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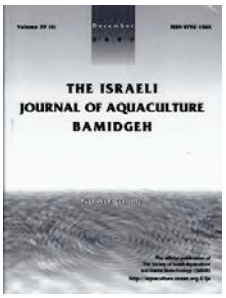

Molecular cloning of Indian hedgehog gene and its expression in golden pompano *Trachinotus ovatus* (Linnaeus 1758) larvae at different water temperatures

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Key words: Indian Hedgehog gene (*Ihh*); expression; ontogeny; temperature; golden pompano *Trachinotus ovatus*.

Abstract

The Indian hedgehog (*Ihh*) gene in golden pompano larvae was cloned and analyzed. The expression of *Ihh* during larval fish ontogeny in the first 18 days was examined, and then the expression of *Ihh* in fish tissues was evaluated on 18 days post hatching (DPH). Subsequently, the response of *Ihh* to water temperatures of 23, 26, and 29^0 C was compared on 12 DPH and 18 DPH. The cDNA sequence length of the golden pompano Ihh gene is 1,484 bp with an open reading frame of 1,314 bp. The *Ihh* gene encodes 437 amino acids and has a calculated molecular weight of 48.175 kDa and a theoretical isoelectric point of 6.22. After hatching, the expression of *Ihh* increased with fish age and peaked at 3 DPH. After fluctuations between 4 and 12 DPH, the expression of *Ihh* reached the second peak at 18 DPH. The highest expression of *Ihh* in tissues occurred in the fish intestine, followed in the stomach on 18 DPH. Water temperature significantly affected *Ihh* expression. On 12 DPH, the expression of *Ihh* at 23°C was significantly higher than that at 26 and 29 °C. In contrast, the expression of *Ihh* at 26 and 29^0 C was significantly higher than that at 23 °C on 18 DPH. This study detected the gene expression of *Ihh* at the early stage of golden pompano development and the time-dependent expression of *Ihh* in fish larvae is important to understand the ontogeny of bone formation in fish larvae.

Introduction

The Hedgehog (*Hh*) gene family codes for a class of secreted proteins that act in a signaling pathway to transmit information for cell differentiation in embryogenesis and development in both vertebrates and invertebrates (Bijlsma et al., 2004; Ingham and McMahon, 2001). Proteins in the *Hh* family of vertebrates control cell growth, survival

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and development in almost all body tissues (Varjosalo and Taipale, 2008). Hh proteins are composed of two distinct domains: an N-terminal “Hedge” domain and a C-terminal “Hog” domain (Burglin, 2008), and separated during an auto-cleavage reaction to generate two similar-sized globular fragments (Burglin, 2008; Lee et al., 1994). Such special structure enables Hh proteins to undergo auto-cleavage, and lipid modifications in the endoplasmic reticulum and produce a mature signaling peptide (Beachy et al., 1997; Chen et al., 2004; Chen et al., 2011; Lee et al., 1994), which can be incorporated into lipoprotein particles or diffuse freely to target cells (Eugster et al., 2007; Ingham and McMahon, 2001). In vertebrates, the *Hh* genes are classified into three subgroups: Desert Hedgehog (*Dhh*), Indian Hedgehog (*Ihh*), and Sonic Hedgehog (*Shh*) (Echelard et al., 1993; Wada and Makabe, 2006). The *Shh* and *Ihh* subgroups are more closely related to each other than to the *Dhh* subgroup that is closest to *Drosophila Hh* (Varjosalo and Taipale, 2008).

The growth of a skeletal element depends on the precise regulation of chondrocyte proliferation and hypertrophy. Appropriate morphogenesis requires integration of proliferation and hypertrophy over the entire element to form the long axis along which the tissue growth follows (Karp et al., 2000). As a member of the conserved hedgehog family of signaling molecules (Echelard et al., 1993), *Ihh* regulates several aspects of bone formation and development (Amizuka et al., 1996; Seki and Hata, 2004; Vortkamp et al., 1996), and play a critical role for skeleton morphogenesis in vertebrates (Karp et al., 2000). For instance, *Ihh* is initially expressed within the cartilage primordium of the long bones in mice, and becomes localized in a zone of postmitotic and pre-hypertrophic chondrocytes immediately adjacent to the region of proliferating chondrocytes at birth (Bitgood and McMahon, 1995; Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). *Ihh* knock-out in the embryogenesis of mice can lead to severe skeletal malformation (St-Jacques et al., 1999).

Golden Pompano 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., 2014a). In golden pompano *Trachinotus ovatus*, over 33% of fish in a population exhibited at least one type of malformation during the larval period (Ma et al., 2014b; Zheng et al., 2014). To understand the cause of malformation in this species, it is necessary to identify an indicator to conduct a rapid and reliable evaluation for predict malformation. As a gene relevant to early bone development, the understanding of the expression of Indian Hedgehog gene (*Ihh*) during fish ontogeny will provide knowledge to rectify fish malformation during early development. This study was designed to explore the expression of *Ihh* during the ontogeny of golden pompano larvae in the first 18 days post-hatch (DPH), and the response of *Ihh* to water temperature at 12 and 18 DPH. The expression pattern of *Ihh* would provide essential information on the osteogenesis of golden pompano larvae. Such knowledge can improve the understanding on the bone formation of golden pompano, and provide a potential indicator to predict skeleton malformation of fish larvae in early life.

Materials and Methods

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

Fertilized eggs of golden pompano were obtained from Guanghui Aquaculture Hatchery, Hainan Provence, P.R. China, and were transported to Lingshui Town and hatched in 500-L fiberglass incubators at 26.5 ◦C with a hatching rate of 97.5 ± 1.5% (mean ± SD). On 2 DPH, larvae were stocked into three 1000-L 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 daily cleaned to reduce clogging. Two air stones were used in each tank to maintain dissolved oxygen close to saturation. Light intensity was maintained at 2,400 lux, and the light regime was controlled at 14 h light and 10 h dark. The salinity was maintained at 33 ± 0.8 ‰ and water temperature was 26.5 ± 1.0 ◦C throughout the experiment.

Rotifers *Brachionus rotundiformis* at a density of 10-20 ind ml^{-1} were used to feed the larvae from 2 DPH to 10 DPH. Rotifers fed with baker 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 larval fish tanks to create a green-water background. *Artemia* nauplii were first introduced at 0.1 nauplii mL^{-1} on 10 DPH, and then added with a daily increment of 90% by number. After five days co-feeding, *Artemia* nauplii were gradually phased out at a daily reduction of 20% by number 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 Ihh gene to rearing temperature

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 500-L incubators and hatched at 26 °C. The experimental design included three constant temperatures 23, 26, and 29 °C 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 500-L fiberglass tanks at a density of 60 fish L^{-1}. Except for the rearing temperature, 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 sampled from rearing tanks in triplicates. Approximately 50 individuals were collected in triplicate on 18 DPH. A total of 100 individuals were collected in triplicate, 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%). Absorbance measured the RNA concentration at 260 nm and the purity was determined at the ratio of absorbance at 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 golden pompano transcriptome sequences measured previously in our laboratory (Illumina HiSeq2000, annotated by NR, KOG, kegg, and Swissprot), the genes cloning primers were designed (Table 1) with Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA). The PCR reaction systems were; 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 denaturation at 94 °C for 1 min, 35-cycles of 94 °C for 30 s, annealing temperature of each gene for 30 s, 72 °C for 4 min, followed by a 10 min extension at 72 °C. The PCR products were cloned into the PMD-19T vector (TAKARA, Japan), and sequenced.

Table 1 Sequences of primers used in this study

Primers	Sequence(5'-3')	Amplicon seizes (bp)
Ihh-F	CAGTCACGGACGATGGAG	1484
Ihh-R	GCTGTGGTCTGTGCTTTAGT	
Ihh-qF	TACGAGTCCAAAGCCCACATT	87
Ihh-qR	AGCATCGCCAGGGAAACA	
EF-1α-qF	CCCCTTGGTCGTTTTGCC	101
EF-1α-qR	GCCTTGGTTGTCTTTCCGCTA	

Quantitative real-time PCR (qPCR) was used to analyze the level of *Ihh* gene expression in golden pompano larvae. Gene-specific primer pairs for the *Ihh* gene (Table 1) were amplified in LightCycler480 II (Roche, Switzerland). EF-1α was used as the

internal reference and amplified. The cycling conditions for *Ihh* genes and EF1α were as follows: 1 min at 95 ◦C, followed by 40-cycles 95 ◦C for 15 s, and 60 ◦C 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 $^{\Delta\Delta}$CT (comparative threshold cycle) method ($^{\Delta}$CT = CT of target gene - CT of EF-1α, $^{\Delta\Delta}$CT = $^{\Delta}$CT of any sample - $^{\Delta}$CT of calibrator sample). The efficiencies of the primers (E) were E_{Ihh} = 0.9999.

Statistical analysis

The data are 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 the 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

BLAST analyzed the Ihh gene cDNA sequences 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). Pairwise deduced amino acids sequence identity and similarity matrices of the *Hh* family sequences from various species were performed using Matgat 2.02 (Campanella et al., 2003). Homology modeling obtained the three-dimensional structures of golden pompano Ihh (http://swissmodel.expasy.org/workspace/index.php).

Results

Cloning and sequencing of golden pompano Ihh gene cDNA

The cDNA sequence length of the golden pompano Ihh gene (GenBank accession: KY039316) was 1,484 bp with an open reading frame (ORF) of 1,314 bp, which encoded 437 amino acids (aa), with a calculated molecular weight (Mw) of 48.175kDa and a theoretical isoelectric point (pI) of 6.22 (Fig. 1). The bioinformatics analysis of the deduced polypeptide sequence revealed several significant domains or motifs (Fig. 2). The deduced amino acid of the *Ihh* gene contained a signal peptide (1-25 aa) a conserved HH-signal domain (26–186 aa), a Hint-N domain (198–331 aa) and Hint-C domain (335-379 aa, Fig. 2). The molecular modeling of the golden pompano *Ihh* is shown in Fig. 3. The golden pompano *Ihh* sequence shared 87.93% identity with human Indian hedgehog protein (PDB ID: 3k7g.1.A, Fig. 3).

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   1 CAGTCACGGACGATGGAGTCGGGAACTGAACCGGGGAGATACTGAAGACATCTCTCTCCC  60
  61 CAGGGAGAGTTCTCCCTCCCCCCCCCGGGGGCatgcggatctccttccttcttctcaccg 120
   1                                   M  R  I  S  F  L  L  L  T  A  10
 121 cctctctgtgtgccttggtcctcctcctcgcacctgcctcggagggctgcgggccgggga 180
  11  S  L  C  A  L  V  L  L  L  A  P  A  S  E  G  C  G  P  G  R  30
 181 gggggtacggcaagaggcggcccccgaagaagctcacgccgctcgcctacaagcagttca 240
  31  G  Y  G  K  R  R  P  P  K  K  L  T  P  L  A  Y  K  Q  F  S  50
 241 gccccaacgttgccgagaagaccctgggagccagcggcagacccgagggcaaaataacgc 300
  51  P  N  V  A  E  K  T  L  G  A  S  G  R  P  E  G  K  I  T  R  70
 301 gcaactccgagcgctttaaagaactgacgccgaattacaacacagacatcatcttcaaag 360
  71  N  S  E  R  F  K  E  L  T  P  N  Y  N  T  D  I  I  F  K  D  90
 361 atgaggaggacacgggcgccgacaggctcatgacccagcgttgtaaagacaagttaaact 420
  91  E  E  D  T  G  A  D  R  L  M  T  Q  R  C  K  D  K  L  N  S 110
 421 ctctggccatctctgtgatgaacatgtggccaggtgtgaagctgagagtgacagagggct 480
 111  L  A  I  S  V  M  N  M  W  P  G  V  K  L  R  V  T  E  G  W 130
 481 gggatgaggacggccatcattcagaggactcgctgcactatgagggacgtgctgtcgaca 540
 131  D  E  D  G  H  H  S  E  D  S  L  H  Y  E  G  R  A  V  D  I 150
 541 tcaccacttcagacagggacagaaataagtatgccatgttggcccggttggctgtagaag 600
 151  T  T  S  D  R  D  R  N  K  Y  A  M  L  A  R  L  A  V  E  A 170
 601 ctggatttgactgggtctactacgagtccaaagcccacattcactgtagcgtcaagtcag 660
 171  G  F  D  W  V  Y  Y  E  S  K  A  H  I  H  C  S  V  K  S  E 190
 661 aacattctgtggcagccaaaactggtggttgtttccctggcgatgctcaggttattctcg 720
 191  H  S  V  A  A  K  T  G  G  C  F  P  G  D  A  Q  V  I  L  E 210
 721 aggacggggctactaaacagatgcgtgaccttcaccctggtgaccgtgtcttagcttcct 780
 211  D  G  A  T  K  Q  M  R  D  L  H  P  G  D  R  V  L  A  S  S 230
 781 caacagcggatggccacggtcctcttctctacagcccagtcttgtccttttggaccgcc 840
 231  T  A  D  G  H  G  P  L  L  Y  S  P  V  L  S  F  L  D  R  Q 250
 841 agcccaacgtcacaaagatcttctacgtcattggcaccgacacaggacttaatattacgc 900
 251  P  N  V  T  K  I  F  Y  V  I  G  T  D  T  G  L  N  I  T  L 270
 901 tcacagcagcccacctgatctttgtcacagactgcactggtggtcagagtgagcctgggt 960
 271  T  A  A  H  L  I  F  V  T  D  C  T  G  G  Q  S  E  P  G  W 290
 961 gggaggagactattgaagagccacttttgggctctatactggggagcaggccgagctggg 1020
 291  E  E  T  I  E  E  P  L  L  G  S  I  L  G  S  R  P  S  W  E 310
1021 aagcaggtctgaggacagttttgccagcgaggtccatccaggacagtgtgtacttacgc 1080
 311  A  G  L  R  T  V  F  A  S  E  V  H  P  G  Q  C  V  L  T  P 330
1081 cgcgagggaaggtggggtcacagacgacattgtcagttgtgacttttgtggaggagcaga 1140
 331  R  G  K  V  G  S  Q  T  T  L  S  V  V  T  F  V  E  E  Q  R 350
1141 ggagcaccggactgtatgccccctcacccagcatggctctgtagtggtgaacggagtgc 1200
 351  S  T  G  L  Y  A  P  L  T  Q  H  G  S  V  V  V  N  G  V  L 370
1201 tcgcatcctgctatgctgctgtggacgatcaccatttggcccactgggtcctggccccac 1260
 371  A  S  C  Y  A  A  V  D  D  H  H  L  A  H  W  V  L  A  P  L 390
1261 tgaggttcttctacagcctgataagaccttcagaactgcagactgacgggctgcactggt 1320
 391  R  F  F  Y  S  L  I  R  P  S  E  L  Q  T  D  G  L  H  W  Y 410
1321 atccttggcttctacagaagctagggcaaatgctgctggatgctggacacttccacccct 1380
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Fig. 1 Nucleotide sequence and deduced amino acid of *Ihh* gene from golden pompano *Trachinotus ovatus* (Linnaeus 1758)

Fig. 2 The prediction of conserved domain in *Ihh* from golden pompano *Trachinotus ovatus* (Linnaeus 1758).

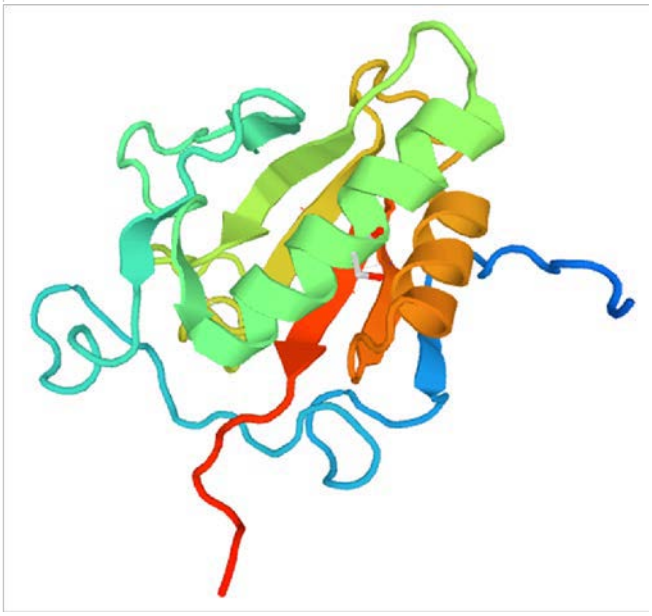

Fig. 3 Predicted tertiary structure of *Ihh* in golden pompano *Trachinotus ovatus* (Linnaeus 1758).

Multiple sequence alignments and phylogenetic analysis

Multiple sequence alignment of the deduced amino acid sequences of the *Ihh* gene with some known *Hh* family amino acid sequences from various species is shown in Table 2. The predicted amino acid sequences of the *Ihh* gene from golden pompano were similar to Lates calcarifer (93.4% and 96.1%, XP_018527527.1) and *Takifugu rubripes* (80.9% and 87%, XP_011609099.1), and different identity (39.9-65%) and similarity (58-78.5%) with other species (Table 2).

Table 2 Identity and similarity between golden pompano Ihh with other Hh family homolog.

Genes	Species	GenBank accession	Identity(%)	Similarity(%)
Ihh	*Lates calcarifer*	XP_018527527.1	93.4	96.1
	Takifugu rubripes	XP_011609099.1	80.9	87
	Larimichthys crocea	KKF30256.1	65	78.5
	Danio rerio	NP_001030165.2	65	76.4
	Homo sapiens	NP_002172.2	60.8	71.4
	Mus musculus	NP_034674.2	60.5	71.6
	Rattus norvegicus	NP_445836.1	60.5	71.6
	Xenopus laevis	NP_001079262.1	59.4	68.2
Dhh	*Danio rerio*	NP_001025286.1	44.1	61.7
	Homo sapiens	NP_066382.1	49.9	64.8
	Rattus norvegicus	NP_445819.1	49.7	65.2
	Mus musculus	NP_031883.1	49.4	65
Shh	*Amazona aestiva*	KQK85399.1	58.7	72.1
	Gallus gallus	NP_990152.1	60	71.6
	Monodelphis domestica	NP_001185482.1	55.7	68.3
	Sus scrofa	NP_001231442.1	57.1	69.7
	Xenopus laevis	NP_001081782.1	54.4	70.4
	Danio rerio	NP_571138.1	57.2	71.2
	Homo sapiens	NP_000184.1	57.2	69
	Rattus norvegicus	NP_058917.1	56.7	71.2
	Mus musculus	NP_033196.1	57.9	71.4
Hedgehog	*Drosophila melanogaster*	NP_001034065.1	39.9	58

Expression of Ihh during fish ontogeny

The phylogenetic tree of hedgehogs comprised three main clusters, contain sonic hedgehog (*Shh*) clusters, Indian hedgehog (*Ihh*) clusters and desert hedgehog (*Dhh*) clusters. Hedgehog of Drosophila melanogaster was in the root by outgroup (Fig. 4) and the deduced *Ihh* amino acid sequences of five fish species contained the signal peptide, and all of them showed high identity and similarity (Fig.5).

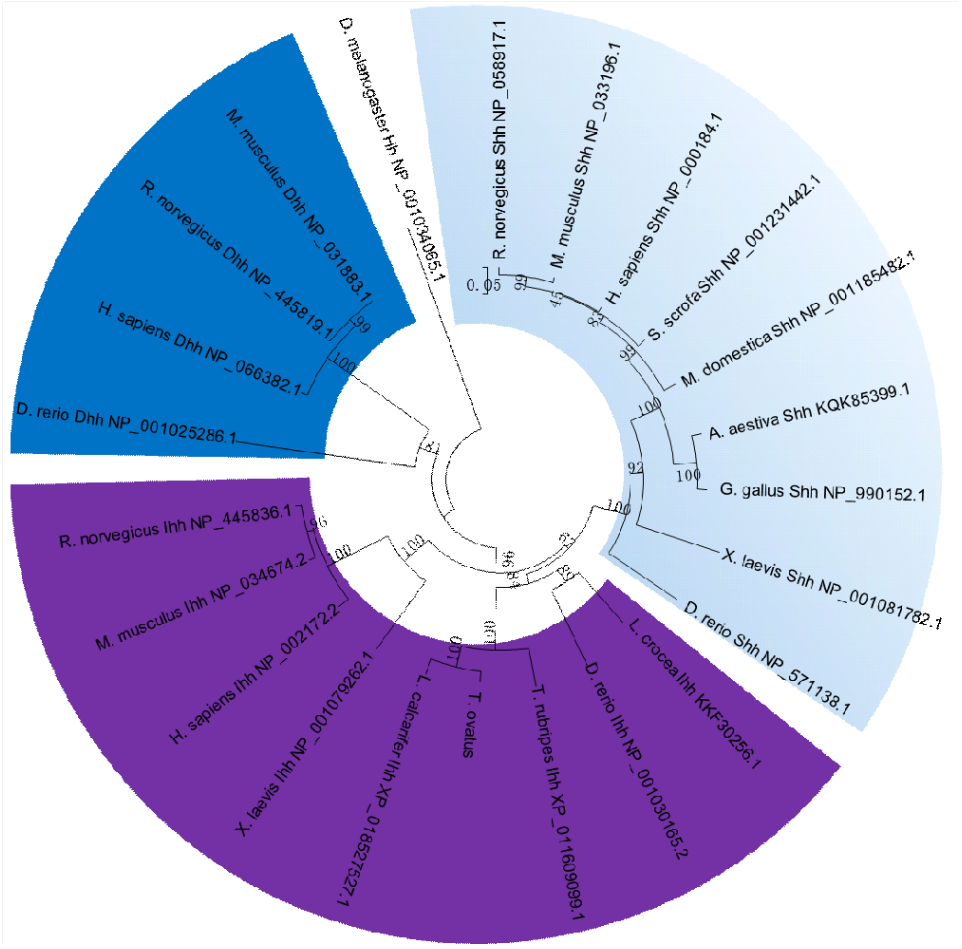

Fig. 4 Phylogenetic tree for amino acid sequences of hedgehog family members.

At hatching, the expression of *Ihh* in fish larvae was low (Fig. 6). The expression level of *Ihh* increased with fish age and reached the first peak level on 3 DPH (Fig. 6). Starting from 4 DPH, the expression levels of *Ihh* in fish larvae fluctuated and remained at a relatively high level. On 18 DPH, the expression of *Ihh* reached the second peak level.

Expression of Ihh in fish tissues

On 18 DPH, the highest expression of *Ihh* in golden pompano was observed in the intestine, followed by the stomach ($P < 0.05$, Fig. 7). The expression of *Ihh* in the liver of golden pompano was significantly lower than the expression in stomach and intestine ($P < 0.05$). The low expression levels of *Ihh* in golden pompano were observed in the brain, gills, head-kidney, spleen, and heart on 18 DPH.

Response of Ihh to water temperature

Water temperature significantly affected the expression of *Ihh* in golden pompano on both 12 and 18 DPH ($P < 0.05$, Fig. 8). On 12 DPH, the expression of Ihh in fish at 23 ◦C was significantly higher than those reared in 26 and 29 ◦C ($P < 0.05$). On 18 DPH, the expression of Ihh in fish at 23 ◦C was significantly lower than fish at 26 and 29 ◦C ($P < 0.05$). From 12 to 18 DPH, the expression of *Ihh* in fish at 26 and 29 ◦C increased significantly, while the expression of *Ihh* in fish at 23 ◦C decreased significantly ($P < 0.05$).

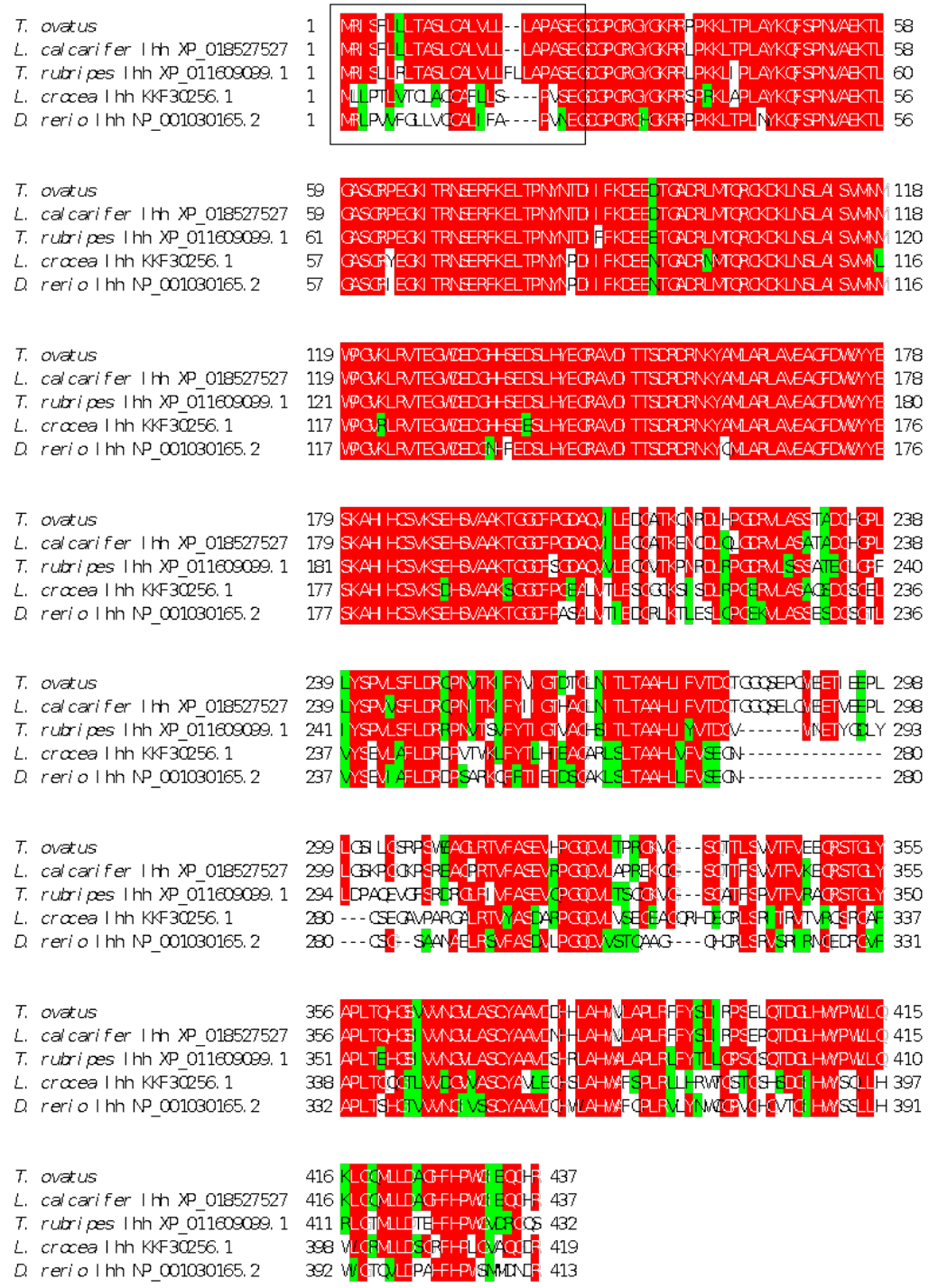

Fig. 5 Multiple sequence alignment of the deduced amino acid sequence of Ihh with other known homologous *Ihh* amino acid sequence.

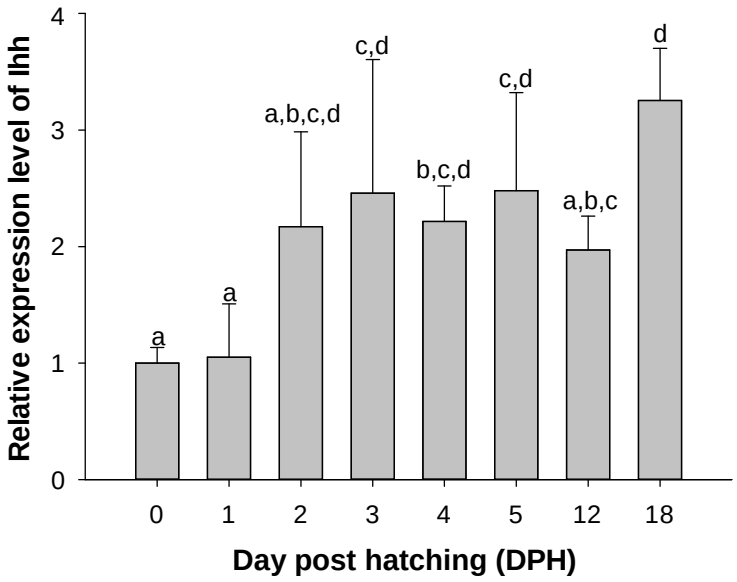

Fig. 6 Relative expression levels of *Ihh* gene during ontogenetic development of golden pompano larvae. Data with different letters were significantly different ($P < 0.05$)

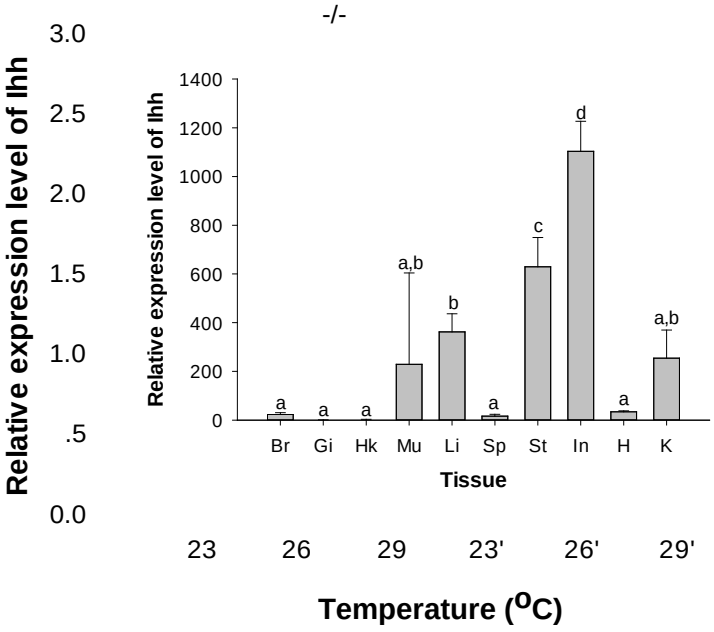

Fig. 7 Relative level of *Ihh* gene mRNA in different tissues of golden pompano *Trachinotus ovatus* larvae on 18 DPH. Data with different letters were significantly different ($P < 0.05$). Abbreviations: Br, Brain; Gi, Gill; Hk, Head-kidney; Mu, Muscle; Li, Liver; Sp, Spleen; St, Stomach; In, Intestine; H, Heart; K, Kidney.

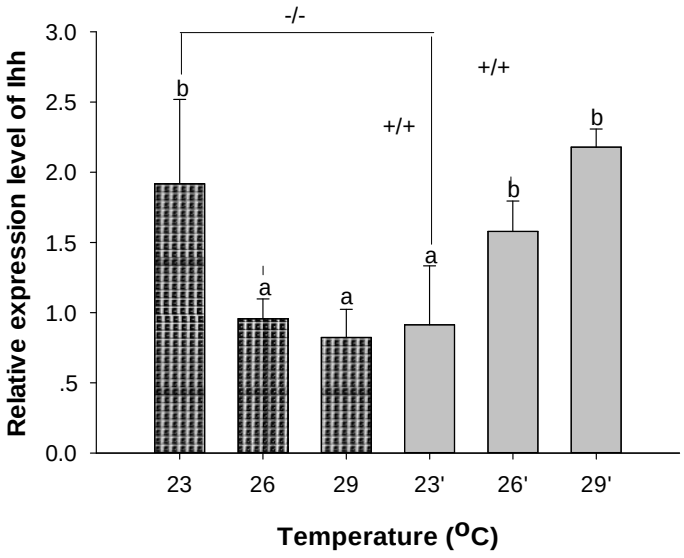

Fig. 8 Relative expression levels of *Ihh* gene of golden pompano *Trachinotus ovatus* larvae development at different temperatures on 12DPH ( ) and 18DPH ( ).Data with different letters were significantly different ($P < 0.05$)

Discussion

In this study, the *Ihh* gene was isolated and identified for the first time in golden pompano larvae. The deduced amino acid of *Ihh* gene in golden pompano contained a signal peptide (1-25 aa), a conserved HH-signal domain (26–186 aa), a Hint-N domain (198–331 aa) and Hint-C domain (335-379 aa). In bony fish, the sequence of the Ihh gene from golden pompano showed 93.4% similarity with Lates calcarifer. Similar to other species. Such unique structure in *Ihh* allows it to produce a mature signaling peptide that can be combined into lipoprotein particles or diffused freely to target cells (Eugster et al., 2007; Ingham and McMahon, 2001). These play a critical role in cell proliferation and cell differentiation in various development processes (Bitgood and McMahon, 1995; Ramalho-Santos et al., 2000; Varjosalo and Taipale, 2008).

Expression of Ihh during fish ontogeny

Previous studies have demonstrated that *Ihh* gene can participate in the regulation of the development and differentiation of many tissues (Bitgood and McMahon, 1995; Hebrok et al., 2000; Iwasaki et al., 1997; Kronmiller and Nguyen, 1996; Ramalho-Santos et al., 2000; Thomas et al., 2000; Vortkamp et al., 1996). Interruption of *Ihh* can lead to malformation of tissue and organs. For example, increased hedgehog signaling in the pancreas might disrupt organogenesis (Hebrok et al., 2000). Furthermore, loss of hedgehog signaling results in ectopic pancreas formation in chicken embryos (Cooper et al., 1998; Kim and Melton, 1998; Roberts et al., 1995). During bone formation, disruption of *Ihh* signaling regulation leads to multiple bone diseases, such as progressive osseous heteroplasia (Yang et al., 2015). The expression pattern of *Ihh* gene during embryonic development has drawn particular attention in the past studies. Bitgood and McMahon (1995) quantified *Ihh* expression in developing mouse embryos from gestational days 11.5 to 16.5. Ihh transcripts present from gestational day 9 to 14 in the mouse mandible (Kronmiller and Nguyen, 1996). In the present study, the expression level of *Ihh* increased sharply from hatching to 2 DPH, and maintained at a relatively high expression level until reaching the peak level on 18 DPH. The high level of Ihh expression during early development of larval golden pompano is consistent with the fast growth of this species (Ma et al., 2014a), indicating a fast formation of organs and tissues during this period.

Tissue expression of Ihh in golden pompano larvae

Ihh is specifically expressed in some tissues (Varjosalo and Taipale, 2008), such as primitive endoderm (Dyer et al., 2001), pancreas (Kayed et al., 2003), gut (van den Brink, 2007), and prehypertrophic chondrocytes in the growth plate of bones (St-Jacques et al., 1999; Vortkamp et al., 1996). Mutation of *Ihh* in mice can lead to 50% mortality rate during early embryogenesis, and surviving embryos exhibit cortical bone defects and aberrant chondrocyte development in the long bones (Colnot et al., 2005; St-Jacques et al., 1999). In humans, mutations of *Ihh* can cause *Ihh* knock-out pitofemoral dysplasia, a congenital condition characterized by bone defects and short stature (Hellemans et al., 2003). The tissue expression of *Ihh* is species dependent and varies among development times and locations. For instance, *Ihh* is expressed mainly in early hypertrophic chondrocytes in the human growth plate (Kindblom et al., 2002). While during the embryonic development of mouse, *Ihh* shows two main sites of expression: gut endoderm and cartilage (Bitgood and McMahon, 1995). Up to date information on the tissue expression pattern of *Ihh* in fish is rare. The present study was the first time reporting the tissue expression of *Ihh* in golden pompano larvae. The highest expression of *Ihh* in golden pompano larvae was observed in the intestine, and followed by stomach on 18 DPH. Higher expression in intestine and stomach of fish was similar to the results observed in mouse (Bitgood and McMahon, 1995).

Response of Ihh to water temperature

The critical function of *Hh* signaling in bone formation was identified. In the early stages of embryonic limb development, *Shh* acts as a major morphogen in patterning the limb buds, while *Ihh* has an essential function in endochondral ossification and induces osteoblast differentiation in the perichondrium (Yang et al., 2015). As primary signaling molecules, *Ihh* along with hormone-related peptide (PTHrP) plays important roles during

cell differentiation and skeletal tissue ontogeny (Hogan, 1996; Karp et al., 2000; Yamaguchi et al., 2000). *Ihh* produced by prehypertrophic and hypertrophic chondrocytes stimulates the production of PTHrP through perichondrial and early chondrocyte cells. PTHrP then maintains chondrocytes in a proliferative and less differentiated state. Once unusually regulation occurs in *Ihh*, the feedback loop that determines the pace of differentiation of chondrocytes during endochondral ossification is interrupted, and may inhibit osteoblasts from propagation and differentiation to form cartilaginous craniofacial parts of the skeleton (Haga et al., 2003). As a repressor of terminal hypertrophic differentiation (Colnot et al., 2005), *Ihh* was found to be highly up-regulated when skeleton malformation occurs in hyperthermic Atlantic salmon (Ytteborg et al., 2010). In the present study, the expression of *Ihh* in fish reared at 26 and 29 ◦C significantly increased as compared to those reared at 23 ◦C. Such up-regulated expression of *Ihh* was consistent with the increasing of malformation of golden pompano larvae reared at 26 and 29 ◦C (Yang et al., 2016). This may suggest that the expression of *Ihh* during fish ontogeny could be used as an indicator for skeleton malformation.

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

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