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Genetic Characteristics and Evolution of *Pitx2* in Sinistral Tongue Sole, *Cynoglossus semilaevis* and Dextral Stone Flounder, *Kareius bicoloratus*

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Abstract The Nodal signaling pathway downstream transcription target *pitx2* has been found to be involved in determination of left-right (L-R) asymmetry in the mouse, chick and *Xenopus* embryos. However, the genetic characteristic and evolution of *pitx2* has rarely been reported in marine fish. In our study, we cloned and analyzed the structure of *pitx2* in tongue sole (*Cynoglossus semilaevis*) (*Cs-pitx2*) and stone flounder (*Kareius bicoloratus*) (*Kb-pitx2*). Phylogenetic and sequences analysis showed that *Cs*-pitx2 and *Kb*-pitx2 had high identity, and homologous to the pitx2 of other vertebrates. A low *Ka/Ks* ratio showed that *pitx2* evolving was under purifying selection. There are several basal core promoter elements and transcription factor binding sites in the promoter region of *Cs-pitx2*. Quantitative RT-PCR analysis showed that the expression of *pitx2* increased during the developmental stages. Interestingly, the expression of *pitx2* in sinistral tongue sole was much higher than in dextral flatfish stone flounder during metamorphosis, which might be one of the reasons of different deflecting direction of them. These data provide the evidence that *pitx2* was a crucial Nodal signaling pathway gene that might mediate L-R asymmetry in flatfish.

Keywords: *pitx2*, tongue sole *Cynoglossus semilaevis*, stone flounder, *Kareius bicoloratus*, gene expression and evolution.

INTRODUCTION

Members of the Pleuronectiformes, including tongue sole (*Cynoglossus semilaevis*) and stone flounder (*Kareius bicoloratus*) are unusual organisms because they show clear external left-right (L-R) asymmetry with both eyes localized on a single side of the body, and melanophores also occurring predominantly on the ocular side (Hashimoto *et al*., 2007). Tongue sole belongs to sinistral flatfish, while stone flounder is one of the dextral flatfish. It is well known that pleuronectiformes eyes first develop symmetrically during embryogenesis, with one on each side of the face, and that one eye then migrates to the other side at metamorphosis, with subsequent pigmentation on the ocular side (Okada *et al*., 2001; Watanabe *et al*., 2008). L/R asymmetry of the external body shape is established long after the internal organs such as heart, dorsal diencephalon are oriented asymmetrically, which is one of the characteristic events specific to the flatfish (Hashimoto *et al*., 2002; Ryan *et al*., 1998). It had been revealed that the L/R asymmetric orientation of the external and internal organs is controlled by the molecular pathways and all the vertebrates examined to date have a common signaling molecule related to Nodal (Capdevila, 2000; Collignon *et al*., 1996; Rebagliati *et al*., 1998).

Nodal signaling pathway plays key roles in patterning the nervous system germ-layer specification and mediating asymmetric organogenesis in the process of embryonic development in vertebrates (Faucourt *et al*., 2001; Schier, 2003; Shen, 2007). Several studies had reported that many genes, such as *activin* and its receptor, *Sonic hedgehog* (*Shh*), *nodal* [*southpaw*

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(*spaw*) and *cyclops* (*cyc*)] in teleost (Levin *et al*., 1995), the *TGF-β* super-family members *lefty-1* and *lefty-2* (Meno *et al*., 1997, 1996), and *cSnR*, a member of the Snail family (Isaac, 1997) were shown to mediate L-R asymmetrical expression in vertebrate embryos. In addition to these, one important mediator is the homeobox gene *pitx2*, which expressed asymmetrically in the left lateral plate mesoderm (LPM), tubular heart and early gut tube (Campione *et al*., 1999; Peyrieras *et al*., 1998) and had been proposed to act downstream of Nodal signaling pathway in the gene cascade providing left-right cues to the developing organs. Meanwhile, it also determined left-right polarity of mesoderm-derived organs such as heart, gut and eyes (Hjalt *et al*., 2000).

Tongue sole and stone flounder are good models for studying the L-R asymmetry, since one is sinistral flatfish and the other is dextral flatfish. Both of these two flatfishes have the original symmetric prototypical structure and later transform into an asymmetric organ. Nodal signaling pathway is a putative candidate pathway that regulates this process. However, the specific regulating mechanism that forms this asymmetry and the relative function of *pitx2* in the system is not clear.

In this study, we cloned the full length tongue sole *pitx2* (*Cs-pitx2*) and stone flounder *pitx2* (*Kb-pitx2*) and compared the sequence with other species through bioinformatics software. Low *Ka/Ks* ratio showed that *pitx2* evolving was under negative selection, which is also supported by the high conservation of this gene. In addition, we analyzed the expression of *pitx2* in different development stages of two flatfishes. Promoter analysis of *Cs-pitx2* also provided evidences that *pitx2* was an important factor in L-R asymmetry. All of the results indicated that *pitx2* functions as an important component of the Nodal signaling pathway which might regulate the L-R asymmetry in flatfish.

MATERIALS AND METHODS

Samples

Fertilized eggs of tongue sole and stone flounder were collected from a pool, which were obtained by artificial fertilization and incubated at 22°C in sea water with aeration. Ten embryonic stages (egg, 16 cells, high, sphere, 30% epiboly, 2-somite, 21-somite, 27-somite, hatching and 1 day after hatching, DAH) were selected at the time before eye migration. 30 embryos were put in one tube with 1 ml RNA wait (Solarbio, Beijing, China) and then stored at −80°C until use. Meanwhile, larvae were selected at the time of stage D (before eye migration) and stage I (end of eye migration) (Okada *et al*., 2001, 2003).

Total RNA extraction, cDNA synthesis

Total RNA was extracted from embryos of different developmental stages with Trizol (Invitrogen) and treated with DNase I (CWBIO) immediately at 37°C to remove DNA contamination. The quality and quantity of total RNA was determined by agarose gel electrophoresis and Nanophotometer Pearl (Implen GmbH, Munich, Germany). First-strand cDNA was prepared from total RNA using M-MLV reverse transcriptase (RNase H^-) (Takara) and random primers.

Primers were designed to get the ORF sequences (Table I) by IDT website (http://www.idtdna.com/Primerquest/Home/Index). All the amplified PCR products were separated by agarose gel electrophoresis, purified, cloned into pMD18-T (Takara) and sequenced.

Alignment and phylogenetic analysis

Homologous nucleotide sequences of flatfish and other vertebrates were confirmed through the BLAST search at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments and phylogenetic tree was constructed using MEGA6.0 with neighbor-joining method with 1,000 bootstrap replicates.

Ka/Ks ratio

In the process of molecular evolution and reconstruction of phylogenetic tree, a reasonable assessment of sequences non-synonymous substitution rate (*Ka*) and synonymous substitution rate (*Ks*) is crucial. The *Ka/Ks* test is also one of the most frequently used tests of adaptive molecular evolution (Eyre-Walker, 2006). Thus, the ratio *w=Ka/Ks* measures the difference between the two rates and is most easily understood from a mathematical description. If an amino acid change is

neutral, it will be fixed at the same rate as a synonymous mutation, with w=1. If the amino acid change is deleterious, purifying selection will reduce its fixation rate, thus w<1. Only when the amino acid change offers a selective advantage is it fixed at a higher rate than a synonymous mutation, with w>1. Therefore, a w ratio significantly higher than one is convincing evidence for diversifying selection (Eyre-Walker, 2006; Yang and Bielawski, 2000).

To test the molecular adaptation of *pitx2* gene in the evolutionary process, we calculated the *Ka/Ks* ratio in all above protein-coding DNA sequences using PAML 4.7 software.

Sequence analysis

The whole genome sequence of tongue sole had been published (Chen *et al*., 2014). So, we truncated 2,500bp upstream sequences of *Cs-pitx2* by comparing tongue sole genome with *Cs-pitx2*. Bioinformatic analysis of promoter sequence and potential transcription factor binding sites within the 5' regulatory region of *Cs-pitx2* was mainly performed by using online program TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and Matinspector (http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html). Neural Network Promoter Prediction (NNPP, http://www.fruitfly.org/ seq_tools/promoter.html) was also used to predict the transcription start site (TSS).

Quantitative real-time PCR (qRT-PCR)

The expression levels of *pitx2* in developmental stages were measured by qRT-PCR. GAPDH was selected as the reference gene (Liu *et al*., 2014). Amplifications were performed in 20 µl volume using LightCycler480 (Roche Applied Science, Mannheim, Germany) which contained 10 µl 2× SYBR® Premix Ex Taq™II (Perfect Real Time) (Takara), 0.4 µM each of specific forward and reverse primers, and 1.0 µl template cDNA (20 ng/µl). Reaction conditions were 95°C (5 min) for pre-incubation, followed by 45 cycles each of 95°C for 15 s, 60°C for 45 s. The melting curve was analyzed to detect single amplification. Triplicate PCRs were run for each developmental sample. The relative expression of *pitx2* was calculated by the formula $2^{-\Delta\Delta Ct}$ and the results were given as the relative expression ratio of each target to reference gene.

Table I. - Primers used in the study.

Primers	Sequence(5'-3')	Function
Cs-pitx2-Fw	ACTTTCCGCTCATCCTTCC	Fragment PCR
Cs-pitx2-Rv	CACGCCACTACAGCCTTTG	Fragment PCR
Kb-pitx2-Fw	ATGAACTCTATGAGGGATCCATTAA	Fragment PCR
Kb-pitx2-Rv	TTAGACGGGTCTGTCCACGG	Fragment PCR
pitx2-RT-Fw	ACTCCTCGGATGACCCTTCG	q RT-PCR
pitx2-RT-Rv	GGCTATCTCCTCCCTCGTGC	q RT-PCR
GAPDH-Fw	GAAGGGCATTCTGGGATACACT	q RT-PCR
GAPDH-Rv	TCAAAGATGGAGGAGCGGC	q RT-PCR

The abbreviations: *Cs-pitx2: Cynoglossus semilaevispitx2* gene; *Kb-pitx2: Kareius bicoloratuspitx2* gene; Fw: Forward primer; Rv: Reverse primer; qRT-PCR: quantitative RT-PCR.

Statistical analysis

The expression data were tested with SPSS 20.0 software (SSPS, IL, USA). Significant differences between samples were analyzed via one-way ANOVA (analysis of variance) using Duncan's test. Data were considered significantly different when $P<0.05$. All data are expressed as mean ± standard error of the mean (SEM).

RESULTS

Cloning and characteristics of pitx2

With specific primers (Table I), we obtained the sequence of *Cs-pitx2*, which contained 939 bp of the ORF (KM667976) (Fig. 1A), and *Kb-pitx2* ORF sequences of 936 bp (KM667975) (Fig. 1B). By multiple alignments, we found a large difference in the 5'-end of *pitx2* gene in different species of vertebrates. But like other vertebrates *pitx2*, both *Cs-pitx2* and *Kb-pitx2* proteins contain two conservative domains: Homeodomain (pos.82-141) and OAR domain (pos.272-287) (Fig. 2) (Gehring *et al.*, 1994; Shiomi *et al.*, 2007). The results indicated that *pitx2* gene showed high identity in the kingdom of vertebrates.

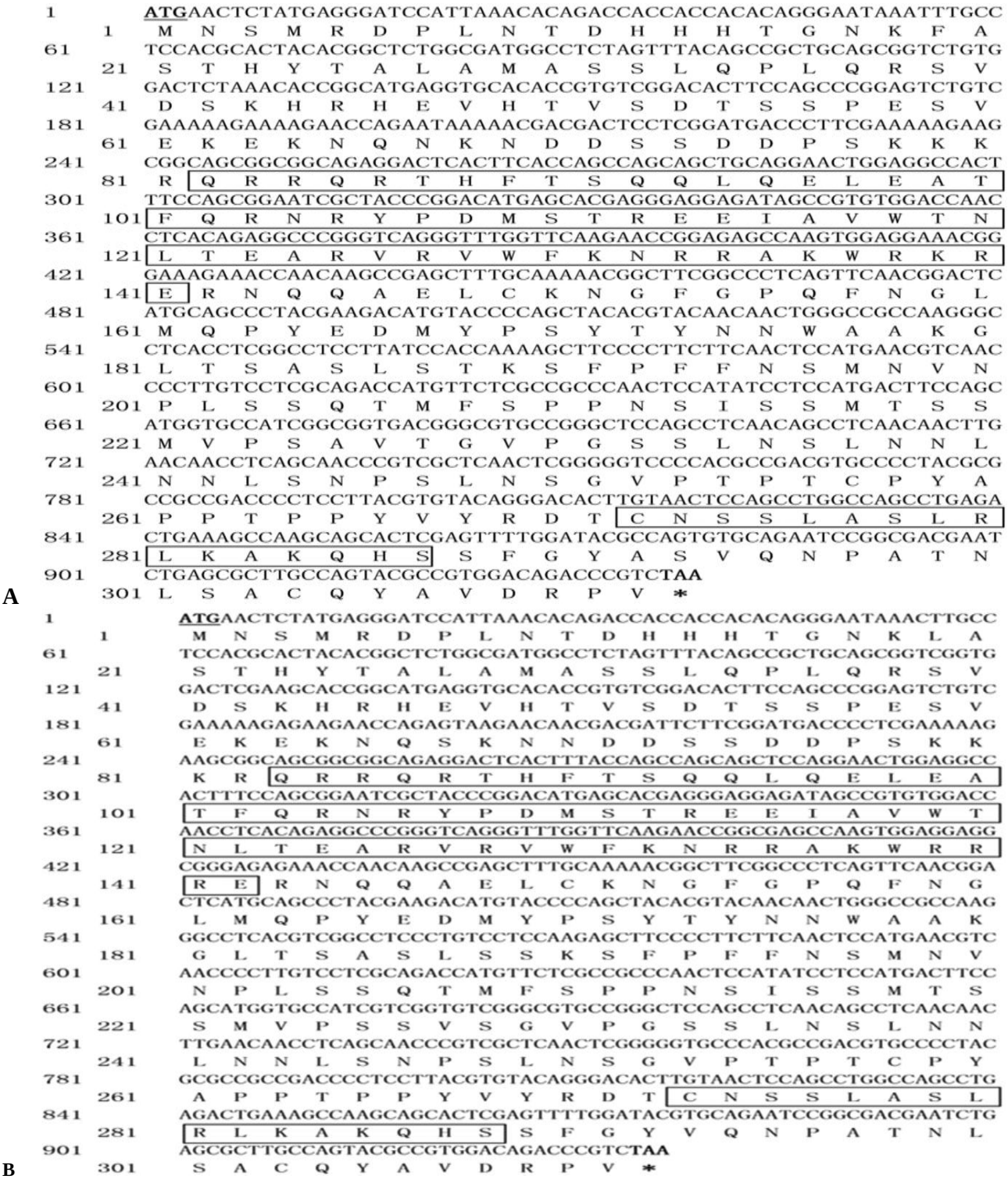

Fig. 1. *Pitx2* sequence, A, *Cs-pitx2* and B, *Kb-pitx2* sequences. Coding sequences (CDS) are shown in uppercase letters. The deduced amino acid sequences were shown underneath the CDS using single letter codes. The first blank frame is the homeodomain and the second blank frame was OAR domain.

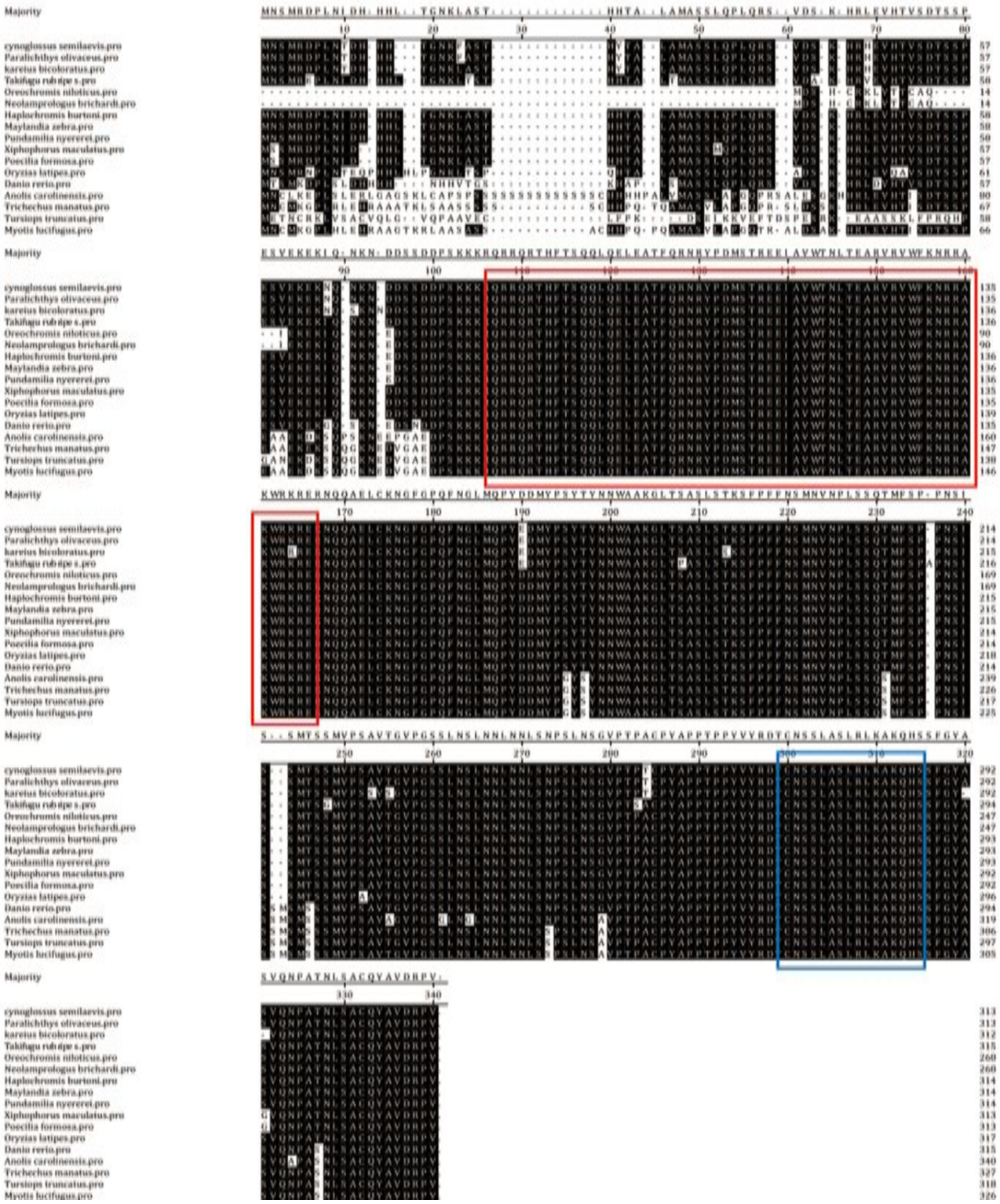

Fig. 2. Alignment of full-length *pitx2* amino acid sequences in different species. The conservative domains, homeodomain and OAR domains were marked with red and blue frame, respectively.

Table II. - Estimation of *Ka* and *Ks* between the tongue sole and other fish.

Species	S	N	*Ka(dN)*	*Ks(dS)*	*Ka/Ks*
Oryzias latipes	116.2	672.8	0.0122	2.1995	0.0056
Oreochromis niloticus	151.8	637.2	0.0306	0.573	0.0533
Maylandia zebra	150.9	638.1	0.0079	0.6637	0.0119
Kareius bicoloratus	120.4	668.6	0.0075	0.1506	0.0499
Haplochromis burtoni	150.1	638.9	0.0079	0.6489	0.0122
Danio rerio	171.1	617.9	0.0266	3.8568	0.0069
Pundamilia nyererei	150	639	0.0079	0.6712	0.0118
Poecilia formosa	125.2	663.8	0.0061	0.8144	0.0075
Takifugu rubripes	137.5	651.5	0.0112	0.6409	0.0175

Abbreviations used: *Ka*, the number of non-synonymous substitutions per site. An alternative symbol is *Ka (dN).Ks*, the number of synonymous substitutions per site. An alternative symbol is *Ks (dS).*

Phylogenetic analysis

To evaluate the evolutionary relationships of *pitx2* between the flatfish and other vertebrates, a genealogical tree was constructed based on the full-length nucleotide sequences using MEGA6.0 with neighbor-joining method (Fig. 3). We found that the genealogical tree polymerized into two clades: teleost and tetrapod. In the teleost group, *Cs-pitx2* was clustered to Japanese flounder (*Paralichthys olivaceus*) and *Kb-pitx2* was clustered to Atlantic halibut (*Hippoglossus hippoglossus*). And all of them are the members of pleuronectiformes and being grouped into the clade of *pitx2* with above 99 bootstrap support value (Fig. 3). All of these suggested that *Cs-pitx2* and *Kb-pitx2* were indeed the orthology genes of the pleuronectiformes. Furthermore, they had a high conservation in vertebrates.

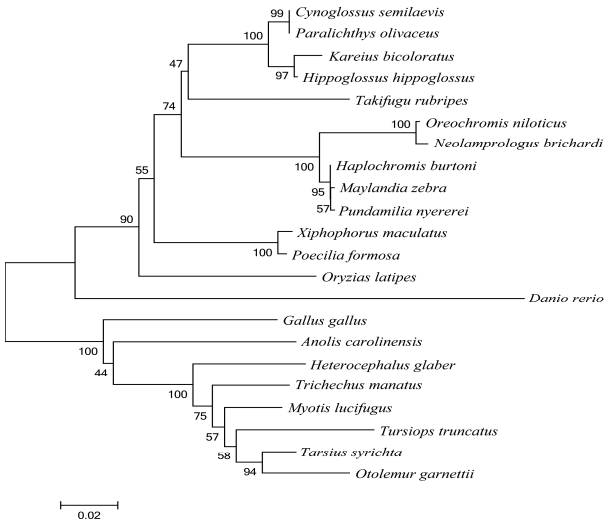

Fig. 3. Molecular phylogenetic analysis of *pitx2* products by neighbor-joining method with 1,000 bootstrap replicates. All the sequences were gained from the NCBI. The accession numbers are as follows: *Paralichthy solivaceus*, AB050722; *Takifugu rubripes*, XM_003970578; *Hippoglossus*, AY999687; *Haplochromis burtoni*, XM_005919426; *Oryzias latipes*, NM_001122916; *Maylandia zebra*, XM_004553612; *Oreochromis niloticus*, XM_005467266; *Xiphophorus maculates*, XM_005815551; *Poecilia formosa*, XM_007551061; *Pundamilia nyererei*, XM_005726475; *Danio rerio*, NM_130975; *Neolamprologus brichardi*, XM_006798846; *Tursiops truncates*, XM_004318743; *Anolis carolinensis*, XM_003221750; *Tarsius syrichta*, XM_008061221; *Trichechus manatus*, XM_004380240; *Gallus gallus*, NM_205010; *Heterocephalus glaber*, XM_004867586; *Myotis lucifugus*, XM_006098040; *Otolemu rgarnettii*, XM_003798155.

Diagnosis of the form of sequence evolution

By the values of *Ka* and *Ks*, we calculated and found that all of the *w=Ka/Ks* less than 1 ($w<1$) or much less than 1 ($w<<1$) (Table II). The results demonstrated that *pitx2* gene underwent a purifying selection during the evolutionary process. In its simplest form, this test is conservative because most non-synonymous mutations had been deleted. Furthermore, the smaller value showed the greater purifying selection and the greater value represented the smaller purifying selection.

Sequence analysis

By comparing the *pitx2* sequence with genomic database of tongue sole, we truncated

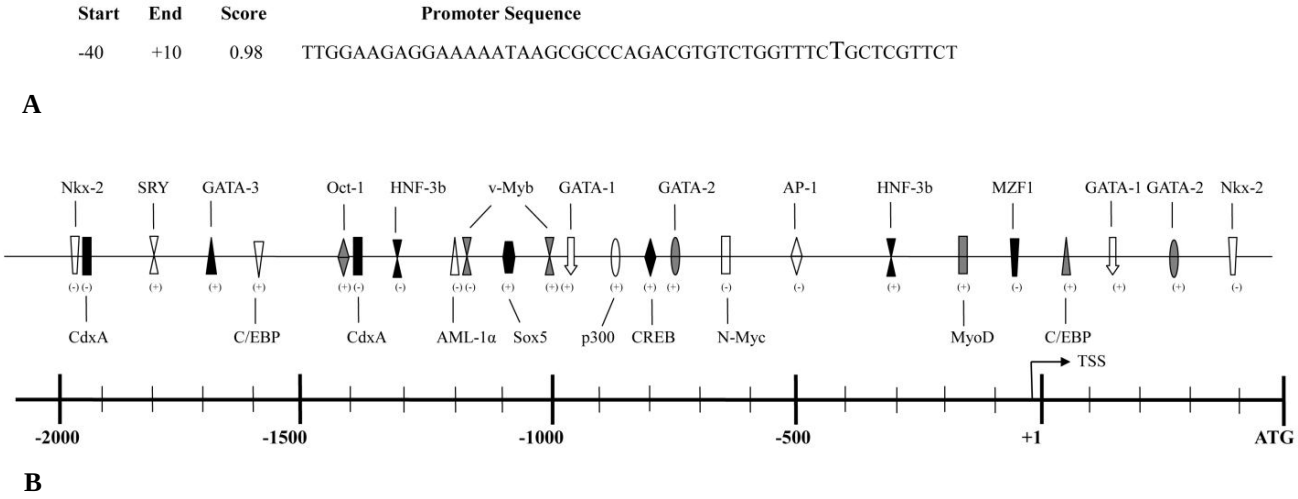

Fig. 4. *Cs-pitx2* promoter sequence features; A,The predicted transcription start site (TSS) by NNPP; B, A schematic diagram of putative regulatory motifs in the *Cs-pitx2* gene. The scale was given (the ATG position=1).

2,500 bp upstream sequences as the gene promoter region. Using the tool of NNPP, the transcription start site (TSS) was identified (Fig. 4A). Sequence analysis shown that the regulatory region contained some essential binding sites for multiple transcription factors (TFs), such as Nkx-2, CdxA, p300, v-Myb, MZF1, Sox5 (Fig. 4B). Additionally, binding sites for CCAAT/enhancer binding protein (C/EBP), GATA binding protein (GATA-1/3),activator protein-1 (AP-1), hepatocyte nuclear factor (HNF-3b), POU domain factor (Oct-1), transcriptional activator (v-Myb) and sex-determining region Y (SRY) were also identified in the promoter region of *Cs-pitx2* (Fig. 4B).

Expression levels of pitx2 in different development stages

We examined the expression patterns of *Cs-pitx2* (Fig.5A) and *Kb-pitx2* (Fig.5B) in different embryonic developmental stages by qRT-PCR. With the cleavage proceeding and embryonic development, the amount of transcript was slightly increased without significant difference until 30% epiboly stage or sphere stage, and then strongly increased to the peak at 27-somite stage, then gradually decreased until 1DAH (day after hatched). Obviously, in the early and later metamorphic stage, the expression of *pitx2* appeared an opposite trend between sinistral tongue sole and dextral stone flounder. The

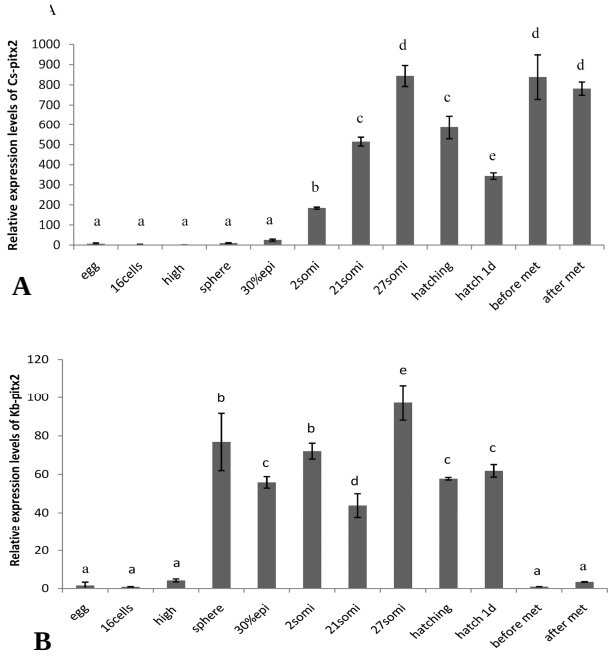

Fig. 5. Relative expression levels of *pitx2* gene. (A) Relative expression levels of *Cs-pitx2* gene during embryo development from unfertilized egg to metamorphic stages. (B) Relative expression levels of *Kb-pitx2* gene during embryo development from unfertilized egg to metamorphic stages. Data were shown as mean ± SEM. Values with different superscripts indicated statistical significance ($p < 0.05$), which were calculated via one-way ANOVA.

transcription levels of *pitx2* showed a second increase in tongue sole after 1 DAH. In stone flounder, however, *pitx2* only had a weak expression after this stage. The inconsistent expression patterns of *pitx2* during metamorphosis between sinistral and dextral fish was also supported by a previous study (Yoshioka *et al*., 1998) which showed that *pitx2* played a key role in asymmetric organogenesis during the metamorphosis in left-deflecting flatfish.

DISCUSSION

Teleost fishes are an extremely diverse group of vertebrate aquatic animals (Kim *et al*., 2008), and flatfish is one of its important species for special body. The symmetrical body of flatfish larvae dramatically changes into an asymmetrical form after metamorphosis, with one eye migrates to the other side. An increase in skin thickness beneath the eye was observed only on the blind side at the beginning of eye migration (stage D)--the first definitive difference between the right and left sides of the body (Okada *et al.*, 2001). The internal organs such as heart, brain and gut also appear asymmetrical phenomena during embryogenesis (Hjalt *et al.*, 2001; Logan *et al.*, 1998; Ryan *et al.*, 1998). Several studies had reported that left-right (L/R) asymmetry in vertebrates was controlled by activities emanating from the left lateral plate mesoderm (LPM) during embryogenesis (Burdine and Schier, 2000; Campione *et al.*, 1999; Lustig *et al.*, 1996; Sampath *et al.*, 1998). In all vertebrate embryonic studies to date, Nodal signaling pathway members of the *nodal*, *lefty* and *pitx2* gene families are asymmetrically expressed on the left side of the embryo and are thought to convey 'left-sidedness' to developing organs (Amack *et al.*, 2007; Yost, 1999). In the present study, several evidences, including protein sequence and phylogenetic analysis, supported the view that *pitx2*is highly conserved in the kingdom of vertebrates. The protein comparison (Fig. 1) further confirmed that *Cs-pitx2* belongs to the paired-bicoid homeobox protein family (Amendt *et al.*, 1999; Brouwer *et al.*, 2003), which includes two conservative domains, homeodomain and OAR domain. Homeodomain, containing 60 conservative amino acids, is an important and conservative DNA binding site in vertebrates such as fish and tetrapods. OAR, a conserved C-terminal amino acid stretch, includes 16 amino acids. These data indicated that *pitx2* gene conceives the conserved function in vertebrates. The phylogenetic tree, based on protein sequences also supported the view (data not shown). When we constructed the phylogenetic tree using protein sequences, the support values became much lower than those of the nucleotide phylogenetic tree. This may be due to the substitutes of individual bases at the third position of codon not causing any change in the relevant amino acids because of degeneracy of codon. According to these results, we may conclude that *Cs-pitx2* and *Kb-pitx2* have a high conservation in evolution and are indeed the orthology genes of the Pleuronectiformes and vertebrates. Orthology describes genes in different species that are similar to each other because they originated from a common ancestor by speciation (Kim *et al*., 2008).

As a following step, we diagnosed the form of *pitx2* sequence evolution primary by *Ka/Ks*, which is the ratio of the number of non-synonymous substitution sites (*Ka*) to the number of synonymous substitutions sites (*Ks*) (Hurst, 2002). By the values of *Ka* and *Ks*, we calculated and found that the ratio $w<1$ or $w<<1$ (Table II). The result demonstrated that *pitx2* was a conservative gene that most of the time selection eliminated deleterious mutations to keep the protein as it was. In other words, *pitx2* underwent a purifying selection during the evolutionary process, which was the same situation for mitochondrial CO1 gene in *Epinephelus septemfasciatus* (Guang *et al*., 2014).

We used the tongue sole genetic database to analyze the promoter region. Sequence analysis showed that the regulatory region contained some essential binding sites for multiple transcription factors (TFs), such as Nkx-2, CdxA, C/EBP, GATA-1/3, AP-1, SRY and so on (Fig. 3B), suggesting that some potential roles of *pitx2*. HNF-3b may have a role because it is transiently asymmetrically expressed in the chick (Levin *et al.*, 1995; Ryan *et al.*, 1998). The homeodomain factor Nkx-2.5 appears to regulate the asymmetric expression of the basic helix–loop–helix (bHLH) factors Dhandande HAND, which are required for correct heart looping and morphogenesis (Biben and Harvey, 1997; Ryan

et al., 1998; Srivastava *et al.*, 1995). However, the prediction is largely based on the sequence similarity. Further experiments are necessary to confirm the functional role of these sites so as to make a better understanding about the transcriptional regulation mechanism of *Cs-pitx2*.

Quantitative RT-PCR analysis revealed that both *Cs-pitx2* and *Kb-pitx2* were maternally inherited and started the zygote expression after 30% epiboly transition, and reached the first peak at 27-somite stage. The expression of *pitx2* is relatively low before hatching period, showing that *pitx2* is active during the later stages of embryonic development. Studies had proved that the Nodal pathway downstream gene *pitx2* had a key role in the formation of internal organs such as brain, heart and gut (Hjalt *et al.*, 2001; Logan *et al.*, 1998). It is a coincidence that the internal organogenesis starts from somite stage. So we can speculate that *pitx2* might play an important role on the formation of internal organs.

By comparing the transcription levels of *pitx2* in tongue sole and stone flounder during metamorphosis, we found that the expression of *Cs-pitx2* appeared a second increase during metamorphosis, but *Kb-pitx2* decreased abruptly during this period. As we all know, one eye will migrate to the other side during metamorphosis in flatfish, to the left in tongue sole and to the right in stone flounder. Meanwhile, we also know that *pitx2* is a gene in Nodal signaling pathway related to left deflection. All phenomena suggested that *pitx2* was a critical transcription target that mediates the deflection of eyes in flatfish. The conclusion was also consistent with some previous studies (Okada *et al.*, 2001; Suzuki *et al.*, 2009).

CONCLUSION

This study provided the full-length cDNA sequences of *pitx2* in tongue sole and stone flounder. Phylogenetic tree and evolutionary analysis demonstrated that *pitx2* was a very conservative gene and had two conservative domains. Quantitative RT-PCR showed that *pitx2* was a crucial gene in Nodal signaling pathway that might regulate the migration of eyes in flatfishes.

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Author contributions

Tiantian Liu, Jingjing Niu, Jie Qi and Yan He conceived and designed the experiments; Tiantian Liu, Jie Cheng, Chunli Wang, Bo Wang and Muhammad Shafi performed the experiments; Tiantian Liu, Jie Cheng and Yan He analyzed the data; Jieming Zhai, Quanqi Zhang and Jie Qi contributed reagents/materials/analysis tools, Tiantian Liu, Jingjing Niu and Yan He wrote the paper.

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