論文 / 著書情報
Article / Book Information

Title	Identification and comparative analyses of Siamois cluster genes in the Xenopus laevis and tropicalis
Authors	Yoshikazu Haramoto, Tomohito Saijyo, Toshiaki Tanaka, Nobuaki Furuno, Atsushi Suzuki, Yuzuru Ito, Mariko Kondo, Masanori Taira, Shuji Takahashi
Citation	Developmental Biology, vol. 426, pp. 374-383
Pub. date	2017, 6
DOI	https://doi.org/10.1016/j.ydbio.2016.07.015
Creative Commons	See next page.
Note	This file is author (final) version.

License

Developmental Biology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Developmental Biology

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

Identification and comparative analyses of *Siamois* cluster genes in *Xenopus laevis* and *tropicalis*

Yoshikazu Haramoto [a], Tomohito Saijyo [b], Toshiaki Tanaka [c], Nobuaki Furuno [b], Atsushi Suzuki [b], Yuzuru Ito [a], Mariko Kondo [d], Masanori Taira [e], Shuji Takahashi [b,*]

[a] *Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), Central 5, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan*
[b] *Institute for Amphibian Biology, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan*
[c] *School of Life Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8501, Japan*
[d] *Misaki Marine Biological Station (MMBS), Graduate School of Science, The University of Tokyo, 1024 Koajiro, Misaki, Miura, Kanagawa 238-0225, Japan*
[e] *Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

ARTICLE INFO

Article history:
Received 12 February 2016
Received in revised form
13 July 2016
Accepted 16 July 2016

Keywords:
Xenopus
Siamois-related gene
Secondary axis
Early development
Genome
Wnt/β–catenin

ABSTRACT

Two *siamois*-related homeobox genes *siamois* (*sia1*) and *twin* (*sia2*), have been reported in *Xenopus laevis*. These genes are expressed in the blastula *chordin*- and *noggin*-expressing (BCNE) center and the Nieuwkoop center, and have complete secondary axis-inducing activity when over-expressed on the ventral side of the embryo. Using whole genome sequences of *X. tropicalis* and *X. laevis*, we identified two additional *siamois*-related genes, which are tandemly duplicated near *sia1* and *sia2* to form the *siamois* gene cluster. Four *siamois* genes in *X. tropicalis* are transcribed at blastula to gastrula stages. In *X. laevis*, the *siamois* gene cluster is present on both homeologous chromosomes, XLA3L and XLA3S. Transcripts from seven *siamois* genes (three on XLA3L and four on XLA3S) in *X. laevis* were detected at blastula to gastrula stages. A transcribed gene, *sia1p. S*, encodes an inactive protein without a homeodomain. When over-expressed ventrally, all *siamois*-related genes tested in this study except for *sia1p. S* induced a complete secondary axis, indicating that *X. tropicalis* and *X. laevis* have four and six active *siamois*-related genes, respectively. Of note, each gene required different amounts of mRNA for full activity. These results suggest the possibility that *siamois* cluster genes have functional redundancy to endow robustness and quickness to organizer formation in *Xenopus* species.

1. Introduction

In *Xenopus*, after the mid-blastula transition, zygotic gene transcription starts in various regions including the blastula *chordin*- and *noggin*-expressing (BCNE) center, which is formed in dorsal-animal region (Kuroda et al., 2004) and the Nieuwkoop center, which is formed in the dorsal-vegetal region (Gimlich and Gerhart, 1984). *Nodal*-related genes are highly expressed in the Nieuwkoop center and cells deriving therefrom (Agius et al., 2000; Takahashi et al., 2000), and induces the Spemann organizer, an essential signaling center for *Xenopus* germ layer specification and axis formation. The BCNE center contains both prospective anterior neuroectoderm and Spemann organizer precursor cells and is required for head formation (Kuroda et al., 2004; Ishibashi et al., 2008). *Siamois*-related homeobox genes *siamois* (*sia1*) (Lemaire et al., 1995) and *twin* (*sia2*) (Laurent et al., 1997) are expressed immediately after the mid-blastula transition in the BCNE and Nieuwkoop centers (Kuroda et al., 2004; Rankin et al., 2011; Sudou et al., 2012; Li et al., 2015) and are directly regulated by Wnt/β-catenin signaling (Carnac et al., 1996; Brannon and Kimelman, 1996; Brannon et al., 1997; Laurent et al., 1997). Siamois and Twin have been shown to function as transcriptional activators (Kessler, 1997). Ectopic expression of *siamois* and *twin* induces an organizer-specific gene, *goosecoid* (*gsc*) and an anterior endodermal gene, *hhex* (Carnac et al., 1996; Laurent et al., 1997; Rankin et al., 2011).

Analysis of the *siamois* promoter and regulatory sequences revealed that Tcf/Lef regulates *siamois* expression by either activation or repression depending on its partner. A β-catenin/Tcf complex activates *siamois* expression (Brannon et al., 1997), whereas Tcf/Lef itself represses it by binding with the co-repressors Groucho and CtBP (Roose et al., 1998; Brannon et al., 1999). The nuclear localization of maternal β-catenin is broadly present in animal to vegetal regions on the dorsal side of the blastula in *X. laevis* (Schneider et al., 1996). β-catenin can cancel the repressive function of Tcf/Lef and activate *siamois* expression (reviewed in Kimelman, 1999).

* Corresponding author.
E-mail address: shujit@hiroshima-u.ac.jp (S. Takahashi).

http://dx.doi.org/10.1016/j.ydbio.2016.07.015
0012-1606/

Because of the similarity in amino acid sequences and expression patterns of *siamois* and *twin*, these genes are thought to function redundantly or act synergistically during *Xenopus* development. To verify the function of *siamois* and *twin in vivo*, several knockdown experiments have been performed. Loss-of-function studies for *siamois* using a dominant repressive form with the engrailed repressor domain (EnR) indicated that Siamois and Twin are essential for organizer formation (Fan and Sokol, 1997; Kessler, 1997). A recent study using Morpholino Antisense Oligos (MOs) for *siamois* and *twin* showed that these genes are necessary for head formation and planar neural induction (Ishibashi et al., 2008). Another knockdown study using MOs against *siamois* and *twin* has reported results consistent with studies using a dominant repressor form of *siamois*, and shown that *siamois* and *twin* have similar functions with regards to promoter binding and transcriptional activation and are required for organizer function (Bae et al., 2011). Another report showed that *siamois* and *twin*-expressing cells in the blastula contributed to both the Spemann organizer and neural ectoderm lineages and directly activated neural genes before gastrulation (Klein et al., 2015). In these reports, the simultaneous knockdown of *siamois* and *twin* (but not individually) showed significant phenotypes, consistent with a redundant role for *siamois* and *twin in vivo*. However, functional differences were also reported; *Foxd4* can be directly induced by Siamois homodimers but not by Twin homodimers, whereas *Gmnn* and *Zic2* can be induced by both (Klein et al., 2015).

Here, we report that *X. tropicalis* has four active *siamois*-related genes and *X. laevis* has six active *siamois*-related genes, which exhibit secondary axis-inducing activity and are regulated by Wnt/β-catenin signaling. Our findings indicate that a comprehensive analysis of *siamois*-related genes is required to understand their precise function in the BCNE and Nieuwkoop centers.

2. Materials and methods

2.1. Embryos

X. tropicalis (Nigerian strain) and *X. laevis* (J-strain) embryos were obtained by artificial fertilization, and cultured in 0.1 × Steinberg's solution. The embryos were staged according to Nieuwkoop and Faber (1994). Synthesized mRNA was microinjected into the two blastomeres of two-cell stage embryos for animal cap assay and the two ventral blastomeres at the four- or eight-cell stage for axis duplication assay.

2.2. RNA synthesis

Capped mRNAs were synthesized by *in vitro* transcription of plasmids using SP6 mMessage mMachine kits (Ambion). The expression vectors used for RNA synthesis were pCS2p-XTsiamois1, pCS2p-XTsiamois2, pCS2p-XTsiamois3, pCS2p-XTsiamois4, pCS2p-XLJsiamois1.L, pCS2p-XLJsiamois2.L, pCS2p-XLJsiamois4.L, pCS2p-XLJsiamois1p.S, pCS2p-XLJsiamois2.S, pCS2p-XLJsiamois3.S, pCS2p-XLJsiamois4.S, pCS2- XTβ-catenin, pCS2-egfp, pCS2p-vegt. Whole mount *in situ* hybridization was performed as previously described (Sive et al., 2000). DIG RNA probes were synthesized using the same plasmids, pCS2p-XTchordin, and pCS2p-XTgoosecoid as templates.

2.3. Genome analysis

Using the Xenbase genome browser (*X. tropicalis* v8.0 and v.9.0 and *X. laevis* J-strain v8.0 and v9.1, http://www.xenbase.org/other/static/ftpDatafiles.jsp) and BLAST analysis with the gene models (*X. tropicalis* v.8.0 and v.9.0 and *X. laevis* J-strain v.1.8, http://www.xenbase.org/other/static/ftpDatafiles.jsp), *siamois*-related sequences were discovered. The methods of RNA-seq and the analysis were previously described in the whole genome paper (Session et al., submitted). The sequences of *siamois*-related genes in the gene models were verified with cloned cDNA sequences (see below) and corrected for the RNA-seq analysis.

2.4. Cloning of siamois-related genes of X. tropicalis (Nigerian) and X. laevis (J-strain)

The open reading frame (ORF) of *X. tropicalis* (Nigerian) and *X. laevis* (J-strain) *siamois*-related sequences were amplified by RT-PCR from total cDNA using gene-specific primers and KOD plus Neo, a high-fidelity DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). Gene-specific primer sequences and cycling numbers are described in Supplementary Table 1. The ORFs of *X. tropicalis sia1*, *sia2* and *sia3* were cloned into a *Stu*I blunt site of the pCS2p vector. The ORF of *X. tropicalis sia4* was cloned into *Bam*HI-*Xho*I sites of the pCS2p vector. The ORFs of *X. laevis siamois*-related genes were cloned into a pGEM-T easy vector. *X. laevis siamois*-related genes were re-amplified using sequence-verified plasmids and primers (F2 and R2, see Supplementary Table 1), and were subcloned into restriction enzyme sites (*Bam*HI or *Eco*RI and *Xho*I) of the pCS2p vector. The cloned sequences of each plasmid construct were verified to be equivalent to genomic sequences using a Genetic Analyzer (3130xl, Applied Biosystems). The neighbor-joining phylogenetic tree and similarities were calculated using MacVector 12.0.2 software.

2.5. RT-PCR

Total RNA was extracted from embryos and animal caps of *X. tropicalis* (Nigerian), and embryos of *X. laevis* (J-strain) using ISOGEN (Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized from 1 μg of total RNA using Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA) and oligo dT_{15}(Roche Diagnostics, Mannheim, Germany). PCR reactions were performed by KOD plus Neo, a high-fidelity DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) with gene-specific primer sets. All steps followed the manufacturer's instructions. *Elongation factor 1 alpha (eef1a1)* was used as an internal control (Fukuda et al., 2010). Reverse transcriptase negative (RT-) reactions indicated the absence of genomic DNA contamination. Primer sequences, sizes of PCR products, and cycling numbers are described in Supplementary Table 1. Sequences of PCR products were verified to be equivalent to genomic sequences using a Genetic Analyzer (3130xl, Applied Biosystems).

3. Results and discussion

The whole genome sequence of *X. laevis* was recently reported (Session et al., submitted), confirming that *X. laevis* contains an allotetraploid genome, and therefore homeologous gene evolution in this genome is of great interest. Here, we focused on the paired homeodomain transcription factor genes, *siamois* (*sia1*), *twin* (*sia2*), and their relatives.

3.1. Siamois-related genes in X. tropicalis

We found four *siamois*-related sequences in the *X. tropicalis* genome using the JGI gene model *X. tropicalis* v.8.0 and v.9.0 genome and BLAST analysis (Fig. 1A). Two previously reported *siamois*-related genes are *siamois* and *twin*. These genes were also called as *siamois1* (*sia1*) and *siamois2* (*sia2*), respectively in Xenbase (http://www.xenbase.org/entry/). We propose to name

Please cite this article as: Haramoto, Y., et al., Identification and comparative analyses of *Siamois* cluster genes in *Xenopus laevis* and *tropicalis*. Dev. Biol. (2016), http://dx.doi.org/10.1016/j.ydbio.2016.07.015

another two genes as *siamois 3* and *4* (*sia3* and *sia4*). *Sia3*, *sia1*, *sia4* and *sia2* were located in this gene order in the *X. tropicalis* genome assembly. *Sia3*, *sia1* and *sia4* transcriptional units were oriented in the same direction and *sia2* was in the opposite direction.

To study the temporal expression of *siamois*-related genes in early development, we analyzed RNA-seq data with the JGI gene model *X. tropicalis* JGI v9.0 (Session et al., submitted). The RNA-seq analysis identified that the transcripts of all *siamois*-related genes were detected from blastula to early gastrula stages. Expression of *sia1* reached a peak later than the other *siamois*-related genes (Fig. 1B). *In situ* hybridization analysis of *siamois*-related genes in *X. tropicalis* indicate that *siamois*-related genes are expressed in both the BCNE center and Nieuwkoop center regions. *Sia1* was mainly expressed in the BCNE center at mid-blastula (st. 8.5) and in the Nieuwkoop center at late-blastula stage (st. 9). *Sia2* and *sia4* are mainly expressed in the Nieuwkoop center at mid- and late-blastula stages. *Sia3* expression was detected only at late blastula stage in the Nieuwkoop center (Fig. 1C and Supplementary Fig S1). There is an argument that *siamois*-related genes are the BCNE and/or Nieuwkoop center genes (Lemaire et al., 1995; Kuroda et al., 2004). Our results suggest that *siamois*-related genes are expressed in both the BCNE and Nieuwkoop centers. It is notable that *sia1* is especially expressed in the BCNE center at the mid-blastula stage and its expression is sustained until the gastrula stage.

We tested whether these genes are functionally equivalent. Over-expression of all *X. tropicalis siamois*-related genes induced secondary axis formation (Fig. 2A), even though the effective concentration of each mRNA was different (Fig. 2B). Secondary axis-inducing activities were approximately 10-fold different among these genes (Fig. 2B). These results indicate that the *X. tropicalis* has four active *siamois*-related genes, which may exhibit functional redundancy.

3.2. *Siamois-related genes in X. laevis*

In the allotetraploid genome of *X. laevis*, four *siamois*-related sequences are also present as clusters on both homeologous sequences, chromosome 3 L (XLA3L) and scaffold20 that has S-chromosome-specific transposon sequences and a similar synteny to XLA3L, indicating that this scaffold may be integrated into chromosome 3 S (Fig. 3A). Genomic PCR analysis showed that *sia4* and *sia2* transcriptional units are in opposite directions (Supplementary Fig S2). One sequence (*sia3p.L*) located on chromosome 3 L, whose position corresponds to *sia3*, consists of exon1, exon2, and a deleted and broken exon3. We cloned the full-length cDNAs of seven *siamois*-related genes from *X. laevis* J-strain embryos to confirm their transcription and the splice site of the gene model. We did not succeed in isolating *sia3p.L* cDNA. One expressed sequence (*sia1p.S*) located on scaffold20, with a position corresponding to *sia1*, had a different splicing site from *X. tropicalis sia1*, *X. laevis sia1.L*, or the expected gene model for *sia1.S*, and encoded an inactive protein without a homeodomain.

To study the expression of whole genes, *X. laevis* J-strain embryos were collected from oocyte to stage 40. RNA-seq polyA+ libraries for Illumina sequencing were constructed from each sample. RNA-seq reads by Illumina were mapped against the gene model of JGI *X. laevis* v.1.8 (Session et al., submitted). RNA-seq analysis revealed that seven *siamois*-related genes were expressed

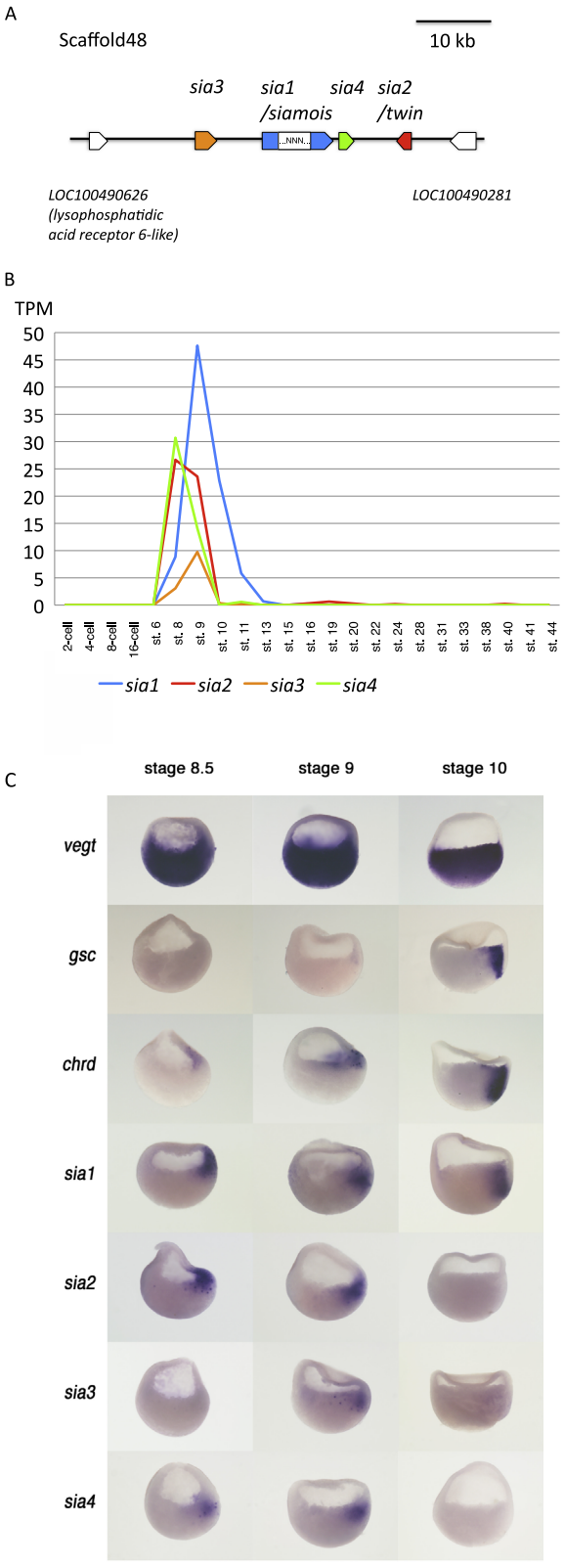


Fig. 1. Genomic organization of *X. tropicalis siamois*-related genes. (A) Genomic clustering of *X. tropicalis siamois* genes. Genomic mapping of the *siamois* genes shows clustering in a short interval of scaffold48 (*X. tropicalis* v.9.0). From the genome assembly, *sia1* was predicted to have a longer intron than the other related genes. This region has a sequence gap, which is an uncharacterized nucleotide stretch of an unknown length. Boat-shaped boxes indicate the direction of the coding frame. (B) Transcriptional profiling in early *X. tropicalis* development based on RNA sequencing data. All *siamois*-related genes are expressed from blastula to gastrula stages. TPM means transcripts per million. st, stage. (C) Whole mount *in situ* hybridization analysis for *X. tropicalis siamois*-related genes, a maternal gene, *vegt*, a BCNE gene, *chrodin* (*chrd*) and an organizer gene, *goosecoid* (*gsc*). Typical expression patterns are shown. Fixed embryos were bisected before hybridization. Images of whole embryos are also shown in Supplementary Fig S1.

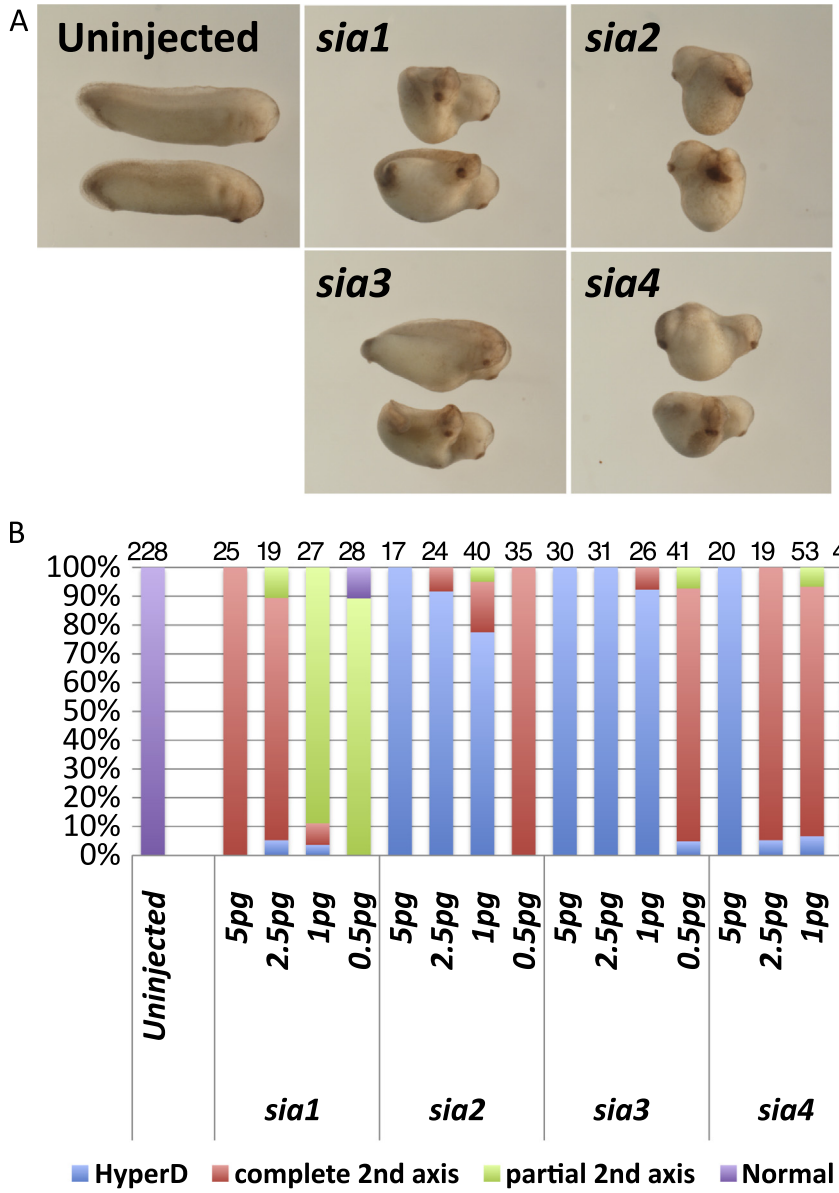


Fig. 2. Activity of *X. tropicalis siamois*-related genes. *X. tropicalis siamois*-related genes (*sia1*, *sia2*, *sia3*, and *sia4*) were over-expressed with total amounts of 0.5, 1, 2.5, 5 pg mRNA into ventral-vegetal cells of *X.laevis* embryos at 4–8-cell stages. (A) Representative embryos injected with 2.5 or 1 pg mRNA of each *siamois*-related gene. (B) The percentage of embryos in which the secondary axis was observed after injection of mRNAs of *siamois*-related genes. The number of embryos analyzed is shown above each bar. All *siamois*-related genes in *X. tropicalis* induced complete secondary axis.

from blastula to early gastrula stages similar to that for *X. tropicalis*. Expression of *sia1.L* reached a peak later than the other *siamois*-related genes (Fig. 3B).

Over-expression of all *X. laevis siamois*-related genes identified in this study except for *sia1p.S* induced secondary axis formation (Fig. 4A), even though the functional concentration of each mRNA was different (Fig. 4B). Secondary axis-inducing activities were about 5-fold different among these genes (Fig. 4B). These results suggest the possibility that *siamois* cluster genes have functional redundancy to endow robustness and quickness to organizer formation in *Xenopus* species.

To date, knockdown experiments using MOs have targeted only two genes, *sia1.L* (*siamois*) and *sia2.S* (*twin*) (Ishibashi et al., 2008; Bae et al., 2011, Klein and Moody 2015). The target sequences of *siamois* MO and *twin* MO used in Ishibashi et al., 2008 and Klein and Moody 2015 had 3–11 and 2–8 mismatches to other *siamois*-related genes, respectively. The target sequences of *siamois* MO and *twin* MO used in Bae et al., 2011 had 3–11 and 1–7 mismatches to other *siamois*-related genes, respectively (Supplementary Fig S3). Because it is unlikely that inactivation of all the active genes has been achieved, it would be worth reexamining whether knockdown of all four genes might lead to a reproducible and more extreme phenotype.

3.3. Conservation of siamois-related genes

Phylogenetic tree analysis of *siamois*-related genes of *X. laevis* and *X. tropicalis* indicated that orthologous and homeologous genes have high similarity, suggesting no translocation and no recombination occurred among homeologous genes (Fig. 5A). Siamois-related proteins also displayed high sequence identity (gray) with their own orthologous and homeologous proteins compared with other Siamois-related proteins (Fig. 5B).

Siamois-related genes were known to be present only in *Xenopus* species. We could not find orthologous sequences or similar types of homeobox genes in syntenic analyses of other vertebrate

Please cite this article as: Haramoto, Y., et al., Identification and comparative analyses of *Siamois* cluster genes in *Xenopus laevis* and *tropicalis*. Dev. Biol. (2016), http://dx.doi.org/10.1016/j.ydbio.2016.07.015

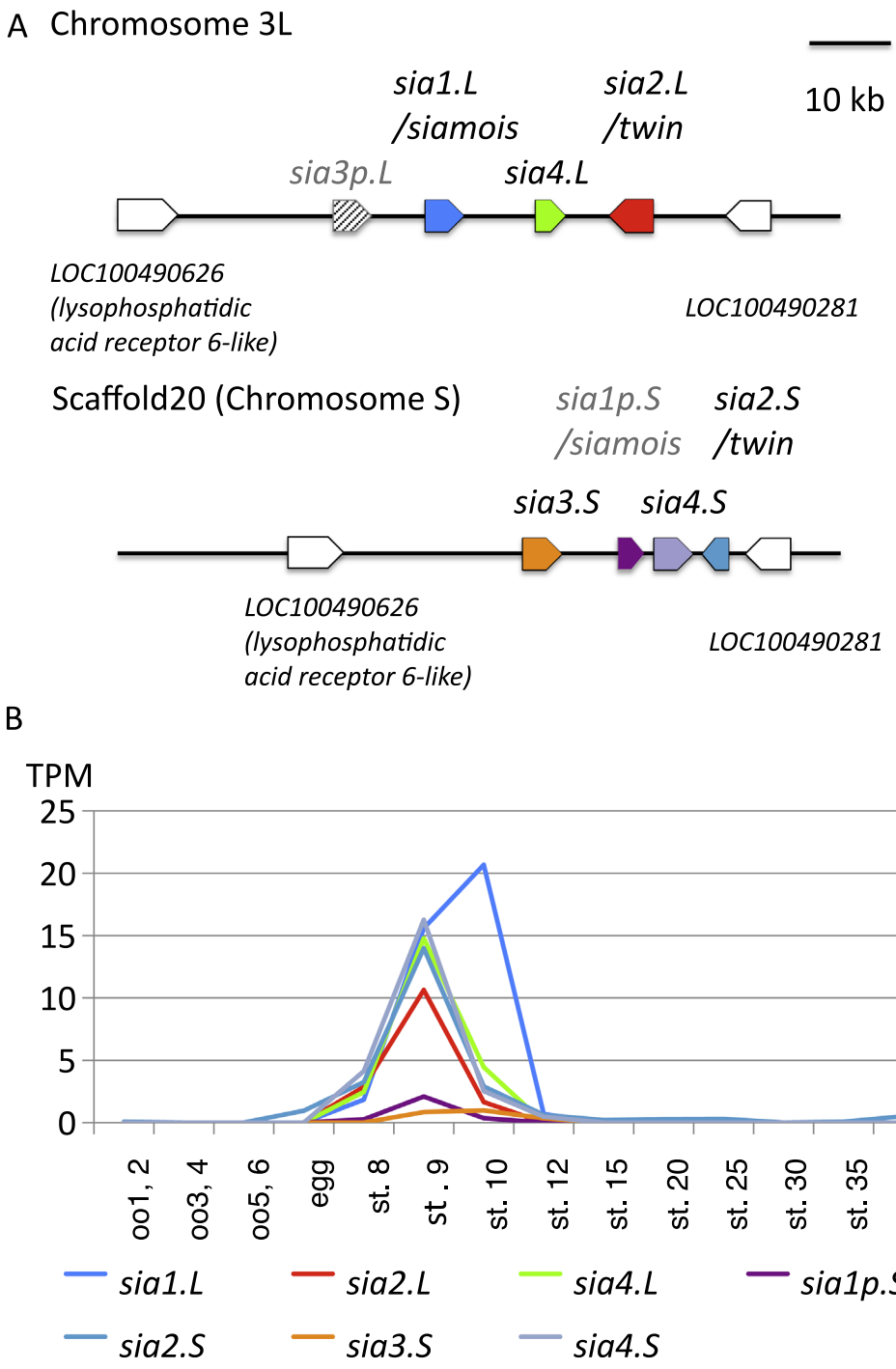


Fig. 3. Genomic organization of *X. laevis siamois*-related genes. (A) Genomic clustering of *X. laevis siamois* genes and pseudogenes. Genomic mapping of the *siamois* genes shows clustering in a short interval of chromosome 3 L and 3 S. Synteny of *siamois* genes is conserved among the *X. tropicalis* genome, *X. laevis* chromosome 3 L, and *X. laevis* chromosome 3 S. Boat-shaped boxes indicate the direction of the coding frame. Black indicates a confirmed active gene, and gray indicates a pseudogene. A striped box indicates that gene expression was not confirmed. (B) Transcriptional profiling in early *X. laevis* development based on RNA sequencing data. Seven *siamois*-related genes are expressed from blastula to gastrula stages. TPM means transcripts per million. oo, oocyte; st, stage.

genomes. However, we found that a predicted gene of a spotted garfish (*Lepisosteus oculatus*, XM_006627494) named *orthopedia-like* is a potential candidate for orthologous gene of *siamois*-related genes. BLAST analysis indicated this gene is the most similar to *Xenopus siamois*-related genes. This registered sequence has a complete duplication in the latter part, suggesting mis-assembly or mis-registration of sequences. Alignment and phylogenetic analysis using this deduced amino acid sequence (XP_006627557) without duplication (Supplementary Text 1) suggest that this protein is more similar to Siamois-related proteins than Orthopedia homeobox (Otp) (Fig. 5C, and D). We consider that this sequence found in a spotted garfish is important to know the evolutionary lineage of *siamois*-related genes. To confirm that this predicted sequence is an ortholog of *siamois*-related genes, it is necessary to analyze the expression pattern and functional equivalence.

Recently, the sequencing of the *de novo* genome of the Tibetan frog, *Nanorana parkeri* (*N. parkeri*), was reported (Sun et al., 2015). We performed tblastn search of the *N. parkeri* assembly using amino acid sequences of *X. tropicalis* Siamois-related proteins

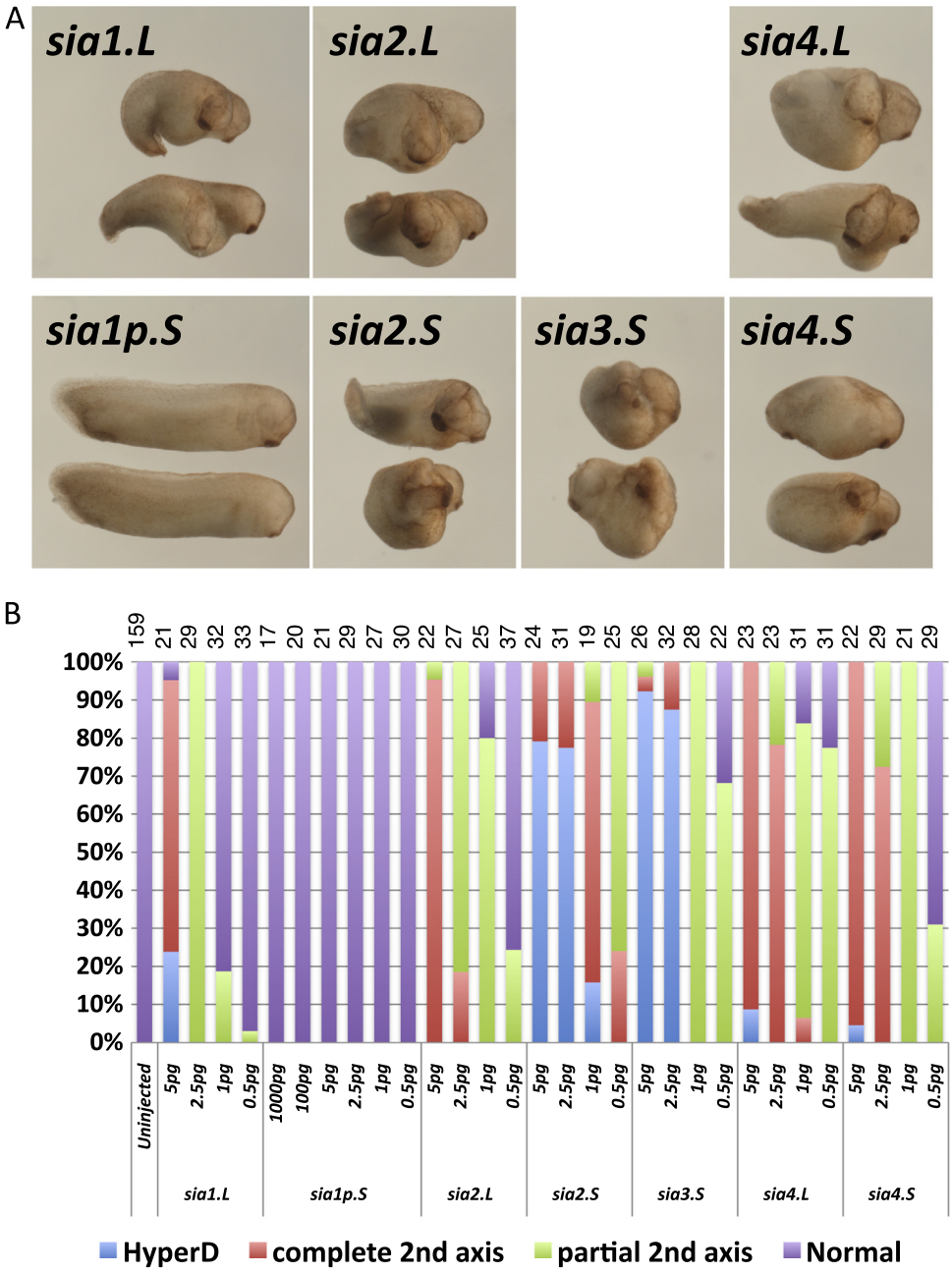
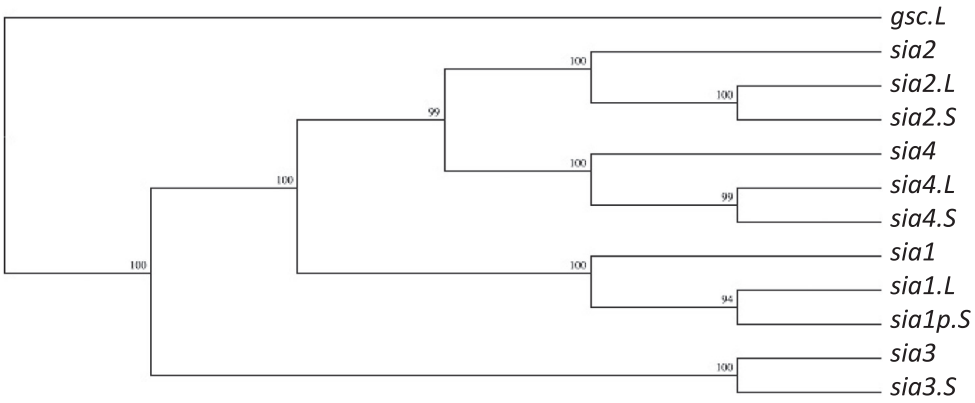


Fig. 4. Activity of *X. laevis siamois*-related genes. Seven *X. laevis siamois*-related genes (*sia1. L*, *sia2. L*, *sia4. L*, *sia1p. S*, *sia2. S*, *sia3. S*, and *sia4. S*) were over-expressed with total amounts of 0.5, 1, 2.5 and 5 pg mRNA, (100 and 1000 pg for *sia1p. S*) into ventral-vegetal cells of X. *laevis* embryos at 4–8-cell stages. (A) Representative embryos injected with 5 pg mRNA of each *siamois*-related gene. (B) The percentage of embryos in which a secondary axis was observed after injection of mRNAs of *siamois*-related genes. The number of embryos analyzed is shown above each bar. *sia1p. S* has no activity. All *siamois*-related genes in *X. laevis* except for *sia1p. S* induced a complete secondary axis.

Fig. 5. Conservation of *siamois*-related genes. (A) Phylogenetic tree of nucleotide sequences of *siamois*-related genes in *X. tropicalis* and *X. laevis*. cDNA sequences were obtained by cloning from *X. tropicalis* Nigerian strain or *X. laevis* J-strain. Accession numbers are described below [*X. tropicalis* (*sia1*: LC167289, *sia2*: LC167290, *sia3*: LC167291, *sia4*: LC167292), *X. laevis* (*sia1. L*: LC167293, *sia2. L*: LC167295, *sia4. L*: LC167298, *sia1p. S*: LC167294, *sia2. S*: LC167296, *sia3. S*: LC167297, *sia4. S*: LC167299)]. The *gsc. L* (Xenbase Gene ID: XB-GENE-6252605) was used as an outgroup. (B) Orthologous and homeologous comparisons of Siamois-related proteins as percent identity using ClustalW alignment. Gray colors indicate high similarity over 65%. (C) Phylogenetic tree of amid acid sequences of *siamois*-related genes and closely related genes. Deduced amino acid sequences were used. Gene accession numbers, Xenbase gene IDs, and protein IDs are described below [*X. tropicalis* (*sia1*: LC167289, *sia2*: LC167290, *sia3*: LC167291, *sia4*: LC167292, *mix1*: XB-GENE-485898, Otp: XP_002940916), *X. laevis* (*sia1. L*: LC167293, *sia2. L*: LC167295, *sia4. L*: LC167298, *sia2. S*: LC167296, *sia3. S*: LC167297, *sia4. S*: LC167299, *gsc. L*: XB-GENE-6252605), Human Otp: XP_942665, Zebrafish Otp: NP_001122175]. A homeobox protein of a spotted garfish (*Lepisosteus oculatus*) is XP_006627557 (1–220aa were used). Gsc. L was used as an outgroup. (D) The amino acid sequence alignments of *siamois*-related proteins. Homeodomain is boxed in red. (E) A schematic representation of the genome scaffolds containing *X. tropicalis* and *N. parkeri siamois*-related genes. Boat-shaped boxes indicate the direction of the coding frame. *Siamois*-related genes are depicted in black. Orthologous sequences are connected with dashed lines. Predicted coding sequences (CDSs) of *N. parkeri sia-a–c* are shown in Supplementary Text 1. (F) Phylogenetic tree of nucleotide sequences of *siamois*-related genes in *X. tropicalis* and *N. parkeri.* A homeobox gene of a spotted garfish (LOC102694986: 1–660, 1258–1260 were used) is shown in Supplementary Text 1. The *gsc* (Xenbase Gene ID: XB-GENE-486771) was used as an outgroup. The phylogenetic trees were calculated by MacVector 12.0.2 software [Method: Neighbor Joining; Bootstrap (1000 reps); tie breaking = Systematic, Distance: Uncorrected ("P"); Gap distributed proportionally].

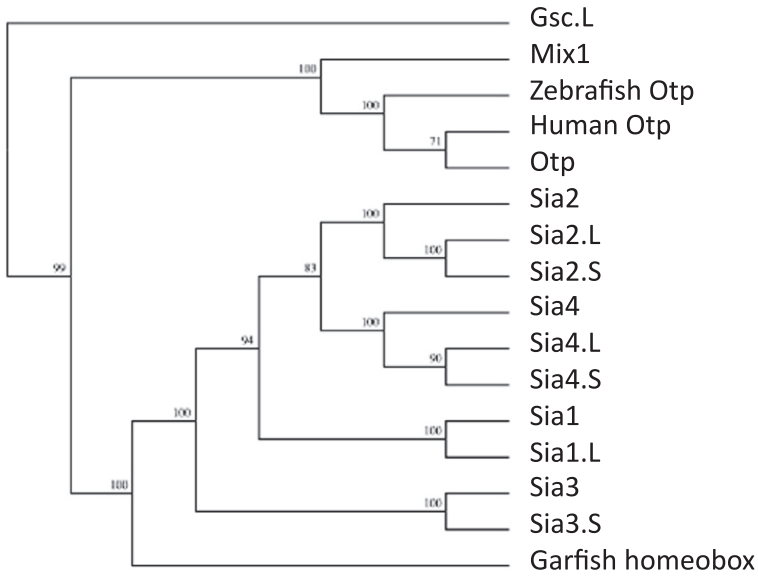
A



B

Sia1	Sia2	Sia3	Sia4	Sia1.L	Sia2.L	Sia4.L	Sia2.S	Sia3.S	Sia4.S	Gsc.L	
	47	41	42	74	46	43	47	38	41	12	Sia1
		44	52	50	75	60	76	45	54	14	Sia2
			40	41	43	41	42	73	38	13	Sia3
				44	52	65	52	41	70	15	Sia4
					50	47	50	41	43	12	Sia1.L
						58	86	45	53	14	Sia2.L
							57	41	70	15	Sia4.L
								44	51	14	Sia2.S
									39	15	Sia3.S
										14	Sia4.S
											Gsc.L

C



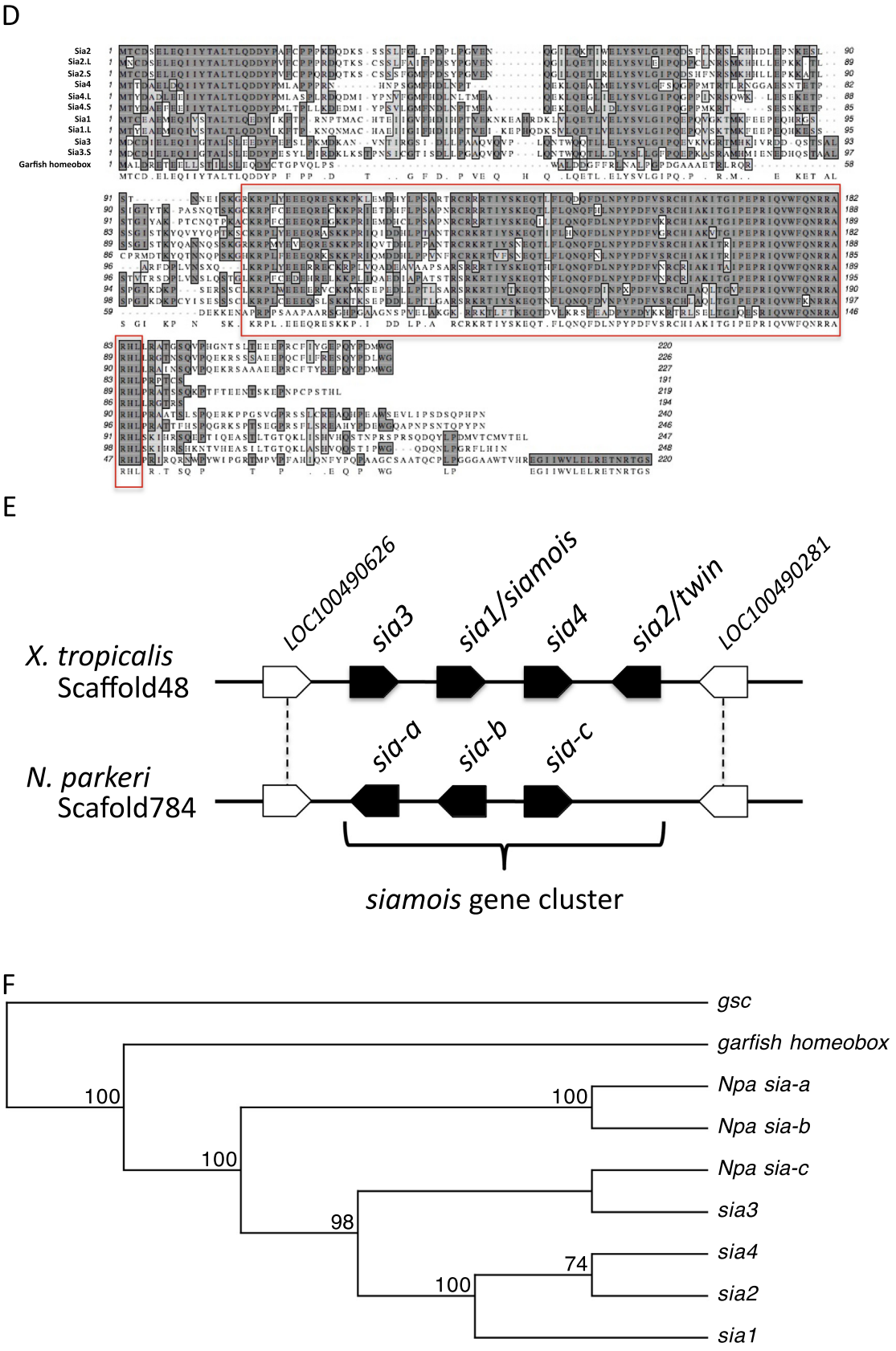


Fig. 5. (*continued*)

(http://blast.ncbi.nlm.nih.gov/Blast.cgi? PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_SPEC=Assembly&ASSEMBLY_NAME=GCA_000935625.1). We found three *siamois*-related sequences that were temporarily called as *sia-a*, *sia-b*, and *sia-c* in the *N. parkeri* genome assembly (Fig. 5E and Supplementary Text 1). *Sia-a*, *sia-b* transcriptional units were oriented in the same direction

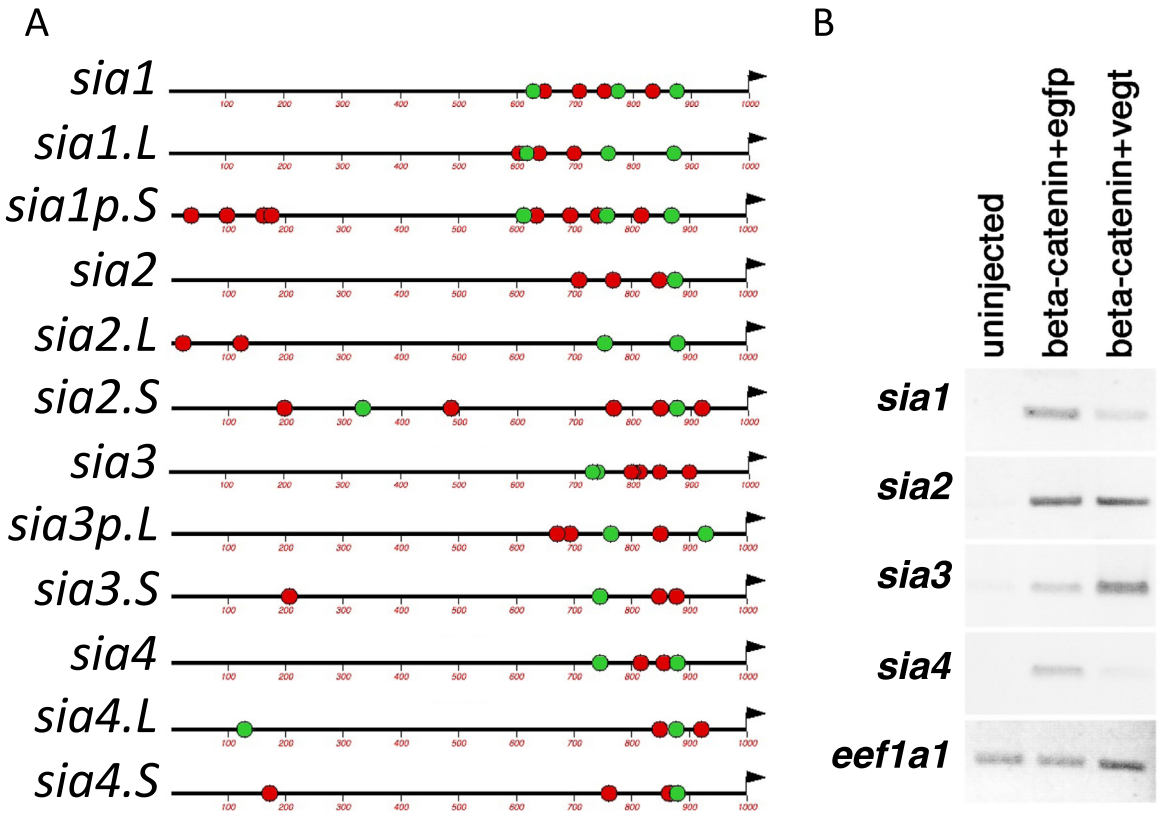


Fig. 6. *Siamois*-related genes are regulated by Wnt/β-catenin signals. (A) Upstream regulatory sequences of *siamois*-related genes in both *X. laevis* and *X. tropicalis*. TCF binding sites (CTTTGAW) are indicated with green circles. T-box binding sites (TNNCACYY) are indicated with red circles. Arrows indicate the first codon. (B) The over-expression of β-*catenin* increased the expression of all *siamois*-related genes and *vegt* affected this induction in *X. tropicalis*. β-*catenin* mRNA (200 pg) was co-injected with *egfp* or *vegt* mRNAs (200 pg) into *X. tropicalis* embryo at the two-cell stage. Animal caps were dissected and were analyzed at stage 9.

and *sia-c* was in the opposite direction.

The estimated divergence time between *X. tropicalis* and *N. parkeri* is 266 millions of years ago, but these two frogs exhibit considerable conserved whole-genome *syn*teny (Sun et al., 2015). The phylogenetic analysis using these sequences (Supplementary Text 1) showed that there are no one-to-one correspondences between *X. tropicalis sia1–4* and *N. parkeri sia-a–c* (Fig. 5F), suggesting that these gene duplications were not derived from common ancestral species. It is possible that *siamois*-related genes were duplicated independently in the genomes of these two frogs or that many intra-cluster recombination events had frequently occurred at the *siamois*-related gene loci. It was suggested here that multiple *siamois*-related genes are present in the genome of anurans and a spotted garfish has at least one orthologous candidate sequence.

3.4. Regulation by Wnt/β-catenin signaling and VegT

Previous reports revealed that both *siamois* and *twin* expressions are regulated by Wnt/β-catenin signaling (Carnac et al., 1996; Brannon and Kimelman, 1996; Brannon et al., 1997; Laurent et al., 1997). The proximal upstream regulatory regions of these two genes have several conserved TCF binding sites, which are required for Wnt/β-catenin signaling (Brannon et al., 1997; Laurent et al., 1997; Fan et al., 1998). Other reports showed that VegT negatively regulates (Ishibashi et al., 2008) or positively regulates (Li et al., 2015) *siamois* expression. We found 1–3 conserved TCF sites (CTTTGAW) and 2–8 T-box sites (TNNCACYY) up to 997 bp upstream regions of all *siamois*-related genes in both *X. tropicalis* and *X. laevis* (Fig. 6A and Supplementary Fig S4A-L). We tested whether other *siamois*-related genes were also regulated by Wnt/β-catenin signaling and VegT in *X.tropicalis*. Over-expression of β-*catenin* mRNA up-regulated all *siamois*-related genes (*sia1*, *sia2*, *sia3*, and *sia4*) in animal caps of *X. tropicalis* (Fig. 6B), suggesting that all *siamois*-related genes are downstream targets of Wnt/β-catenin signaling. Addition of *vegt* mRNA with β-*catenin* showed complex results that *sia1* and *sia4* were down-regulated, that *sia3* was up-regulated, and that *sia2* was not affected (Fig. 6B).

The regulation of *siamois* expression by VegT is controversial. Previous reports showed that VegT negatively regulates (Ishibashi et al., 2008) or positively regulates (Li et al., 2015) *siamois* expression. In these studies, VegT function was inhibited by MO (Ishibashi et al., 2008) or *VegT-EnR* (Li et al., 2015). In their over-expression experiments, different doses of *vegt* mRNA were used. The differences of experimental condition between these two studies may result in different conclusions. It is reported that the most proximal T-box site is essential for positive regulation of *sia1. L* by VegT (Li et al., 2015). However, this reported site (5′-AGAGTGGA-3′) is not T-box binding consensus sequence (5′-TNNCACYY-3′), and we could not find T-box sites around this region (Supplementary Fig S4). Our results that the area and timing of expression of *siamois*-related genes are slightly different from each other and their expressions were differently regulated by *vegt* over-expression suggest that VegT contribution to the expressions of *siamois*-related genes should be analyzed individually in consideration of *in vivo* conditions.

4. Conclusion

X. tropicalis has four active *siamois* cluster genes in the genome. *X. laevis* has an allotetraploid genome, and has at least six active *siamois*-related genes, three of which are located as a cluster on each homeologous sequence (XLA3L and scaffold20 that is

Please cite this article as: Haramoto, Y., et al., Identification and comparative analyses of *Siamois* cluster genes in *Xenopus laevis* and *tropicalis*. Dev. Biol. (2016), http://dx.doi.org/10.1016/j.ydbio.2016.07.015

expected to be located on XLA3S by transposon analysis) (Session et al., submitted). These *siamois*-related genes are similarly expressed between the blastula and early gastrula stages. We report that all expressed *siamois*-related genes except for *sia1p.S* induced secondary axis when over-expressed on the ventral side of embryos at four- or eight-cell stages (Figs. 2A and 4A). In *X. tropicalis*, all four *siamois*-related genes (*sia1*, *sia2*, *sia3*, and *sia4*) were regulated by Wnt/β-catenin signaling. *Sia1* was mainly expressed in the BCNE center at mid-blastula and in the Nieuwkoop center at late-blastula stage. The others are mainly expressed in the Nieuwkoop center.

This study shows that *siamois*-related genes are present as clusters on the *Xenopus* genome, and *siamois*-related genes have redundant functions but their spatial expression patterns and regulation by VegT are different from each other, suggesting that a comprehensive analysis of *siamois*-related genes is required to understand their precise function in the BCNE and Nieuwkoop centers.

Author contributions

Conceived and designed the experiments: NF, AS, YI, MK, MT, and ST. Performed the experiments: YH, TS, TT, and ST. Wrote the paper: YH, MT, and ST.

Acknowledgment

X. tropicalis was provided by the National Bio-Resource Project (NBRP) of the Ministry of Education, Culture, Sports, Science and Technology, Japan. This work was supported by JSPS KAKENHI Grant Number JP24590232. We thank Dr. Yuuri Yasuoka for an important information and discussion about a garfish gene. The authors report no conflicts of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.07.015.

References

Agius, E., Oelgeschläger, M., Wessely, O., Kemp, C., De Robertis, E.M., 2000. Endodermal nodal-related signals and mesoderm induction in Xenopus. Development 127 (6), 1173–1183.

Bae, S., Reid, C.D., Kessler, D.S., 2011. Siamois and Twin are redundant and essential in formation of the Spemann organizer. Dev. Biol. 352 (2), 367–381.

Brannon, M., Kimelman, D., 1996. Activation of Siamois by the Wnt pathway. Dev. Biol. 180 (1), 344–347.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R.T., Kimelman, D., 1997. A-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. Genes Dev. 11 (18), 2359–2370.

Brannon, M., Brown, J.D., Bates, R., Kimelman, D., Moon, R.T., 1999. XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development. Development 126 (14), 3159–3170.

Carnac, G., Kodjabachian, L., Gurdon, J.B., Lemaire, P., 1996. The homeobox gene Siamois is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm. Development 122 (10), 3055–3065.

Fan, M.J., Sokol, S.Y., 1997. A role for Siamois in Spemann organizer formation. Development 124 (13), 2581–2589.

Fan, M.J., Grüning, W., Walz, G., Sokol, S.Y., 1998. Wnt signaling and transcriptional control of Siamois in Xenopus embryos. Proc. Natl. Acad. Sci. USA 95 (10), 5626–5631.

Fukuda, M., Takahashi, S., Haramoto, Y., Onuma, Y., Kim, Y.J., Yeo, C.Y., Ishiura, S., Asashima, M., 2010. Zygotic VegT is required for Xenopus paraxial mesoderm formation and is regulated by Nodal signaling and Eomesodermin. Int. J. Dev. Biol. 54 (1), 81–92.

Gimlich, R.L., Gerhart, J.C., 1984. Early cellular interactions promote embryonic axis formation in Xenopus laevis. Dev. Biol. 104 (1), 117–130.

Ishibashi, H., Matsumura, N., Hanafusa, H., Matsumoto, K., Robertis, E.M., De, Kuroda, H., 2008. Expression of Siamois and Twin in the blastula Chordin/Noggin signaling center is required for brain formation in Xenopus laevis embryos. Mech. Dev. 125 (1–2), 58–66.

Kessler, D.S., 1997. Siamois is required for formation of Spemann's organizer. Proc. Natl. Acad. Sci. U S A 94 (24), 13017–13022.

Kimelman, D., 1999. Transcriptional regulation in Xenopus: a bright and froggy future. Curr. Opin. Genet. Dev. 9 (5), 553–558.

Klein, S.L., Moody, S.A., 2015. Early neural ectodermal genes are activated by siamois and twin during blastula stages. Genesis 53 (5), 308–320.

Kuroda, H., Wessely, O., De Robertis, E.M., 2004. Neural induction in Xenopus: requirement for ectodermal and endomesodermal signals via Chordin, Noggin, -Catenin, and Cerberus. PLoS Biol. 2 (5), e92.

Laurent, M.N., Blitz, I.L., Hashimoto, C., Rothbächer, U., Cho, K.W., 1997. The Xenopus homeobox gene twin mediates Wnt induction of goosecoid in establishment of Spemann's organizer. Development 124 (23), 4905–4916.

Lemaire, P., Garrett, N., Gurdon, J.B., 1995. Expression cloning of Siamois, a xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. Cell 81 (1), 85–94.

Li, H.Y., El Yakoubi, W., Shi, L., 2015. Direct regulation of siamois by VegT is required for axis formation in Xenopus embryo. Int. J. Dev. Biol. 59, 443–451.

Nieuwkoop, P.D., Faber, J., 1994. Normal Table of Xenopus laevis (Daudin): A Systematical & Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis. Garland Science, New York.

Rankin, S.A., Kormish, J., Kofron, M., Jegga, A., Zorn, A.M., 2011. A gene regulatory network controlling hhex transcription in the anterior endoderm of the organizer. Dev. Biol. 351 (2), 297–310.

Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destrée, O., Clevers, H., 1998. The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature 395 (6702), 608–612.

Schneider, S., Steinbeisser, H., Warga, R.M., Hausen, P., 1996. β-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. Mech. Dev. 57 (2), 191–198.

Sive, H.L., Grainger, R.M., Harland, R.M., 2000. Early development of Xenopus laevis: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Sudou, N., Yamamoto, S., Ogino, H., Taira, M., 2012. Dynamic in vivo binding of transcription factors to cis-regulatory modules of cer and gsc in the stepwise formation of the Spemann-Mangold organizer. Development 139 (9), 1651–1661.

Sun, Y.B., Xiong, Z.J., Xiang, X.Y., Liu, S.P., Zhou, W.W., Tu, X.L., Zhong, L., Wang, L., Wu, D.D., Zhang, B.L., Zhu, C.L., Yang, M.M., Chen, H.M., Li, F., Zhou, L., Feng, S.H., Huang, C., Zhang, G.J., Irwin, D., Hillis, D.M., Murphy, R.W., Yang, H.M., Che, J., Wang, J., Zhang, Y.P., 2015. Whole-genome sequence of the Tibetan frog Nanorana parkeri and the comparative evolution of tetrapod genomes. Proc. Natl. Acad. Sci. USA 112 (11), E1257–E1262.

Takahashi, S., Yokota, C., Takano, K., Tanegashima, K., Onuma, Y., Goto, J., Asashima, M., 2000. Two novel nodal-related genes initiate early inductive events in Xenopus Nieuwkoop center. Development 127 (24), 5319–5329.