testis (Zhang et al., 1999). The alleles of the *Gadd45g* gene in the mouse were reported to play a role in the regulation of body weight (Balliet et al., 2003). It is possible that the specific physical location of *Gadd45g1* in the W chromosome causes it to have a female-specific distribution (highly expressed in liver and ovary) in the different tissues of *C. semilaevis*. The high expression levels of *Gadd45g1* in the female liver suggested that it may play an important role in energy metabolism because the female half-smooth tongue sole grows faster and attains a higher body weight than the male. This result is consistent with the idea that *Gadd45g1* and *Gadd45g2* have different functional roles in *C. semilaevis*. The high expression of *Gadd45g1* in ovary may imply a great function of *Gadd45g1* in maintaining the sex character in *C. semilaevis*. Similarly, the expression of *Gadd45g2* was extremely high in ovary but negligible in the other female and male tissues tested, although it was detected on the Z chromosome, suggesting that the Gadd45g1 and Gadd45g2 proteins could trigger and maintain the female character in *C. semilaevis*.

The expression profiles of *Gadd45g1* in the female ovary during the different developmental stages showed that *Gadd45g1* may participate in the gender decision-making process. Generally, gonad differentiation has been found to occur at the 56–62-day stage in half-smooth tongue sole (Chen et al., 2014). In the present study, the high expression of the *Gadd45g1* detected in the females at 50 days after hatching suggested that *Gadd45g1* may play an important role in gonadal differentiation in half-smooth tongue sole. This is consistent with *Gadd45g* expression levels peaking at the time of primary sex differentiation (11.5 dpc, or 18 tail somites) in mice (Johnen et al., 2012). However, *Gadd45g2* apparently plays different roles, and is involved only in maintaining the female characters in the later developmental stages. The significant variance in expression levels between *Gadd45g1* and *Gadd45g2* might suggest that the functional divergence has occurred between two *Gadd45g* homologs in *C. semilaevis*.

In summary, two full-length *Gadd45g* genes were cloned successfully and mapped on W and Z chromosomes, respectively, in half smooth tongue sole. The results show that the two *Gadd45g* homologous genes exhibit apparent evolutionary variation and also display different expression patterns, indicating their different functions in sex determination and differentiation in *C. semilaevis*. These results will provide basic information for further study of sex determination and differentiation mechanism in half-smooth tongue sole and other related species.

Acknowledgments

This work was supported by State 863 High-Technology R&D Project of China (2012AA092203, 2012AA10A403-2), National Nature Science Foundation (31130057, 41006107, 31472269), and Taishan Scholar Project Fund of Shandong of China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpb.2014.09.001.

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