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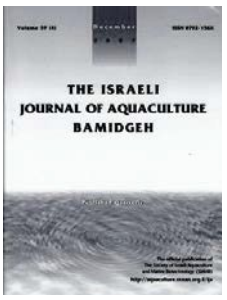

Molecular Cloning of Twist Gene and its Expression in Golden Pompano *Trachinotus ovatus* (Linnaeus 1758) Larvae at Different Water Temperatures

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Keywords: twist gene; expression; ontogeny; temperature; golden pompano *Trachinotus ovatus*

Abstract

The twist gene in golden pompano *Trachinotus ovatus* larvae was cloned and analyzed in this study. in the first 18 days the expression of twist during larval fish ontogeny was explored, then on 18 day post hatch (DPH) the expression of twist in fish tissues was evaluated. Subsequently, the response of twist to water temperatures of 23, 26, and 29ºC was compared on 12 DPH and 18 DPH. The cDNA sequence length of the twist gene in golden pompano is 880 bp with an open reading frame of 507 bp. The twist gene encodes 168 amino acids and has a calculated molecular weight of 18.93 kDa and a theoretical isoelectric point of 9.14. After hatching, the expression of twist increased with fish age, and peaked at 3 and 4 DPH. The highest expression of twist in fish tissue occurred in the spleen and stomach, followed by the brain and kidney on 18 DPH. On 12 DPH, the highest expression of twist was observed in fish reared at 26ºC, and lowest expression was observed in fish reared at 29ºC. On 18 DPH, the expression of twist was not significantly affected by the rearing temperatures. This study identified the gene expression of twist at the early developmental stage of golden pompano; the time dependent expression of twist in fish larvae may improve knowledge of bone ontogeny and formation in fish larvae.

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Introduction

Twist protein contains a basic helix-loop-helix (bHLH) structure that belongs to the nuclear transcription factors. Twist protein plays a critical role in cell type determination and differentiation and can regulate osteogenesis at the early developmental stage (Germanguz and Gitelman, 2012; Hornik et al., 2004; Lee et al., 1999). Twist genes are a small family presented across the metazoan, ranging from cnidarians to humans (Castanon and Baylies, 2002). The embryonic lethality of twist mutants of both vertebrates and invertebrates indicates that a functioning twist gene is essential for development and survival (Germanguz et al., 2007; Yeo et al., 2009). The twist gene was originally identified in Drosophila melanogaster, in which it plays an important role in gastrulation and mesoderm formation, and in the establishment of dorso-ventral polarity (Simpson, 1983; Thisse et al., 1987). Several studies have shown that the twist gene plays an important role in bone development and is expressed in primary osteoblastic cells and preosteoblasts (Murray et al., 1992; Rice et al., 2000). The expression of twist gene functions in vertebral column formation, an expression that is occasionally used as a marker of axial mesoderm development (Halpern et al., 1995; Yan et al., 1995).

Golden pompano (*Trachinotus ovatus*) belongs to the family of Carangidae and is a good candidate species for aquaculture due to fast growth and suitability for cage culture (Ma et al., 2014; Lin et al., 2018). In golden pompano, over 33% of the fish in a population exhibited at least one type of malformation during the larval period (Ma et al., 2016; Zheng et al., 2016; Ma et al., 2017). To understand the cause of malformation in this species, it is necessary to identify a potential indicator to allow a rapid and reliable evaluation and predict malformation. As a gene relevant to early bone development, the understanding of the expression of twist during fish ontogeny will improve our knowledge to rectify fish malformation during early development. This study was designed to explore the expression of twist during the ontogeny of golden pompano larvae in the first 18 days post-hatch (DPH), and the response of twist to water temperature on 12 and 18 DPH. The expression pattern of twist could provide useful information on the osteogenesis of golden pompano larvae.

Materials and Methods

In this study, the handling of fish was carried out in strict accordance with the recommendation in the Animal Welfare of Chinese Academy of Fishery Sciences Animal Welfare Committee. The protocol, species and number of animals used in this study were approved by the Animal Welfare Committee (Approved Number: 2014YJ01).

Expression of twist gene in the first 18 days of golden pompano larvae

Experiment I

Fertilized eggs of golden pompano were obtained from a local hatchery, Hainan Provence, P.R. China, and were transported to Lingshui Town and hatched in 500L fiberglass incubators at 26.5ºC with a hatching rate of 97.5 ± 1.5% (mean ± SD). On 2 DPH, larvae were stocked into three 1000L larval rearing tanks. Larval rearing tanks were supplied with filtered seawater (5-μm pore size) from the bottom of each tank through upwelling with a daily exchange rate of 200% tank volume. Water was discharged through an outlet screen (300 μm) on the upper side of each tank, and the screen was cleaned daily to reduce clogging. Two air stones were used in each tank to maintain dissolved oxygen close to saturation. Light intensity was maintained at 2400 lux, and the light regime was controlled at 14 h light and 10 h dark. Salinity was maintained at 33±0.8 ‰ and water temperature was 26.5±1.0ºC throughout the experiment.

Rotifers *Brachionus rotundiformis,* at a density of 10-20 ind/ml were used to feed the larvae from 2 DPH to 10 DPH. Rotifers fed with bakers yeast were enriched with DHA protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) for 12 h before they were added into the larval rearing tanks. Instant microalgal paste (Nannochloropsis sp.) was also added into the larval fish tanks to create a green-water background. *Artemia* nauplii were first introduced at 0.1 nauplii/mL on 10 DPH, and then added with a numbered daily increment of 90%. After five days co-feeding, *Artemia* nauplii were gradually phased out at a daily numbered reduction of 20% until the co-feeding period ended. Artemia nauplii were enriched with DHA Protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) following the manufacturer's instruction.

Response of twist gene to rearing temperature

Experiment II

Fertilized eggs of the same batch were obtained from Lingshui, Hainan Province, and transported to the Tropical Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Xincun Town. Upon arrival, all eggs were transferred into 500L incubators and hatched at 26ºC. The experimental design included three constant temperatures 23, 26, and 29ºC with three replicates each. On 2 days post hatch (DPH), yolk sac larvae were acclimatized at each desired temperature for 5 h, and then stocked in 500L fiberglass tanks at a density of 60 fish/L. Apart from the rearing temperatures, all the feeding protocols and rearing conditions were the same as in experiment I.

Total RNA extraction and reverse transcription

On 0, 1, 2, 3, 4, 5, 12, and 18 DPH, approximately 300 mg (wet weight) fish larvae were taken in triplicate samples from the rearing tanks. Approximately 50 individuals were in each sample 18 DPH. A total of 100 individuals were and examined under a dissecting microscope for tissue expression analysis. Total RNA was extracted using TRIzol (Invitrogen, USA). RNA integrity was verified by electrophoresis on a formaldehyde-agarose gel (1.2%). The RNA concentration was measured by absorbance at 260 nm and the purity was determined using the ratio at the absorbance of 260 nm and 280 nm (260/280) and agarose gel electrophoresis. RNA was reverse-transcribed to cDNA with oligo (dT) primers using a PrimeScript 1st strand cDNA synthesis kit (TaKaRa Biotechnology, Dalian Co., Ltd). The cDNA was used as a template in subsequent PCR.

Cloning of the gene cDNA and real-time PCR

Based on unpublished sequences of golden pompano transcriptome measured previously in our laboratory (Illumina HiSeq2000, annotated by NR, KOG, kegg, and Swissprot), the gene cloning primers were designed (Table 1) with Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA). The PCR reaction systems were as follows: 1 μL of golden pompano larval cDNA, 1 μL of gene-specific forward primer (F), 1 μL of gene-specific reverse primer (R), 0.5 μL of ExTaq, 5 μL of PCR buffer, 4 μL of dNTP mixture (2.5 μM) and 37.5 μL of ddH_2O were mixed in a total volume of 50 μL. The PCR conditions were as follows: DNA denaturation at 94ºC for 1 min, 35 cycles of 94ºC for 30 s, annealing temperature for 30 s, 72ºC for 4 min, followed by a 10 min extension at 72ºC. The PCR products were cloned into the PMD-19T vector (TAKARA, Japan), and then sequenced.

Quantitative real-time PCR (qPCR) was used to analyze the level of twist gene expression in golden pompano larvae. Gene specific primer pairs for the twist gene (Table 1) were amplified in LightCycler480 II (Roche, Switzerland). EF-1α was used as the internal reference and amplified. The cycling conditions for twist gene and EF1α were as follows: 1 min at 95ºC, followed by 40 cycles at 95ºC for 15 s, and 60ºC for 1min. Dissociation curves were employed to ensure that only one single PCR product was amplified in each gene reaction. For each test, three replicates were performed. The relative quantification (RQ) was calculated using the ΔΔCT (comparative threshold cycle) method (ΔCT = CT of target gene - CT of EF-1α, ΔΔCT = ΔCT of any sample - ΔCT of calibrator sample). The efficiencies (Etwist) of the primers (E) were 1.0002.

Table 1 Sequences of primers used in this study

Primers	*Sequence(5'-3')*	*Amplicon sizes (bp)*
Twist-F	TCCCAGGAACCCATCTAT	852
Twist-R	CAACACGAAAGCACATCAG	
Twist-qF	GTGGGAGGAAGAGGAGACCG	215
Twist-qR	CGAGGGCAAAGTGGGGAT	
EF-1α-qF	CCCCTTGGTCGTTTTGCC	101
EF-1α-qR	GCCTTGGTTGTCTTTCCGCTA	

Statistical analysis

The data were all expressed as mean ± SD and compared with one-way ANOVA (PASW Statistics 18.0, Chicago, SPSS Inc.). Tukey's test was used for multiple range comparisons with the level of significant difference set at $P < 0.05$. All data were tested for normality, homogeneity and independence to satisfy the assumptions of ANOVA.

Sequences and phylogenic analysis

The twist gene cDNA sequences were analyzed by BLAST at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The complete ORF regions and amino acid sequences were deduced with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight (Mw) and isoelectronic point (pI) of deduced amino acids were computed by the pI/Mw tool of ExPASy (http://web.expasy.org/compute_pi/). Protein domains were predicted using SMART (http://smart.embl-heidelberg.de/). Multiple sequence alignments of amino acids were performed by ClustalX 2.1. The phylogenetic tree was constructed by the neighbor-joining (NJ) method in MEGA 6.0, and the bootstrap values were replicated 1000 times to derive the confidence value for the analysis (Tamura et al., 2013). The sequences for pairwise deduced amino acid sequence identity and similarity matrices were performed using Matgat 2.02 (Campanella et al., 2003). The three-dimensional structures of golden pompano twist were obtained by homology modeling, (http://swissmodel.expasy.org/workspace/index.php).

Results

The length of the cDNA sequence of golden pompano twist gene (GenBank accession: KY204035) was 880 bp with an open reading frame (ORF) of 507 bp, which encodes 168 amino acids (aa), with a calculated molecular weight (Mw) of 18.93kDa and theoretical isoelectric point (pI) of 9.14 (Fig. 1). The bioinformatics analysis of the deduced amino acids sequence contained Granins signature sequence (19DSLSNSEgEL28), nuclear localization signals (34RCGRKRRPSRK44), helix loop helix domain (80N-S131) (Fig. 2).

```
  1 ATCAGAAAAGTCTTGTCCTCCCAGGAACCCATCTATGGATACTTTGGTGTCTTCAAAGCC 60
 61 ACATAGACCTACAAGTGGACTAATGATTTGACTTCATGAGAGGGAAATAATTTTTCGGCA 120
121 CCCTTTCTGGCCGCCATCTACCCAAGTGGAGatgtctgaggaaaatatgggggaagattc 180
  1                              M  S  E  E  N  M  G  E  D  S  10
181 gagcagctcccctgtctctcctgtggacagcctgagcaacagcgaggggggagctggacag 240
 11  S  S  S  P  V  S  P  V  D  S  L  S  N  S  E  G  E  L  D  R  30
241 acaaccgaagagatgtgggaggaagaggagaccgagcaggaaaaacggggaggactcaga 300
 31  Q  P  K  R  C  G  R  K  R  R  P  S  R  K  N  G  E  D  S  D  50
301 tagcccgacccctgggaaaagagggaagaagtccagcagcagcagcccacagtctttcga 360
 51  S  P  T  P  G  K  R  G  K  K  S  S  S  S  S  P  Q  S  F  E  70
361 ggagctccagtcacagcggatcatggccaacgtccgggagcgacagaggacccagtctct 420
 71  E  L  Q  S  Q  R  I  M  A  N  V  R  E  R  Q  R  T  Q  S  L  90
421 caacgaggcgttcgcagccttgcggaaaattatccccacttttgccctcggacaaactcag 480
 91  N  E  A  F  A  A  L  R  K  I  I  P  T  L  P  S  D  K  L  S  110
481 caaaatacagaccctaaagcttgcagccagatacatcgacttcctctaccaggtgctgca 540
111  K  I  Q  T  L  K  L  A  A  R  Y  I  D  F  L  Y  Q  V  L  Q  130
541 gagcgatgagctggactccaaaatgtcaagttgtagttatgtggctcacgagaggctgag 600
131  S  D  E  L  D  S  K  M  S  S  C  S  Y  V  A  H  E  R  L  S  150
601 ttacgccttctctgtatggaggatggagggcgcttggtccatgtcaacatctcactagCA 660
151  Y  A  F  S  V  W  R  M  E  G  A  W  S  M  S  T  S  H  *     168
661 TCTGGAGAAATTATGCCCAAAATGGTGACTGCTGAATCTAATTATTACACTCTGACGGGA 720
721 CGAATCTGGAGTCCAGTGCTGGATACATGGGATCACTCTATTTAAGCCAAAGACGACAGA 780
781 AGGTCTGGGGATCACTCCTGCAGAGGCCCGATAGGGACTTGCAGTCGTGCGTTAGTTCCG 840
841 TACACCCCATTCTGATGTGCTTTCGTGTTGTCGACGACGA 880
```

Fig. 1. Nucleotide sequence and deduced amino acid of twist gene from golden pompano Trachinotus ovatus. The helix loop helix domain (HLH) are underlined. Predictions of nuclear localization signals (NLSs) are double-underline. Granins signature is boxed.

Fig. 2. The prediction of conserved domain in twist from golden pompano Trachinotus ovatus showing granins signature sequence (19DSLSNSEgEL28), nuclear localization signals (NLS: 34RCGRKRRPSRK44), and helix loop helix domain (80N-S131).

The predicted secondary structure of golden pompano twist contained two helixes and one stand (Fig 3). The molecular modeling of golden pompano twist was shown in Fig. 3, containing two α-helixes. The deduced protein subcellular localization predicted in the nucleus (0.960) by the PSORT II Server (http://psort.hgc.jp/form.html).

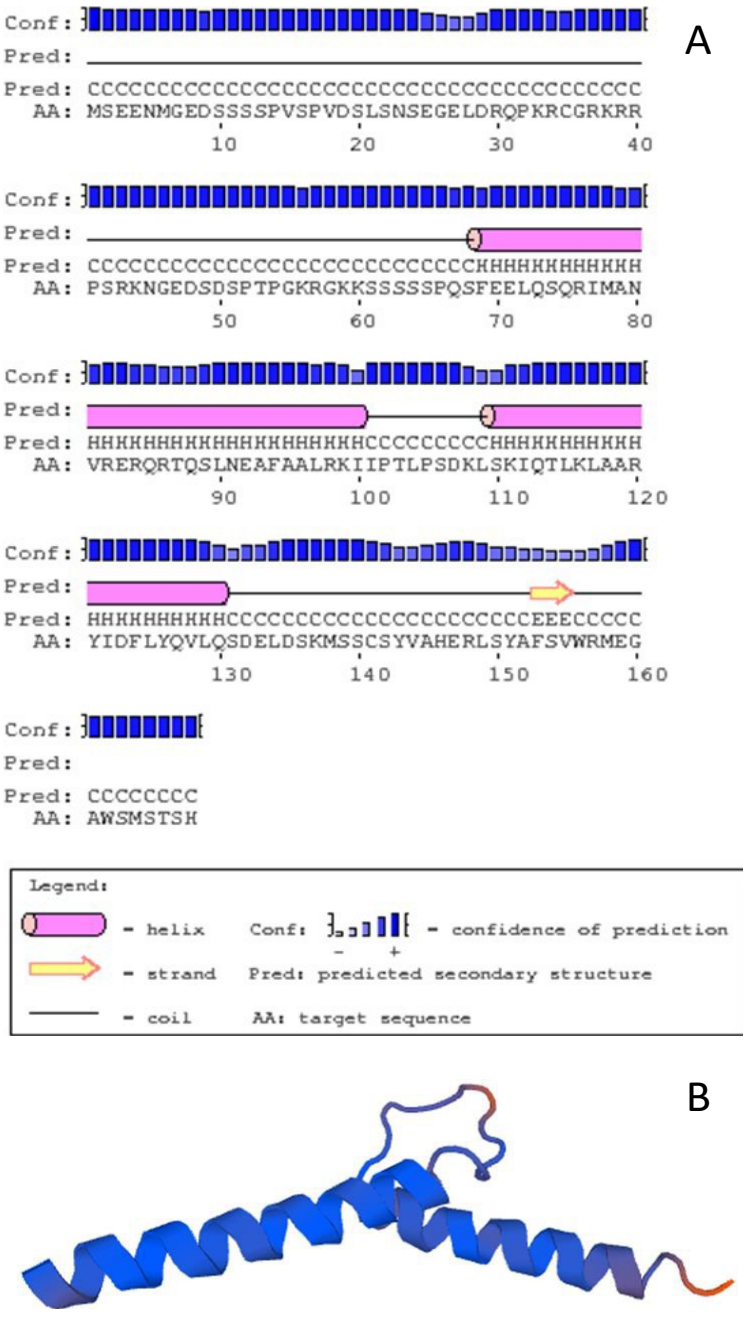

Fig. 3 Predicted secondary structure (A) and tertiary structure (B) of the twist gene in golden pompano Trachinotus ovatus.

Multiple sequence alignment of the deduced amino acid sequences of the golden pompano twist and some known twist family amino acid sequences of other species are shown in Table 2. The predicted amino acid sequence of the twist gene from golden pompano showed high identity and similarity with the twist 1 gene of *Oryzias latipes* (95.8% and 98.8%, NP_001098177.1) and *Takifugu rubripes* (93.5% and 98.2%, NP_001098069.1), different identity (68.6-87.1%) and similarity (74.8-91.1%) with twist 1 gene of other species, and different identity (77.1-84.5%) and similarity (84.5-89.3%) with twist 2 gene of other species. The deduced twist amino acid sequences of all species contained the conserved helix loop and helix domain.

Table 2. Identity and similarity between golden pompano twist with other twist family homologue

Species	*Homologues*	*Accession NO.*	*AA*	*Similarity (%)*	*Identity (%)*
Trachinotus ovatus	Twist	Present study	168	-	-
Oryzias latipes	Twist 1	NP_001098177.1	168	98.8	95.8
Takifugu rubripes	Twist 1	NP_001098069.1	168	98.2	93.5
Danio rerio	Twist 1	NP_001017820.1	169	91.1	87.1
Gallus gallus	Twist 1	NP_990070.1	190	78.9	72.2
Rattus norvegicus	Twist 1	NP_445982.1	203	73.9	67.8
Mus musculus	Twist 1	NP_035788.1	206	72.8	66.8
Homo sapiens	Twist 1	NP_000465.1	202	74.8	68.6
Oryzias latipes	Twist 2	NP_001295933.1	164	84.5	77.6
Takifugu rubripes	Twist 2	NP_001098070.1	163	86.3	77.1
Danio rerio	Twist 2	NP_001005956.1	160	86.3	79.9
Gallus gallus	Twist 2	NP_990010.1	160	89.3	84.5
Mus musculus	Twist 2	NP_031881.1	160	88.7	83.3
Rattus norvegicus	Twist 2	NP_067723.1	160	88.7	83.3
Homo sapiens	Twist 2	NP_476527.1	160	88.7	83.3

At hatch, the expression of the twist gene in fish larvae was low (Fig. 4). The expression level of twist increased with fish age and reached the first peak level on 3 DPH ($P < 0.05$, Fig. 4). The expression level of twist was not significantly different between 3 and 4 DPH. Stating from 5 DPH, the expression levels of twist in fish larvae gradually reduced. On 18 DPH, the expression level of twist was similar to the expression level on 1 DPH ($P > 0.05$).

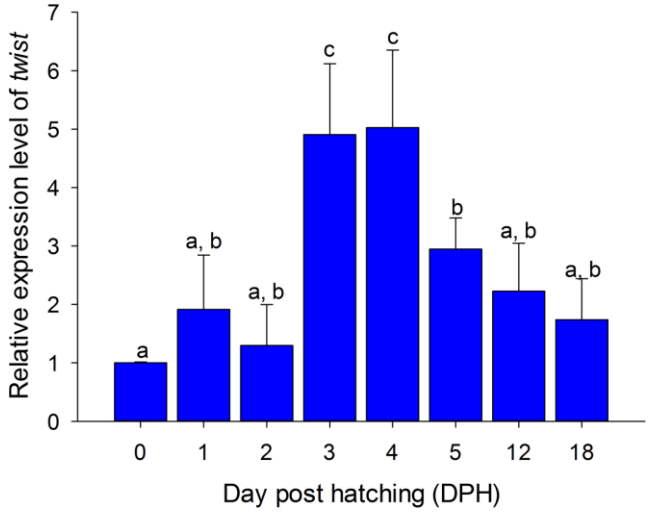

Fig. 4. Relative expression levels of the twist gene during golden pompano larvae development. Data with different letters were significantly different ($P < 0.05$).

On 18 DPH, the highest expression of twist in golden pompano was observed in spleen and stomach ($P < 0.05$, Fig. 5), followed by brain and kidney. The expression of twist in the gills, head-kidney, liver, and intestine of golden pompano was significantly lower than the expression in brain and kidney ($P<0.05$). The low expression levels of twist in golden pompano were observed in the heart of golden pompano larvae on 18 DPH ($P<0.05$).

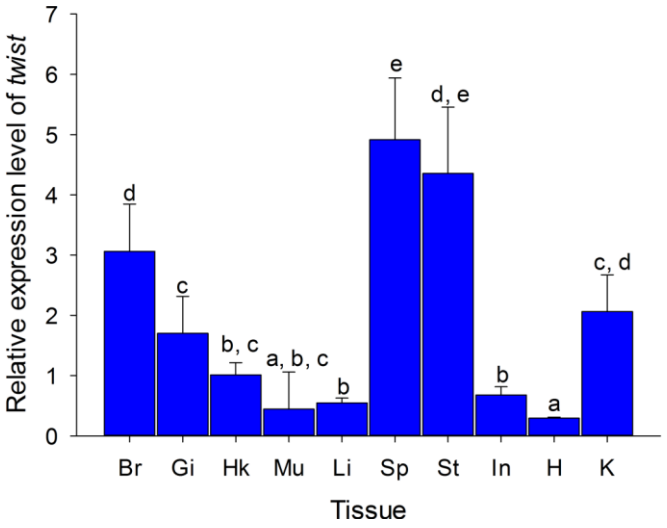

Fig 5. Relative level of twist gene mRNA in different tissues of golden pompano *T.ovatus*. Data with different letters were significantly different (P < 0.05). Abbreviations: Br, brain; Gi, gill; Hk, head-kidney; Mu, muscle; Li, liver; Sp, spleen; St, stomach; In, intestine; H, heart; K, kidney.

Water temperature significantly affected the expression of twist in golden pompano on both 12 and 18 DPH ($P < 0.05$, Fig. 6). On 12 DPH, the highest expression of twist was observed in fish at 26 ºC ($P < 0.05$), and lowest expression of twist was observed in fish at 29ºC ($P < 0.05$). On 18 DPH, the expression of twist in fish was not significantly affected by the rearing temperature ($P > 0.05$). In each temperature treatment, the expression of twist was not significantly different between 12 and 18 DPH ($P > 0.05$).

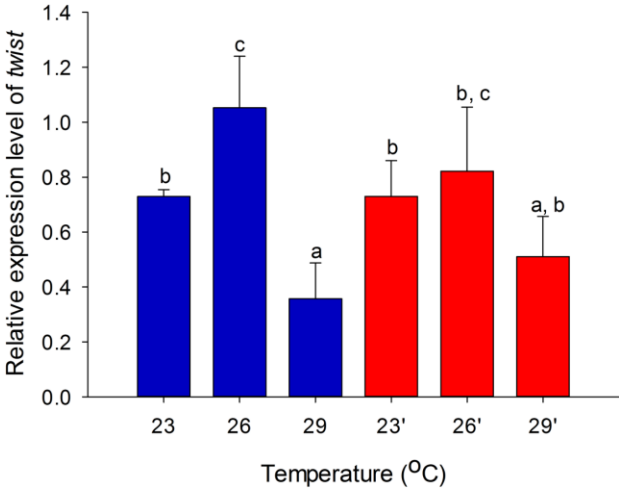

Fig 6. Relative expression levels of the twist gene at different temperatures on 12DPH and 18DPH in golden pompano larvae. Data with different letters were significantly different (P<0.05).

Discussion

All twist proteins are in the group “A” basic helix-loop-helix (bHLH) domain (Atchley and Fitch, 1997), and are considered the nuclear transcription factors. As the nuclear transcription factors, the function of twist proteins is cell-autonomous and is reflected in their expression patterns. Developmental expression histories of conserved genes can illustrate the evolution of gene functions (Germanguz et al., 2007). Twist genes belong to the highly-conserved family of important transcription factors whose functions in directing developmental programs have diverged during evolution. In the present study, phylogenetic analysis pointed to two main clusters: twist 1 and twist 2. However, the twist gene in golden pompano was more closely related to twist 1 in species such as *Oryzia latipes*, *Takifugu rubripes*, and *Danio rerio*. It shows the most restricted patterns in both space and time in the species under comparison. The deduced twist amino acid sequences of all compared species contain conserved helix loop and helix domain.

Previous studies have demonstrated that twist genes can participate in the regulation of the development and differentiation of many tissues and organs (Hebrok et al., 1997; Komori, 2006; Singh et al., 2011; Soo et al., 2002). In mouse and chicken embryos, expressions of twist genes suggest a dual role during development: the first role is to prevent premature differentiation, and the second role is tissue specification (Fuchtbauer, 2002; Scaal et al., 2002; Soo et al., 2002). In this study, expression level of the twist gene increased significantly from fish hatch to 3 DPH, and gradually decreased until 18 DPH. The high level of twist gene expression during early development of larval golden pompano is in accordance with the period of quick fish growth (Ma et al., 2014), indicating rapid formation of organs and tissues during this period.

Due to their functional importance, the expression pattern of twist genes during embryonic development has drawn particular attention, but most studies have been conducted during the embryonic stage (Dill et al., 2007; Germanguz and Gitelman, 2012; Germanguz et al., 2007; Yeo et al., 2009). The present study is the first to report tissue expression of the twist gene in a fish species with economic value to aquaculture. The highest expression of twist in golden pompano larvae was observed in the spleen and stomach, followed by its expression in the brain and kidney on 18 DPH. The twist gene is specifically expressed in tissues where they regulate mesoderm patterning and muscle differentiation (Baylies and Bate, 1996; Castanon and Baylies, 2002; Cripps et al., 1998). In *B. mori*, expression of the twist gene can be observed in the hemolymph, testis, ovary, epidermis, silk gland, and midgut (Guo et al., 2011). In frogs, chicks and mice, twist genes are expressed in pharyngeal arches (Hopwood et al., 1989; Scaal et al., 2001; Tavares et al., 2001), and somites or sclerotome (Hopwood et al., 1989; Li et al., 1995; Tavares et al., 2001; Wolf et al., 1991). In humans, twist mutations may be an essential underlying factor in the development and pathophysiological changes in tumors that lead to arrested osteoblastic differentiation and maintenance of an immature and neoplastic phenotype (Singh et al., 2011). Information on the tissue expression pattern of twist in fish, especially in early ontogeny, is scarce. In zebrafish, the expression of twist genes is found in head mesenchyme, intermediate mesoderm, somite, caudal gut, olfactory placode, branchial arch, caudal notochord, tail bud, hypochord, dorsal aorta, body wall, and heart valve (Germanguz and Gitelman, 2012; Yeo et al., 2007; Yeo et al., 2009). It is unclearly why higher expression was observed in the spleen and stomach of golden pompano larvae. Such higher expression may suggest rapid development in these organs as observed in other studies (Nieto et al., 1996; Shishido et al., 1993; Tavares et al., 2001). But there is no direct evidence to prove this, therefore further investigation is needed.

Twist gene controls skeletogenic mesenchyme in zebrafish (Germanguz and Gitelman, 2012). Temperature is a primary rearing condition influencing bone development as organ development and differentiation in fish are temperature-dependent (Lein et al., 1997; Yu et al., 2017). Twist was found to be up and down-regulated when skeleton malformation occurs in hyperthermic Atlantic salmon (Ytteborg et al., 2010). In the present study, the expression of twist in fish reared at 26ºC significantly increased when compared to those reared at 23ºC, and its expression in fish reared at 29ºC was significantly increased. Such up and down-regulated expression of twist was consistent with increasing malformation of golden pompano larvae reared at 26 and 29ºC (Yang et al., 2016). This may suggest that the expression of twist during fish ontogeny could be used as an indicator for skeleton malformation.

In summary, the twist gene cDNA of golden pompano larvae was cloned and analyzed in this study. The present study indicates that the expression of twist in golden pompano larvae was significantly affected by water temperature. The time-dependent expression of twist genes in fish larvae is essential to understand the ontogenetic development and growth of fish larvae in early life. The monitoring of twist gene expression in golden pompano larvae may serve as a useful indicator in the field and in fish farming, leading to rapid assessment of environmental conditions affecting fish skeletal development.

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