

Teleost Type 2 Interleukin-1 Receptor (IL-1R2) from the Spotted Halibut (*Verasper variegatus*): 3D Structure and a Role in Immune Response¹

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Abstract—The type 2 interleukin-1 receptor (IL-1R2) is one of natural IL-1 β signaling inhibitors in mammals. We cloned and sequenced the *IL-1R2* gene in *V. variegatus* (*VvIL-1R2*). The phylogenetic analysis showed that the molecular structure *VvIL-1R2* is similar to that of its orthologues in other vertebrates. The expression levels of *VvIL-1R2* are relatively high in the peripheral blood leukocytes (PBLs), gill, and spleen. In addition, peculiar expression patterns for this molecule were detected at various developmental stages, implying that in flatfishes the *IL-1R2* may have been important for embryonic development and metamorphosis. In PBLs, the treatment with pathogen-associated molecular patterns (PAMPs) induced a significant and rapid up-regulation of *VvIL-1R2*, pointing at its involvement in the immune responses against bacterial and viral pathogens.

Keywords: *IL-1R2*, *Verasper variegatus*, metamorphosis, 3D modeling, PAMPs

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INTRODUCTION

Interleukin-1 β (IL-1 β), which is a member of the interleukin-1 cytokine family [1], plays a pivotal role in inflammatory responses and some related diseases. All of the known biological activities of IL-1 β are mediated by its type 1 receptor (IL-1R1) [2]. In course of inflammation, IL-1 β induces the expression of a large number of proinflammatory genes and proteins, therefore, enhancing inflammatory responses further. These genes and proteins include IL-1 β itself, IL-6, IL-8, monocyte chemoattractant protein 1, and cyclooxygenase 2, which are usually not expressed in healthy body [3]. Due to associated damage to the tissue, excessive inflammatory cytokine production is harmful to the host [1, 2]. Accordingly, overexpression of IL-1 β induces several natural IL-1 β signaling inhibitors, in particular IL-1 receptor antagonist (IL-1Ra) and IL-1 receptor type 2 (IL-1R2) [4]. In mammals, IL-1Ra competes for IL-1R1 binding with IL-1 β . Moreover, the recombinant human IL-1Ra analog, anakinra, is utilized to block the effect of IL-1 β in patients [4]. IL-1R2, which lacks the Toll/interleukin-1 receptor (TIR) domain and, therefore, does not transduce signals,

also competes with IL-1R1, thus serving as a decoy receptor for IL-1 β [5].

IL-1R1 is expressed at low levels in nearly all cells [6, 7], whereas expression of IL-1R2 is predominant in neutrophils, B cells, and monocytes [8], pointing at the role of IL-1R2 as a naturally occurring inhibitor of IL-1 β activity in mammals.

Among teleosts, IL-1R2 cDNA was first isolated from *Oncorhynchus mykiss*, where its inducible expression by LPS and TNF- α has been shown [9]. Subsequently, IL-1R2 sequences have been identified in *Sparus aurata* [10], *Paralichthys olivaceus* [11], *Salmo salar* [12] and *Ctenopharyngodon idella* [13]. In both *S. aurata* and *P. olivaceus*, a bacterial challenge markedly stimulates the IL-1R2 mRNA expression in immune-related tissues [10, 11], which also suggests the involvement of IL-1R2 in inflammatory responses. Importantly, the putative proteins of fish IL-1R2 also lack an intracellular TIR domain, implying the decoy function of fish IL-1R2.

Due to its high market value, *Verasper variegatus* has been widely recognized as a promising candidate for aquaculture and fishery enhancement in Asia. However, spontaneous maturation and ovulation of its broodfish have not yet been achieved in captivity [14].

¹ The text was submitted by the author(s) in English.

Table 1. Primers used in this study

Primers	Sequence (5'–3')	T_m , °C	Usage
IL-1R2-Fw	GAAAGAYGGCTGCTWMCTGG	57.4	Degenerate PCR
IL-1R2-Rv	HGCVKKTGACATYCGGTGA	57.8	Degenerate PCR
IL-1R2-5'Rv1	CCAGTGATACAGTAGGTGTCATTCC	59.7	5'RACE1
IL-1R2-5'Rv2	TCTGTGGGTCTGGTTCTTGC	58	5'RACE2
IL-1R2-3'Fw1	AGTACAAAGTCAGCAGGGTTCG	57	3'RACE1
IL-1R2-3'Fw2	GATTGAGTTGAGGCTGGTTGT	56.6	3'RACE2
IL-1R2-RT-Fw	TCATGGCGATAACGGAAGAAG	60	qRT-PCR
IL-1R2-RT-Rv	TGGGTTTGAAGAGGACGTAAG	60	qRT-PCR

Moreover, *V. variegatus*, which is an endangered flounder fish species, is susceptible to bacterial and virus infections. To improve the survival rate of *V. variegatus* in aquaculture stations, the function of immunity genes in this fish should be understood. For a better understanding of the function of *VvIL-1R2*, a full-length cDNA of this gene was reverse transcribed from the mRNA pool of the spleen and cloned, its expression levels were profiled in different adult tissues and at different developmental stages, including metamorphosis. In PBLs of *V. variegatus*, stimulated by pathogen-associated molecular patterns (PAMPs), the immune challenge responses were recorded.

EXPERIMENTAL

Collection of fish embryos and adult tissues. Fish samples of *V. variegatus* were obtained from a commercial hatchery in Weihai, Shandong Province, China. Adult tissues (heart, liver, spleen, kidney, brain, gill, muscle, and intestine) and PBLs were collected from five 1-year-old samples. The adult tissue and PBL samples were snap frozen in liquid nitrogen and then stored at -80°C until further use. Each of these samples was collected in triplicate. Embryos were collected from the same farm. Fertilized eggs were obtained by artificial fertilization and incubated at $11 \pm 1^{\circ}\text{C}$ in sterile seawater with an open recirculation water system and sufficient air supply. The different embryonic stages were observed under a stereomicroscope. Three pools of samples at different embryonic stages (i.e., unfertilized egg, 1-cell, 4-cell, morula, blastula, gastrula, neurula, somite, tail bud, and hatching stages) were separately collected from mixed families with a nylon net (100 mesh). The embryos were immersed in 1.5 mL of RNAlater liquid (Solarbio, Shanghai, China) overnight at 4°C and then stored at -80°C until further use.

RNA extraction and first-strand cDNA synthesis of *VvIL-1R2*. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The extracted total RNA was treated with RNase-free DNase I (TaKaRa, Dalian, China) to remove DNA

contamination and then frozen at -80°C . Reverse transcription and cDNA synthesis were performed with 1 μg of total RNA and random hexamer primers using a reverse transcriptase M-MLV kit (TaKaRa) in accordance with the manufacturer's protocol. The quality and quantity of the total RNA were evaluated by 1.5% agarose gel electrophoresis and spectrophotometry with the NanoPhotometer Pearl.

Molecular cloning and sequence analysis of *VvIL-1R2*.

No genome information is available for *V. variegatus*. Thus, a pair of degenerate primers (Table 1) was designed based on the conserved sequences of *IL-1R2* in other teleosts to find the conserved region of *VvIL-1R2*. PCR amplification was performed with an initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. The 5'- and 3'-rapid amplification of cDNA ends (RACE) were performed using the SMART RACE cDNA Amplification Kit (Clontech, CA, USA), in accordance with the manufacturer's protocol, to isolate the full-length cDNA of *IL-1R2* from the *V. variegatus* spleen samples. Gene-specific primers (GSPs) were designed based on the known cDNA sequence. The GSPs for the nested PCR assay were 5'RACE1 and 5'RACE2 for the 5'-RACE (Table 1) and 3'RACE1 and 3'RACE2 for the 3'-RACE (Table 1). The PCR assay was conducted following the SMART RACE amplification method. The PCR products were separated by 1.5% agarose gel electrophoresis, purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, CA, USA), cloned into the pMD-18T vector (TaKaRa), and then sequenced. The CDS region and open reading frame (ORF) were predicted by the ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>).

Quantitative real-time PCR of *VvIL-1R2* in different tissues and at different developmental stages. A specific primer pair (IL-1R2-RT-FW/RV, Table 1) was designed based on the characteristics of *IL-1R2*. Pre-experiments were conducted to confirm the generation of single cDNA PCR products. The evaluation of the housekeeping genes of *V. variegatus* showed that *β -actin* and *18S rRNA* were the two most stable reference gene in different tissues and at different develop-

mental stages. Thus, the relative expression of *VvIL-1R2* was determined using both β -actin and *18S rRNA* as the reference genes.

Total RNAs were extracted from different adult tissues and developmental stages, and cDNA synthesis was performed. Three biological replicates of each sample, running in triplicate, were analyzed. Quantitative real-time PCR (qRT-PCR) was performed in a 20 μ L solution containing 10 ng of template cDNA and SYBR Premix Ex Taq II (TaKaRa) by using LightCycler 480 at 95°C for 5 min pre-incubation, followed by 45 cycles of 95°C for 15 s and 60°C for 45 s. Finally, the melting curve was analyzed to detect single amplification. Fluorescent signal accumulation was recorded at the 60°C 45 s phase during each cycle under the control of LightCycler 480 Software 1.5. The relative quantities of the target *VvIL-1R2* expressed as fold variation over both β -actin and *18S rRNA* were calculated using the $2^{-\Delta\Delta C_t}$ comparative Ct method.

Sequence alignment and phylogenetic tree reconstruction. Homologous nucleotide and protein sequences were confirmed through a BLAST search against the NCBI and Ensembl databases. Multiple sequence alignments were conducted using ClustalX 2.1 and DNAMAN 7.0. A phylogenetic tree was constructed using MrBayes 3.2.3.

PAMPs-induced *VvIL-1R2* expression in PBLs. Blood was collected from the caudal veins of the *V. variegatus* samples, and the PBLs were prepared with Percoll as previously reported [15]. *V. variegatus* PBLs were cultured at 24°C in a 24-well plate (Thermal Scientific, 3.6×10^4 cells/well) overnight and then stimulated with 50 μ g/mL lipopolysaccharide (LPS), 50 μ g/mL polyinosinic: polycytidylic acid (poly(I:C), Sigma), and PBS (control). The samples were obtained after 0, 0.5, 1, 2, 4, 6, 12, and 24 h of PAMPs administration. RNA isolation and cDNA synthesis were performed as described previously. The changes in *VvIL-1R2* expression in response to LPS, poly(I:C), and PBS challenge were determined by qRT-PCR, with both β -actin and *18S rRNA* as reference genes.

Protein structure analysis of *VvIL-1R2*. Pfam and CCD databases were used for searching the motifs of *VvIL-1R2* and other vertebrate *IL-1R2* sequences. The protein secondary and 3D structure of *VvIL-1R2* were predicted using online prediction bioinformatics tools, such as Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) and PDBsum Generate (<http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html>). Protein docking simulation was conducted with the ZDOCK server (<http://zdock.umassmed.edu/>).

Statistical analysis. qRT-PCR data were statistically analyzed using a one-way ANOVA followed by an LSD test using SPSS 20.0 (IBM, New York, USA). A difference of $P < 0.05$ between groups is considered statistically significant. The data are expressed as mean \pm SD ($n = 3$).

RESULTS AND DISCUSSION

VvIL-1R2 Is Highly Conserved among Vertebrates

Given that no genome sequencing information for *V. variegatus* is available, we started with designing a pair of degenerate primers (Table 1) to the conserved region of *VvIL-1R2* after aligning the sequences of *IL-1R2* in other vertebrates. The 5'- and 3'-regions of the cDNA sequence of *VvIL-1R2* were cloned using the RACE-PCR. Entire sequence of *VvIL-1R2* cDNA was obtained using SeqMan by assembling all the cloned sequences (NCBI accession no. KY038172). The complete mRNA sequence of *VvIL-1R2* consists of 1852 bp nucleotides with a 106 bp 5'-untranslated region (UTR), a 471 bp 3'-UTR including a poly(A) tail, and a 1275 bp ORF (Fig. 1). *VvIL-1R2* has a complete polyadenylation (AATAAA) signal and TA-rich motifs (ATTTA) in the 3'-UTR (Fig. 1) [10, 11]. A previous research reported that the consensus sequence ATTTA is present in the 3'-UTR of both mouse and human IL-1 mRNAs, as well as the mRNAs encoding human and mouse TNF, human CSF, human lymphotoxin, human and rat fibronectin, and a majority of the sequenced human and mouse IFNs [16]. All these mRNAs lack homology to the IL-1R mRNAs in the coding region [16]. TA-rich motifs are particularly prevalent among mRNAs encoding proteins related to inflammatory responses [16]. The ATTTA instability motif is a characteristic feature of inflammatory mediator genes [17], and its presence suggests the transient expression of an inflammatory mediator for *VvIL-1R2*.

The ORF of *VvIL-1R2* was predicted to encode a 424 amino acid residues long protein with a molecular weight of 47.38 kDa and a theoretical isoelectric point of 5.78. As in other vertebrates, a putative single transmembrane region with 23 amino acid residues (residue numbers 382–405, Fig. 1) is predicted at the C-terminus by using Singer's classification for membrane topology [18]. The transmembrane region separates the extracellular domain of 364 amino acids from the short intracellular domain of 19 amino acids. The predicted protein domains reveal a potential signal peptide, which comprises 18 amino acid residues at the N-terminus, a splicing site between amino acid residues 18 and 19 (VCG-KP), and a mature peptide of 406 amino acid residues (Fig. 1). The sequence identities of *VvIL-1R2* with the *IL-1R2* of *P. olivaceus*, *Oreochromis niloticus*, *Cynoglossus semilaevis* and *Homo sapiens* are 91, 80, 74, and 46%, respectively. These results imply that the *IL-1R2* protein may be highly conserved among vertebrates.

Domain Structure of *VvIL-1R2* Protein Is Consistent with that of the *IL-1R2* Other Teleosts

The alignment of *VvIL-1R2* with other vertebrate *IL-1R2*s shows that the translated *VvIL-1R2* sequence possesses signature features of the *IL-1R* family of fish and higher vertebrate *IL-1R*s. These fea-

1 acatggggaggagacgaccacatgctgctccactccacactctcatcacatctgcctgacctcacactgccggggccgctcactgtacaccatTTTTgtgctcat
106 cATGTCCTCTGGGCTCTGATGTTTGTGTGGTCATCGTTGAATATGTGTGTGAAAACCTTGGTTACCACCTCTGACCATGAAAGACGGCTGCTACCTGGTGAC
1 M V L W A L M F A V V I V E Y V C G K P W L P P L T M K D G C Y L V T
211 TCCAGAGGTGGAGATATTCAGTGTGGAGGTGAAGGTGTATCATCTCCTTCCCGGTGTTAAGAGCGTCCTCAGATTCTAAACATCGCCCCCAACAGCAAG
36 P E V E I F S V E G E A V I I S F P V F K S V L R F L N I A P P T A R
316 GTTCCTCATACCAAGAACAATGAGACAGGGGCGTGGCCGATGAGAGCGACAGGCGCTCCAGCAGCGTGAGAAGCAGCTGTGGCTCTCCAGCTCAGGCTTC
71 F L I T K N N E T G G V A D E S D R R V Q Q R E K Q L W L L P A Q A S
421 AGACTCAGGGGAATATATCTGCACTTACAGGAATGACACCTACTGTATCACTGGGAATATCATGCTAGAAGTGTTCGAGTCCAGCTCTGTGGATATGGAGAAGT
106 D S G E Y I C T Y R N D T Y C I T G N I M L E V F E S S S V D M E K V
526 GTCTTATCCAATCTCAGCCATGGTGGGAGAGAGGCTGACGTACAGATGCCCTTCGTGAGTGACTCAACAGTACAGACAGACTGATAGAGTGGTACAAGGAAAA
141 S Y P I S A M V G E R L T Y R C P S L S D F N S T D R L I E W Y K E N
631 CAGCTCCACTGCTTTTCAGTCAGGCAGGGTGGGCTCCGTCGCCGGGACAGTGTAGCTGATGATCCCTGCAGTGAGCTGCTCGCATGCAGGCATGTACACCTG
176 S S T A F Q S G R V G S V R R D S A S L M I P A V S C S H A G M Y T C
736 TCAGCTCAGAGTGTCTATCGACCAGCAGCAGTACAAAGTCAGCAGGGTGTCTGCTCCACGTGCAAGAACCAGACCCACAGATCAGTACCCTGCACCTGATGT
211 Q L R V L I D Q Q Q Y K V S R V V L L H V Q E P D P Q I S T T A P D V
841 CTCAGTGACCTCTGACCTGAGAGAAAACAGCAGCAGCAGCTACAGCACTGATCAAGCTTACCACCTGTGATTGTTTCGCCGTTGAACGGGAGTATTTTTGAAAG
246 S V T S D P E R N S S S S Y S T D Q A S P P V I V S P L N G S I F E S
946 TCCACATGGTTCAGGACTGGAGCTGTCTGCAAAAGTCTTACCAGATGTACCAGGCAGATTCCACTGAGGTCAAATGGCTCGTCAACGGCCAATCAGTGGAGTC
281 P H G S G L E L F C K V L T R C H Q A D S T E V K W L V N G Q S V E S
1051 ATCGTACCTGGAAGGGCGGCACTGCAGGGGGAAGAAGGGTAACCAAGGTGACAGAAGGCTGCCAGATTGAGTTGAGGCTGGTTGTCATGGCGATAACGGAAGA
316 S Y L E G R A L Q G G R R V T K V T E G C Q I E L R L V V M A I T E E
1156 AGATGTGAAGACGGAGCTGAAGTGTGTCACTCAGAACCAAGCAGGAAGACAGGAAGTAGTGGCCAGCTTCGTCTGGAGAACTCCACATTCACATGGCTGGTGGT
351 D V K T E L K C V T Q N Q A G R Q E V V A Q L R L E N S T F T W L V V
1261 CGCTGCAGTGGCTGTGCTCTGCTTCTCAGTGTGGTTTCTGTTTCTTTACGCTCTTCAAACCCAAAAGGAAAAGGAACTGGATTACTTTCTGGCTCGACA
386 A A V A V S C F L S V V S V F L Y V L F K P K R K R K L D Y F L A R Q
1366 GAACAGCACCTTCAAActccacaacacctctttgtatgctacatagaataaatctgcatgcatggggtgcatgtgatgttatgcatTTTTTgaccaactctc
421 N S T F *
1471 tttcttacagggatttaatgTTTTtaaatcaggagctttattcagtgacagttacaagctgtctccatttcgccatctcatctgcgcaaggacttagagacac
1576 tttatgttctcatgtggcaggtacatgcaatttggttgcgtgcaagttttctatcatgcatctattctgatataatggtaaattctgtgagggtgcaacatct
1681 gcatgtacatgtaaacgacgcagaagctaaagtactttatttgtttatttggtagtttaaacctccgctcaggaatacacactcaggggcaactttgttagaca
1786 ctctcacagactgtaataaaagatagacaatgtgtctctaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Fig. 1. The mRNA structure of *VvIL-1R2*. Small letters stand for the UTR regions. Capital letters is used to indicate the CDS region. The predicted protein sequence is shown by single letter code of amino acids below the CDS region. Start and stop codons are in the frame and the stop codon is marked with the *. One instability motif (ATTTA) is underlined, two Ig-like domains are represented with wavy line, the transmembrane domain is noted with grey highlighting and polyadenylation signal (AATAAA) is represented with double line. The N-terminus signal peptide is marked with grey capital letters.

tures include two Ig-like domains in its extracellular region, one N-terminus signal peptide, one transmembrane domain, one short cytoplasmic tail of seventeen amino acids, four conserved proline residue sites, and six conserved cysteine residues [11]. The two Ig-like domains of *VvIL-1R2* are observed in the extracellular region, but they lack an intracellular signaling Toll-interleukin-1 receptor (TIR) domain. The same set of features has been observed in *S. salar*, *P. olivaceus*, *S. aurata* and *O. mykiss* [9–12]. Notably, human and mouse IL-1R2s also lack an intracellular signaling TIR domain, but they have three Ig-like domains in the extracellular region. Unlike the IL-1R2s of the compared species, the IL-1R1 of humans has a long intracellular signaling TIR domain and three Ig-like domains in the extracellular region. Thus, further studies should be conducted to confirm whether all

teleost IL-1R2s possess two Ig-like domains. Moreover, in an extramembrane region, six cysteine residues are conserved at positions 112, 120, 156, 210, 290, and 358 in all of the species compared. However, among the six conserved cysteine residues, the last four residues are the backbone residues that form the interchain disulfide bonds of the Ig-like (C-x*-C) domains, and both domains form stable antiparallel β hairpin structures with disulfide bonds [19]. In addition, four proline residues at positions 101, 157, 267, and 272 are conserved in all the species examined.

Few studies have reported on the biological functions of IL-1R2. As IL-1R2 lacks the TIR domain, which is present in IL-1R1 and involved in signal transduction, it is believed to act as a decoy receptor for IL-1R1 [5]. However, the role of IL-1R2 in signal transduction cannot be discounted. A previous study

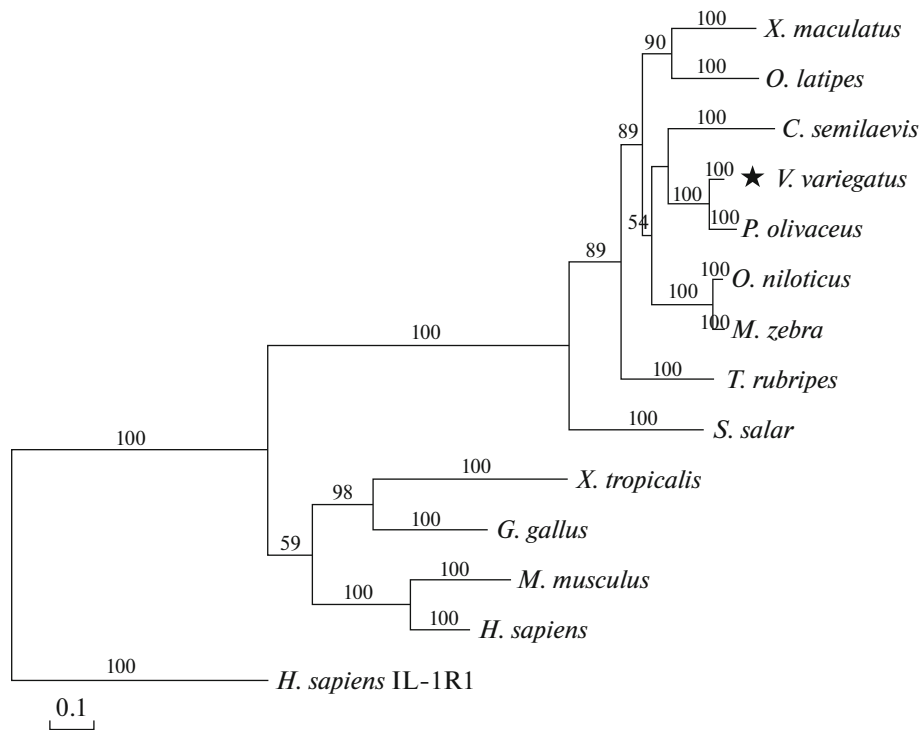


Fig. 2. The protein sequence phylogenetic tree of IL-1Rs. Phylogenetic tree showing the relationship between *V. variegatus* IL-1R2 and other vertebrates proteins. A phylogram was constructed using MyBayes (mcmc = 200000 generations, samplefreq = 10). The IL-1R1 sequence of *H. sapiens* served as the out-group. The species and GenBank accession numbers were as follows: *H. sapiens* IL-1R2 (NP_004624.1); *M. musculus* IL-1R2 (NP_034685.1); *G. gallus* IL-1R2 (XP_416914.3); *X. tropicalis* IL-1R2 (NP_001015713.1); *P. olivaceus* IL-1R2 (ABP99035.1); *C. semilaevis* IL-1R2 (XP_008326152.1); *O. latipes* IL-1R2 (XP_011487540.1); *S. salar* IL-1R2 (NP_001138892.1); *X. maculatus* IL-1R2 (XP_014329400.1); *T. rubripes* IL-1R2 (XP_003962211.1); *O. niloticus* IL-1R2 (XP_013132819.1); *M. zebra* IL-1R2 (XP_004569045.1); *H. sapiens* IL-1R1 (NP_000868.1). The Ig-like domains and other domains of IL-1Rs were showed by the CDD server [27–30].

showed that a monoclonal antibody against IL-1R2 could inhibit the thermogenic and pyrogenic responses to IL-1 β released within the *Rattus norvegicus* brain [20]. The same antibody could weaken the IL-1 β -induced prostaglandin E2 released from *R. norvegicus* hypothalamic explant [21]. These results suggested that IL-1R2 participated in the signal transduction through IL-1 β in the brain and it was able to regulate the process. Moreover, the function of IL-1R2 in signal transduction is also confirmed in these studies [22, 23]. The mechanism for IL-1 β signal transduction mediated by IL-1R2 may be similar to that proposed for IL-1R1. This mechanism may be accomplished by the binding of IL-1R2 to other transmembrane proteins [24]. The present study found that VvIL-1R2 possesses four conserved proline residues that are related to the signal transduction in IL-1R1. The signal-transducing capability of IL-1R1 significantly decreases when these conserved proline residues are mutated [25, 26]. We predict that the IL-1R2s in fishes probably have a signal-transducing function; however, further studies are required to confirm this hypothesis.

Analysis of MrBayes phylogenetic tree shows that IL-1R2 proteins of the vertebrates cluster into one group, which is separate from the *H. sapiens* IL-1R1

out-group (Fig. 2). This indicates that the ancestor gene of *IL-1R* has undergone gene duplication, leading to the formation of *IL-1R1* and *IL-1R2* before the species formation. The *IL-1R2* encoding genes of the fishes cluster as a group, and the IL-1R2 of *V. variegatus*, *C. semilaevis*, and *P. olivaceus* cluster into/group. This result implies that these flounder fishes have a close evolutionary distance from one another.

VvIL-1R2 Expression Exhibits a Significant Up-regulation in the PBLs, Gill, and Spleen and at the Hatching Stage

IL-1R2 expression can be regulated at the transcription level [31]; thus, we investigated the expression level of *VvIL-1R2* in different tissues and at different embryonic developmental stages. The expression level was analyzed by qRT-PCR. The gene expression was normalized to the housekeeping genes, namely, *18S rRNA* and β -actin. Consistent with the tissue distribution results for *P. olivaceus*, *C. idella*, and *S. aurata* [10, 11, 13], *VvIL-1R2* is ubiquitously expressed in all the tested tissues (Fig. 3). The highest expression level is observed in the PBLs, followed by those in the gill and spleen. The other tissues, such as heart, liver, kidney,

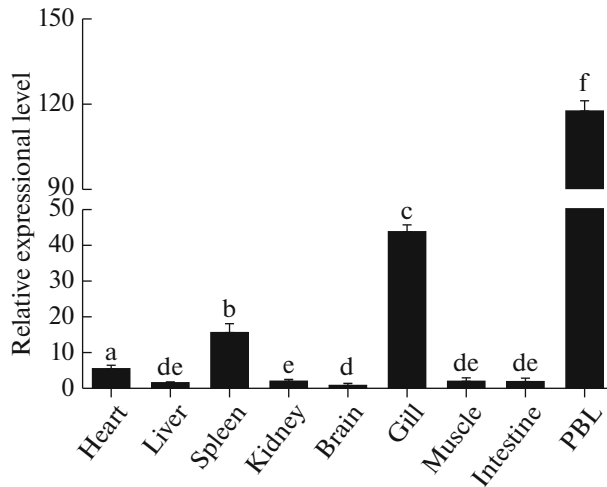


Fig. 3. Quantitative analysis of *VvIL-1R2* expression in adult tissues. The relative expression variance is presented as a ratio (the amounts of *V. variegatus IL-1R2* were normalized to the corresponding *18S rRNA* and *β-actin* genes values). The data are shown as mean ± SD ($n = 3$). Columns with different letters show a significant difference ($p < 0.05$).

muscle, and intestine shows moderate expression levels of *VvIL-1R2* transcripts. This result is similar to the phenomenon in *C. idella* and *O. mykiss* [9, 13]. The presence of the *VvIL-1R2* transcript in the brain is of interest, given that a monoclonal antibody against IL-1R2 can inhibit the thermogenic and pyrogenic responses to IL-1 β released within the *R. norvegicus* brain [20]. However, our result shows that, among the tested tissues, the brain exhibited the lowest expression level of *VvIL-1R2*. Our data indicate that *VvIL-1R2* may be one of the very important cytokines to maintain the immunological functions of the important fish immune organs, i.e., spleen, gill, and the PBLs.

During embryonic development, fish embryos are usually protected by the egg envelope before they are hatched and exposed to the environment after hatching. Currently, our knowledge is rather limited regarding the defense mechanisms of fish against pathogenic attacks in their hostile environment at the earliest stages of their lives. Thus, we investigated how the expression pattern of *VvIL-1R2* changes across the different embryonic stages. mRNA level was detected in the unfertilized eggs of *V. variegatus*, and it gradually decreased to an undetectable level until the blastula stage (Fig. 4). This phenomenon indicates that *VvIL-1R2* is a maternally expressed gene. According to the literature, IL-1 β may have several roles during development [32, 33]. As a receptor of IL-1 β , IL-1R2 also plays an important role in cell proliferation and apoptosis [34–36]. After the blastula stage, the embryonic cells are induced to differentiate. So we hypothesized that IL-1R2 might play a role in the differentiation of

embryonic cells. The expression level maintains a relatively low level before the hatching stage. Interestingly, the highest expression level of *VvIL-1R2* was observed at the hatching stage among the tested stages (Fig. 4). The embryos and hatchlings of most fishes are exposed to their aquatic environment, which is filled with thousands of various microorganisms, including potential pathogens, before their immune systems have fully developed [37]. Furthermore, at the hatching stage, embryos lose the protection of the egg envelope; consequently, they are more vulnerable to pathogens in their external environment. Therefore, the high expression of *VvIL-1R2* in this period implies that this gene may have a role in combating the pathogenic factors in the environment. *VvIL-1R2* has not been identified in the eggs of other fishes; thus, its role at the early developmental stages remains largely unclear. In the current study, we demonstrated that *VvIL-1R2* is a maternal factor stored in the eggs of *V. variegatus*. Furthermore, its mRNA level gradually increases and peaks at the hatching stage. These results are similar to those for fish-egg lectin (FEL), which is an important immunity gene in fishes, in zebra fish and rock bream [37, 38].

The presented results show that the expression of *VvIL-1R2*, which may act as an inflammatory factor, is tissue and stage specific.

VvIL-1R2 Shows the Highest Expression at the Mid-Metamorphosis Stage

As the transition from larva to juvenile, metamorphosis is a crucial developmental phase in fish. Pleuronectiformes is an interesting group of teleosts for the study of metamorphosis, as members of this order undergo a dramatic morphological reorganization during this stage and change from a symmetrical larva to an asymmetrical juvenile. *V. variegatus* has evolutionary relatives with *P. olivaceus* [14, 39, 40], and they are all flatfish that undergo the metamorphic stage. We are interested in whether the expression patterns of *VvIL-1R2* are associated with T3, T4, and TR at this developmental stage. The *VvIL-1R2* expression gradually decreased during metamorphosis, then it increased sharply and transiently in mid-metamorphosis stage followed by another decline in late-metamorphosis stage (Fig. 5). *VvIL-1R2* expression was predominantly detected in mid-metamorphosis, which is the most violent period of metamorphosis (Fig. 5).

Metamorphosis arises from a series of regulated processes that involve cellular differentiation, apoptosis, biochemical, molecular, and physiological changes [41]. Thyroid hormones (T3 and T4) and thyroid hormone receptor (TR) have been reported to drive flatfish metamorphosis, and they are associated with apoptosis and cellular differentiation [41–49]. Similarly, IL-1R2 also plays an important role in the cell proliferation, and apoptosis [34–36]. More importantly, IL-1R2 has an interaction with T3, T4, and TR [50–52].

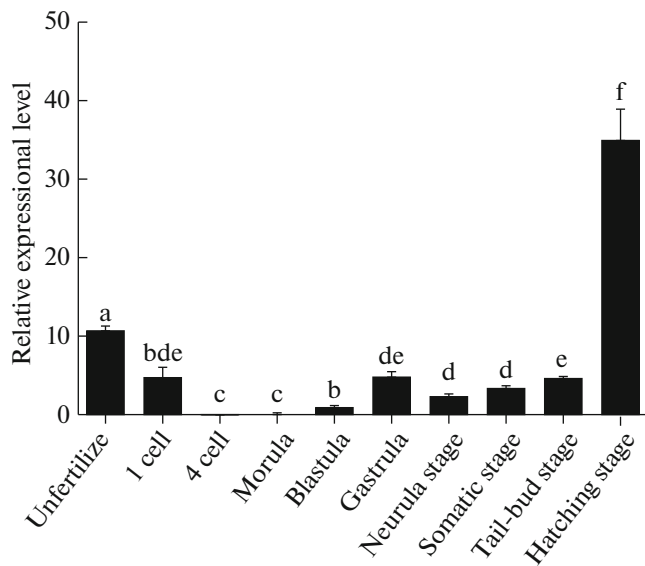


Fig. 4. Quantitative analysis of *VvIL-1R2* expression at different embryonic developmental stages. The relative expression variance is presented as a ratio (the amounts of *V. variegatus IL-1R2* were normalized to the corresponding *18S rRNA* and β -actin genes values). The data are shown as mean \pm SD ($n = 3$). Columns with different letters show a significant difference ($p < 0.05$).

Combined with the results of previous studies, the current results show that *VvIL-1R2* has a basically consistent expression pattern compared with T3, T4, and TR in *Scophthalmus maximus* and *P. olivaceus* at the metamorphic stage [43, 44]. Furthermore, our results strongly imply that *VvIL-1R2* may be involved in a series of regulated processes during flatfish metamorphosis. To the best of our knowledge, this current study is the first to show the relationship among the *IL-1R2* gene, thyroid hormones, and metamorphosis in the development of pleuronectiform fishes. These results contribute to the further understanding of the molecular mechanism of flatfish metamorphosis.

Poly(I:C) and LPS Induce Significant and Rapid Up-Regulation of VvIL-1R2

IL-1R2 is an immunity gene that is involved in the immune defense against pathogens. Evidence has shown that mammalian IL-1 β is a critical cytokine for the antibacterial immune response induced by various bacterial pathogens, including *Listeria monocytogenes*, *Mycobacterium bovis*, and *Mycobacterium tuberculosis* [53–55]. This gene is known to be released from macrophages, monocytes, neutrophils, NK cells, and T-cells [56]. Thus, we investigated the expression level changes in *IL-1R2* which is a receptor of IL-1 β when the PBLs are exposed to PAMPs. PBLs of *V. variegatus* were co-incubated with LPS or poly(I:C). The expression level of *VvIL-1R2* did not change significantly with PBS treatment except for 24 h. The incubation

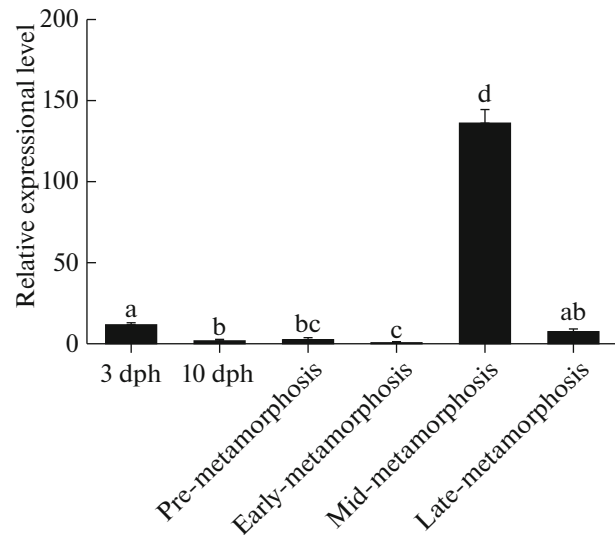


Fig. 5. Quantitative analysis of *VvIL-1R2* expression during metamorphosis. The relative expression variance is presented as a ratio (the amounts of *V. variegatus IL-1R2* were normalized to the corresponding *18S rRNA* and β -actin genes values). The data are shown as mean \pm SD ($n = 3$). Columns with different letters show a significant difference ($p < 0.05$).

with PBS does not have a significant influence on *VvIL-1R2* expression in the PBLs (Fig. 6). By contrast, LPS or poly(I:C) treatment has certain influences on the expression of this gene, as demonstrated by the differences between the experimental groups and the control groups. When the expression level was normalized to the control, the fold change levels of *VvIL-1R2* were significantly and rapidly up-regulated within .5 h and 1 h with LPS and poly(I:C) treatments, respectively. This result indicates that *IL-1R2* expression may be more sensitive to the PAMPs of LPS than to those of poly(I:C).

Studies in human models showed that PAMPs stimulated *IL-1R2* expression in the human peripheral blood mononuclear cells, monocytes/macrophages, and lymphocytes [57]. Recent studies showed that LPS, poly(I:C), bacteria, virus, and parasites induced the high-level transcription of *IL-1R2* in the different fishes [9–11, 13]. Similarly, in our study, the *VvIL-1R2* expression was detected in a wide range of tissues under normal physiological conditions, and *VvIL-1R2* expression was significantly and rapidly up-regulated by LPS and poly(I:C) challenges. These results indicate that the *VvIL-1R2* may be involved in host immune responses against bacterial and viral pathogens. In addition, the expression level began to decrease when it reached its peak within 24 h after co-incubation with the PAMPs. *IL-1R2* can induce apoptosis [34]. Thus, we speculate that the decrease in the *IL-1R2* expression may indicate the protection of PBLs from damage.

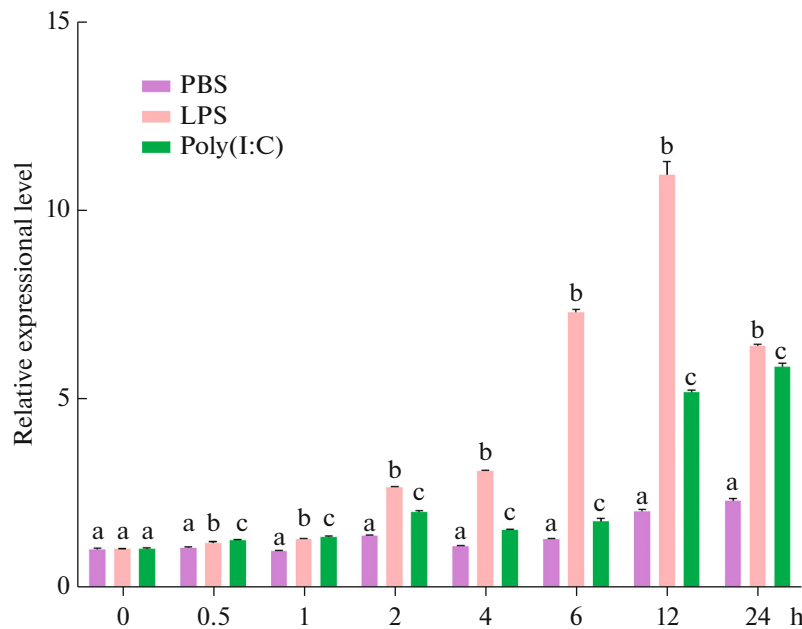


Fig. 6 Quantitative analysis of *VvIL-1R2* expression in peripheral blood leucocytes treated with 50 $\mu\text{g}/\text{mL}$ (final concentration) LPS or poly(I:C). Total RNA was extracted at different time points (0, 0.5, 1, 2, 4, 6, 12, and 24 h post-stimulation). The relative expression variance is presented as a ratio (the amounts of *V. variegatus IL-1R2* were normalized to the corresponding *18S rRNA* and β -actin genes values). The data are shown as mean \pm SD ($n = 3$). Columns with different letters show a significant difference ($p < 0.05$).

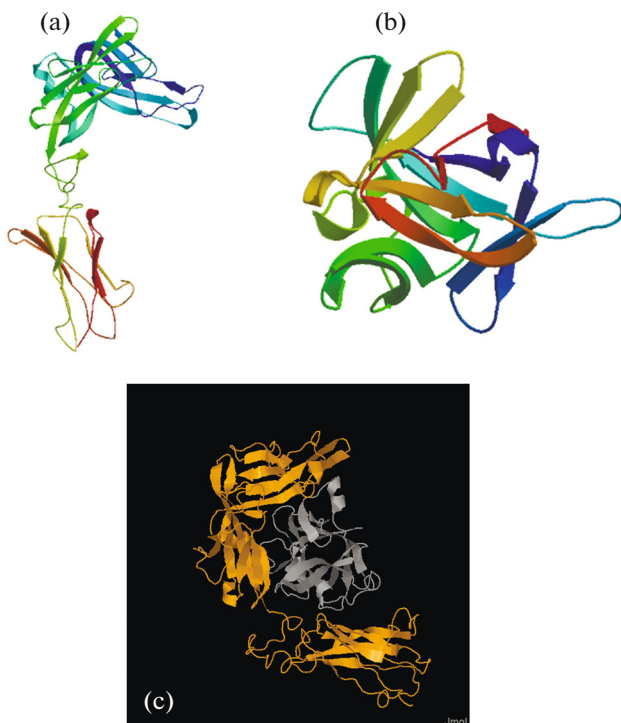


Fig. 7. The 3D structure, and protein docking simulation of the *VvIL-1β* and *VvIL-1R2*. (a) The 3D model of *VvIL-1R2*. (b) The 3D model of *VvIL-1β*. (c) Protein docking simulation between *VvIL-1R2* (yellow) and *VvIL-1β* (grey).

Protein 3D Modeling Analysis and Protein Docking Simulation of VvIL-1R2 and VvIL-1β Predict Its Potential Function

Given that IL-1 β is the ligand of IL-1R2, we tested whether *VvIL-1R2* and *VvIL-1β* interact with each other. We first predicted the secondary structures of *VvIL-1R2* and *VvIL-1β* with Phyre2 tools. With the sequences of *VvIL-1R2* and *VvIL-1β*, we predicted the 3D structures of *VvIL-1R2* and *VvIL-1β* with SWISS-MODEL and illustrate the model with Phyre2 tools (Figs. 7a, 7b). The coverage of IL-1R2 and that of IL-1 β could reach 72 and 60%, respectively. The PDBsum Generate software was used to illustrate the Ramachandran figure and predict the stability of the model. Only several amino acid residues were located in the forbidden area. The assessment indicates the high quality of the predicted 3D structure.

IL-1 β can interact with IL-1R2 and IL-1R1, and this interaction is important for the immune response. IL-1R1 mediates all of the known biological responses to IL-1 [58], while IL-1R2 acts as a decoy receptor of IL-1 and appears to inhibit the activity of IL-1 [59, 60]. Although IL-1R2 is structurally similar to type 1 IL-1 receptor (IL-1R1), which is responsible for IL-1 signal transduction, its truncated cytoplasmic domain and lack of Toll-IL-1 receptor (TIR) region render IL-1R2 incapable of transmembrane signaling [61]. Thus, we tested the interaction of the predicted *VvIL-1R2* and *VvIL-1β* by performing a protein–protein docking analysis. As shown in Fig. 7c, *VvIL-1R2*

could interact with VvIL-1 β through the predicted receptor binding sites.

These results show that the 3D structure of VvIL-1R2 is similar to those of other vertebrate IL-1R2s and indicate that the interaction between VvIL-1R2 and VvIL-1 β may mediate the reduction in immunological responses through the ligand–receptor interaction and dampen the signal transduction [62].

CONCLUSION

In this research, we cloned the mRNA of *VvIL-1R2* from *V. variegatus*, and characterized its domain structure. The six conserved cysteine residues, four conserved proline residues, two Ig-like domains, N-terminus signal peptide sequence, and transmembrane domain of this gene, as well as the TA-rich motifs of its proteins related to inflammatory responses were similar to other reported fish IL-1R2 and mammalian IL-1R2. Interestingly, the spatial and temporal expression levels of *VvIL-1R2* strongly imply that it may have some universal immunological functions and perform certain novel actions at the embryonic and metamorphic stages. The *VvIL-1R2* expression level could significantly and rapidly respond to PAMPs, such as LPS or poly(I:C), thereby pointing that this gene may be involved in host immune responses against bacterial and viral pathogens. The 3D structure prediction and protein docking analysis provided additional information about how VvIL-1R2 mediates its function with VvIL-1 β . This result is consistent with that obtained in a set of previous works, which asserts that the function of IL-1R2 relies on the interaction with IL-1 β .

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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