

Spatio-temporal expression of blunt snout bream (*Megalobrama amblycephala*) mIgD and its immune response to *Aeromonas hydrophila*

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Abstract

The function of IgD in fish and mammals has not been fully understood since its discovery. In this study, we have isolated and characterized the cDNA that encodes membrane-bound form of the immunoglobulin D heavy chain gene (mIgD) of blunt snout bream (*Megalobrama amblycephala*) using RT-PCR and rapid amplification of cDNA ends (RACE). The full-length cDNA of mIgD consisted of 3313 bp, encoding a putative protein of 943 amino acids. The structure of blunt snout bream mIgD is VDJ- μ 1- δ 1- δ 2- δ 3- δ 4- δ 5- δ 6- δ 7-TM. Multiple alignment and phylogenetic analyses indicated that blunt snout bream mIgD clusters with the homologues of cyprinid fish and that its highest identity is with that of *C. idella* (82%). The mIgD expression in early different developmental stages showed that the level of mIgD mRNA decreased dramatically from the unfertilized egg stage to the 32-cell stage, suggesting that mIgD mRNA was maternally transferred. As cell differentiation initially took place in the blastula stage, the mIgD expression increased significantly from the blastula stage to prelarva, which might be attributed to embryonic stem cell differentiation processes. Compared with juvenile fish, the expression and tissue distribution patterns of mIgD in adult individuals exhibited considerable variation. After the injection of *Aeromonas hydrophila*, mIgD expression was up-regulated in various tissues, reaching the peak expression at 5 d, 14 d or 21 d (depending on the tissue type). The present study provides a theoretical basis for further research of the teleost immune system.

Key words: blunt snout bream (*Megalobrama amblycephala*), mIgD, cDNA, *Aeromonas hydrophila*, maternal transfer, spatio-temporal expression.

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Introduction

In all species that possess Igs, the only class of antibody universally found is IgM, which has been well characterized at both the protein and molecular levels and is considered to be the predominant teleost serum Ig [1, 2]. However, there are considerably fewer reports about the teleost homolog(s) of IgD, which was initially discovered in humans from serum of a myeloma patient [3]. For about 30 years, IgD was considered to be a relatively recently evolved class of immunoglobulins as it had been described only in primates and rodents [3-6].

However, in 1997, a new immunoglobulin H-chain gene with some homology to mammalian IgD was cloned from the channel catfish, *Ictalurus punctatus* L. [7]. Since then, IgD genes, albeit with some diversity, have been

identified in a large number of species, including Atlantic salmon, *Salmo salar* [8], Atlantic cod, *Gadus morhua* L. [9], Japanese flounder, *Paralichthys olivaceus* [10] and grass carp, *Ctenopharyngodon idella* [11]. The discoveries of an IgD-like gene in teleost species have changed the evolutionary view, and suggest that the gene existed early in vertebrate evolution.

The role of IgD in teleost in vertebrate immune systems is not fully understood. In channel catfish, transcripts encoding both membrane and secreted IgD have been identified [7]. The IgD heavy chain cDNA clones existed only as the membrane form in both Atlantic salmon and Atlantic cod [8, 9]. In most species, the IgD-encoding gene (C δ) is located downstream of the IgM-encoding gene (C μ) and is co-expressed with IgM on the surface of the majority of mature B cells before antigenic stimulation

[12]. IgD seems to play an important role as an antigen receptor optimized for efficient recruitment of B cells into antigen-driven responses [8].

The structures of teleost IgD genes are different from those of mammals. Human IgD has three constant domains, while there are only two constant domains in the mouse; further, both human and mouse delta constant regions have a flexible hinge region [13]. In contrast, there is no hinge region and there are seven constant domains for both catfish and salmon IgD [7, 8]. The initial discovery of IgD in teleosts also found that IgD was a chimeric protein containing a C μ 1 domain followed by a number of C δ domains [7]. This chimeric structure was later found in grass carp [11], Atlantic salmon [8] and Atlantic cod [9]. Until now, no complete fish IgD heavy chain without C μ 1 has been reported. In addition, the structure of the fish IgD gene is different in various species. For instance, a duplication of domains δ 2- δ 3- δ 4 has been reported in grass carp [11], salmon [8], halibut [14] and catfish [15], but not in flounder [10]. Further, in cod, domains δ 3- δ 6 are absent, and there is a tandem duplication of domains δ 1 and δ 2 [9].

So far, the information obtained indicates that teleosts do not share a common IgD structure. To further our understanding of the immune development of teleosts, it is important to obtain more information on this gene in additional fish species from different families. *Megalobrama amblycephala*, commonly known as blunt snout bream or Wuchang fish, belongs to *Megalobrama*, *Cyprinidae*, *Cypriniformes*, *Actinopterygii*. As a species in *Cyprinidae*, the largest family of freshwater fish, *M. amblycephala* is closely related to the commonly known fish such as zebrafish (*Danio rerio*) and carp. Although the gene encoding IgD has been identified in bony fish by database mining, the biological functions of IgD is yet unknown in *Megalobrama amblycephala*. Therefore, the aim of the present study was to isolate and to characterize the IgD gene in blunt snout bream, *Megalobrama amblycephala*, which is an economically important freshwater fish species in the aquaculture industry in China. In addition, to understand the fish immune system, we examined the spatio-temporal expression of mIgD and investigated the immune response of the mIgD gene after *Aeromonas hydrophila* infection in *M. amblycephala*.

Material and methods

Fish and sampling

Healthy *M. amblycephala* of juvenile (body weight: 45-55 g) and adult fish (body weight: 400-500 g) were collected from the fish base of Huazhong Agricultural University (Wuhan, China). Before experiments, fish were acclimatized in quarantine plastic tanks in aerated freshwater at 24 \pm 2°C for two weeks. After acclimation, each fish was anesthetized with MS-222 (Sigma, USA). To avoid individual differences, tissues were extracted from 30 juvenile and 30 adult *M. amblycephala*. Tissue samples (including

head kidney, trunk kidney, liver, spleen, gill, intestine, muscle and brain) were immediately collected and frozen in liquid nitrogen, and then stored at -80°C until the RNA was extracted. To determine the expression of the mIgD gene in different developmental stages, samples were collected from unfertilized eggs, zygotes, 2-cell embryos, 4-cell embryos, 32-cell embryos, blastula, gastrula and prelarva. Due to the vast differences in the size amongst the developmental stages, samples totaling ~100 mg from six parents were pooled for each stage for RNA isolation. All samples were flash-frozen in liquid nitrogen immediately and then stored at -80°C until RNA isolation. All the experimental procedures involving fish were approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University, Wuhan, China.

Challenge experiment

For the *A. hydrophila* challenge experiment in *M. amblycephala*, all the tested fishes (15 \pm 2 g) were inoculated by intraperitoneal injection. The bacteria *A. hydrophila* was isolated from diseased *M. amblycephala* in Dongxi Lake (Wuhan, China) by our laboratory. A single colony was cultured in LB medium at 28°C to mid-logarithmic growth. In a pre-challenge experiment prior to the challenge trial, the concentration 1 \times 10⁷ colony forming units/ml (CFU/ml) was determined as LD₅₀. The treatment group was injected with 0.1 ml (1 \times 10⁷ CFU/ml) bacterial suspension per individual, while the control group was injected with the same volume of phosphate-buffered saline (PBS, pH 7.2). After the treatment, the fish were returned to tanks with water temperature of 27 \pm 0.5°C. Thirty injected individuals (3 pools) from treated and control groups were randomly dissected at 4 h, 1, 3, 5, 14, and 21 d post injection. Thirty without injected fishes were sampled as a blank control (0 h). Fish were euthanized by exposure to 300 mg/l of MS-222 (Sigma, USA) before dissection, and tissues (including trunk kidney, spleen, gill and liver) were sampled, frozen immediately in liquid nitrogen and then stored at -80°C until RNA extraction.

RNA isolation and cDNA synthesis

Samples, including different tissues and embryos of different developmental stages, were homogenized using a mortar and pestle under liquid nitrogen; total RNA was extracted using Trizol reagent (TaKaRa, Japan), according to the manufacturer's instructions. Total RNA was treated with gDNA Eraser (TaKaRa, Japan) to avoid the contamination of genomic DNA. The quantity and quality of the RNA was determined using agarose gels and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). First strand cDNA was synthesized using PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa, Japan), according to the manufacturer's instructions.

Table 1. The primer sequences for cloning and expression.

Primer name	Primer sequence (5'–3')
Partial cDNA fragments of mIgD	
mIgD-M-F	TGATGTCGGCACAGTTGG
mIgD-M-R	AGAGGGCGAGAAAGGCAC
mIgD specific primers for 3' RACE	
Outer	CCATAGAGCCCAACAACC
Inner	GTTGGCAACTAAATGGGACG
mIgD specific primers for 5' RACE	
Outer	TGTTTCCAGTGGGTTGTG
Inner	GCAATAGTCTTCAGTTTCG
mIgD specific primers for ORF	
ORF-F	GAGGTAAACGTGCCATAA
ORF-R	TAGCAAAGGAAACCAGAG
mIgD primers for real time PCR	
Forward	TGGCTGCTTGGAAACGACT
Reverse	ATGTTATGCGACTGGGTA
Reference gene primers for real time PCR	
18S rRNA-F	CGGAGGTTCTGAAGACGATCA
18S rRNA-R	GGGTCCGCATCGTTTACG
ACTB-F	ACCCACACCGTGCCCATCTA
ACTB-R	CGGACAATTTCTCTTCGGCTG
EF1 α -F	CTTCTCAGGCTGACTGTGC
EF1 α -R	CCGCTAGCATTACCCTCC

Cloning of full-length cDNA by rapid amplification of cDNA ends (RACE)

To amplify partial cDNA fragments of mIgD, amplified primers (Table 1) were designed based on conserved regions of reported Cyprinidae fish IgD sequences (Xiao *et al.*, 2010). Using trunk kidney cDNA as the template, the main part of mIgD cDNA was amplified via PCR reaction. The mIgD cDNA fragment amplification conditions consisted of initial denaturation at 95°C for 5 min, 30 cycles of 30 s at 95°C, 30 s at 56°C and 3 min at 72°C, and final extension at 72°C for 10 min. The PCR fragments were ligated into pMD19-T vector (TaKaRa, Japan) and transformed into *Escherichia coli* DH5 α competent cells. The positive clones were examined by PCR and sequenced commercially.

The primer pairs used for 5' RACE and 3' RACE are listed in Table 1. The 5' and 3' ends of the mIgD were amplified using the 5'-/3'-Full RACE kit (TaKaRa, Japan) following the manufacturer's protocol. Primers (Table 1) for 5'-RACE and 3'-RACE PCR were designed according to the sequence of the cDNA fragment determined as described above. The 5' and 3'-RACE products were purified and ligated into pMD19-T vector (TaKaRa, Japan); then, several random clones were selected and sequenced commercially. The full-length mIgD cDNA sequence was compiled using DNASTar software. For confirmation, the full-length open-reading frame was amplified with specific

primers (ORF-F and ORF-R, Table 1). The PCR reaction was performed using the trunk kidney cDNA with LA Taq (TaKaRa, Japan), according to the manufacturer's protocol.

Sequence analysis

The amino acid sequence of mIgD gene was predicted using a translator program at open reading frame finder on NCBI (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The calculated molecular weight and theoretical isoelectric point from the deduced amino acid sequence were obtained by the online software ProtParam (<http://www.expasy.ch/cgi-bin/protparam>). The protein domains were marked according to the UniProt (<http://www.uniprot.org/>) and SMART (<http://smart.embl-heidelberg.de/>) database. Phylogenetic analysis of the putative amino acid sequence of mIgD was carried out by the neighbor-joining method using MEGA 5.0 program, and the reliability of the estimated tree was evaluated by the bootstrap method with 1000 pseudo-replications.

Quantitative real-time PCR (qRT-PCR)

Based on quantitative real-time PCR (qRT-PCR), expression patterns of mIgD were analyzed using cDNA from different tissues and embryos of different developmental stages of blunt snout bream.

Three reference genes, 18S rRNA, EF1 α (elongation factor 1 α) and ACTB (β -actin) were selected based on expression stability, and all primer sequences were described in Table 1. Products of the qRT-PCR primers (Table 1) were sequenced to confirm specificity. To select the reference genes with the most stable expression, the relative stability measure (M) of the reference genes was calculated by GeNorm (<http://medgen.ugent.be/genorm/>) as described in our previous studies [16]. The value M represents an average pairwise variation of a reference gene with all other reference genes and a lower M value corresponds to the higher expression stability [17]. According to this rule, the most stable gene was 18S rRNA in analysis samples. In addition, PCR amplification efficiency of 18S rRNA is much closer to mIgD.

The qRT-PCR was carried out in triplicate on a Rotor-Gene Q real-time PCR Detection System (QIAGEN, Dusseldorf, Germany) using the SYBR® Premix Ex Taq™ II (TaKaRa, Japan) according to the manufacturer's instructions. The total reaction volume of 20 μ l contained 10 μ l SYBR qPCR Mix, 1 μ l of each primer (10 μ M), 2 μ l cDNA and 6 μ l ddH₂O. Real-time PCR conditions were as follows: initial denaturation at 94°C for 30 s, followed by 40 cycles of 10 s at 94°C, 30 s at 54°C and 30 s at 72°C. The PCR reaction carried out without DNA sample was used as a negative control. The PCR specificity was verified by a melt-curve analysis. Gene expression values were calculated as fold-change in the target gene relative to the reference gene (18S rRNA): fold change = $E^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (Ct \text{ target gene} - Ct \text{ 18S rRNA})$ [18].

Statistical analysis

All the data obtained from the real-time PCR were expressed as mean \pm standard error (M \pm SE) and subjected to a One-way Analysis of Variance (ANOVA), followed by Duncan's test to determine differences among treatments. Statistical significance was set at $p < 0.05$, with $p < 0.01$ being considered highly significant. Statistical analyses were performed using SPSS 13.0.

Results

Analysis of nucleotide and deduced amino acid sequences of mIgD

The full-length cDNA of mIgD gene (GenBank accession no. KC894947) was 3313 bp, consisting of a 5'-untranslated region (5'-UTR, 119 bp), a 3'-untranslated region (3'-UTR, 362 bp) and 2832 bp open reading frame (ORF), encoding a protein of 943 amino acids with a calculated molecular weight of 103.89 kDa and theoretical isoelectric point of 7.49 (Fig. 1). The mIgD deduced an amino acid sequence of *M. amblycephala* shared amino acid identity with orthologous loci of other species: *C. idella* (81%); *C. carpio* (67%); *Salmo trutta* (47%); *Ictalurus punctatus* (41%); *Salmo salar* (41%); and *O. mykiss* (32%).

M. amblycephala mIgD is composed of one variable domain (VH), one μ 1 domain, seven constant domains (δ 1– δ 7) and one transmembrane domain (TM) (Fig. 1). Amino acid analysis of the mIgD revealed that there were 31 cysteine (Cys) residues, which might be involved for the formation of intra-domain and inter-domain disulfide bridges. In addition, 10 putative N-linked glycosylation sites (one in δ 4, four in δ 5, two in δ 6 and three in δ 7) were found in mIgD of *M. amblycephala*.

The amino acid sequences of immunoglobulin from other species based on the closest homologues by running BLASTP search were taken for phylogenetic analysis. Multiple protein sequence alignment revealed that *M. amblycephala* mIgD was clustered with the homologues of other vertebrate species. The mIgD of *M. amblycephala* revealed high identity with the homologues of cyprinid fish, especially with *C. idella* (Fig. 2).

Spatio-temporal expression of *M. amblycephala* mIgD

In the present study, mIgD was determined in different embryo developmental stages (Fig. 3). The level of mIgD mRNA was highest in unfertilized eggs and decreased dramatically to a low point in the 2-cell through 32-cell stages. The level of mIgD mRNA then increased significantly from the blastula stage through the prelarva stage, although it never achieved the level seen in the unfertilized egg.

Transcriptional levels of mIgD were detected in juvenile and adult *M. amblycephala*. mIgD was expressed in head kidney, trunk kidney, spleen, liver, intestine, gill, brain and muscle of both juvenile and adult fish. For juvenile individuals, the expression of mIgD was mainly detected in the head kidney, trunk kidney, spleen and liver, with low levels in other tissues (Fig. 4). For adult individuals, the mIgD expression level was the highest in the head kidney, moderate in trunk kidney and muscle, and low in other tissues (Fig. 4). Compared with juvenile individuals, the expression level of mIgD in adult individuals was higher in head kidney and muscle, and lower in all other tissues (Fig. 4).

Expression of mIgD gene after *A. hydrophila* challenge

At 4 h, 1, 3, 5, 14 and 21 d post-challenge with *A. hydrophila*, the mRNA expression level of mIgD in trunk kidney, spleen, gill and liver of *M. amblycephala* was quantified by qRT-PCR (Fig. 5). Compared with the control group, the expression pattern of the mIgD gene was found first down-regulated at 4 h, then up-regulated and reached the peak at 5 d after injection in trunk kidney of the treatment group. In the spleen, the level of mIgD mRNA was found first down-regulated at 4 h and 1 d, then increased gradually and reached the peak at 21 d in the treatment group. In gill and liver, the expression pattern of mIgD gene was up-regulated by the challenge. Expression reached a peak at 14 d in gill and 21 d in liver. Changes in peak expression relative to the control group were most dramatic in gill (~16-fold) and liver (~80-fold), and lower in trunk kidney (~4.5-fold) and spleen (~3.5-fold). In trunk kidney, spleen and gill, the expression of the mIgD gene was down-regulated at 4 h in the control group compared to the blank control group (0 h).

Discussion

M. amblycephala mIgD transcripts correspond to the membrane form and, just as in Japanese flounder *Paralichthys olivaceus* [10] and mandarin fish, *Siniperca chuatsi* [19], are chimeric, with the inclusion of C μ 1, seven C δ (C δ 1-C δ 2-C δ 3-C δ 4-C δ 5-C δ 6-C δ 7) exons and TM regions. This new H-chain gene from *M. amblycephala* is also homologous to the previously reported delta genes from catfish [7] and salmon [8], albeit with some diversity. The similarities with catfish and salmon IgD are sequence homology and the chimeric nature of its expression, as the δ 1 domain is spliced to the μ 1 domain which permits covalent association with light chains [7]. A previous genome-wide survey of the zebrafish [20, 21] was helpful in identifying the gene segments encoding antibodies in this animal model. Zebrafish IgD was also identified to be a chimeric immunoglobulin, with C μ 1 splicing to the third IgD exon

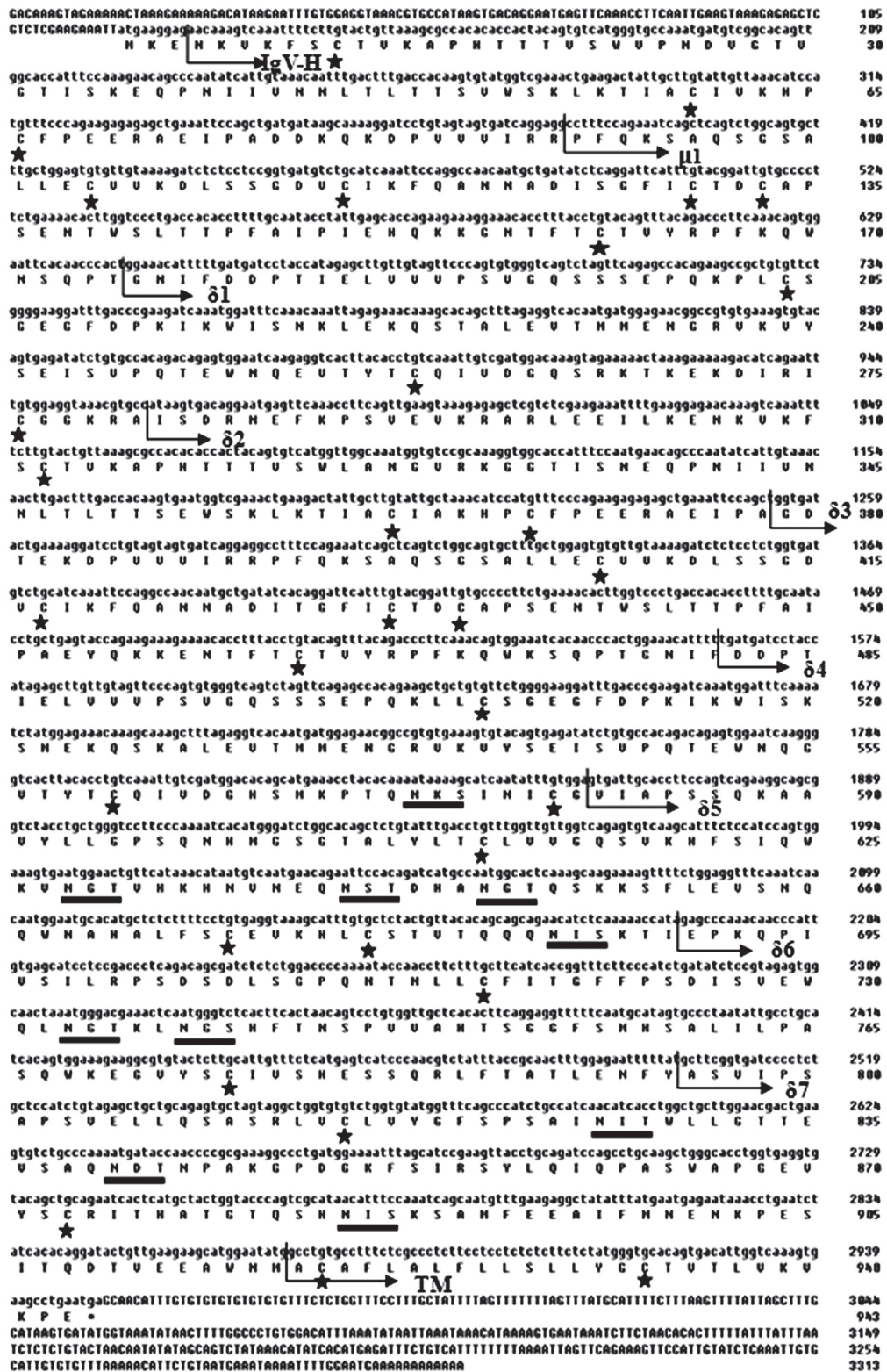


Fig. 1. Nucleotide (upper row) and deduced amino acid (lower row) sequences of the *M. alycephala* mIgD (Genbank accession number KC894947). The sequence was divided into VH, $\mu 1$ domain, seven constant domains ($\delta 1$ - $\delta 7$) and transmembrane domain (TM) on the basis of sequence comparisons with the IgH chains of other teleosts. The cysteines (C) are denoted by pentacle. The putative N-glycosylation sites are designated by the underline. A sequential numbering is used

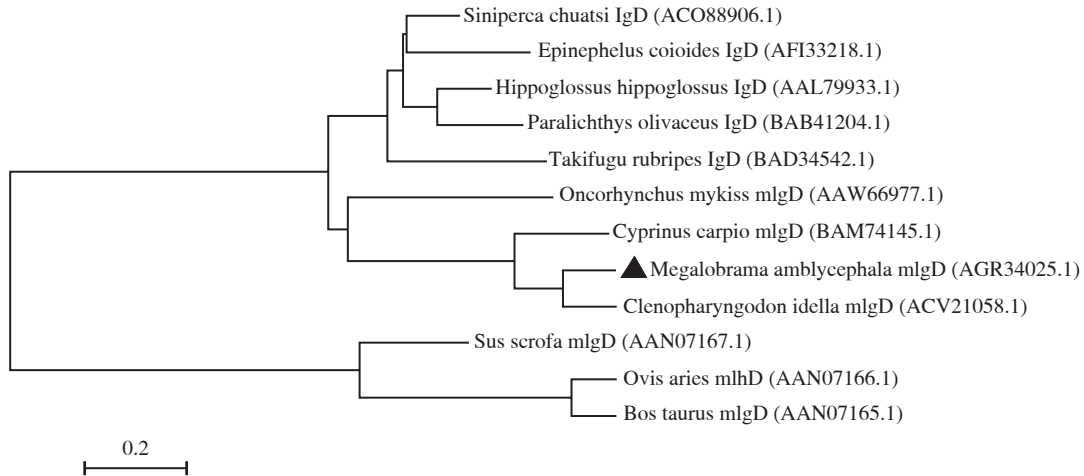


Fig. 2. Polygenetic tree showing the relationship of the deduced *M. amblycephala* mIgD molecule with representative mIgD proteins of other species. The tree was constructed using a neighbor-joining method. The bootstrap support (MEGA5.05 software) for each branch (1000 replications) was shown. The bottom scale refers to percentage divergence

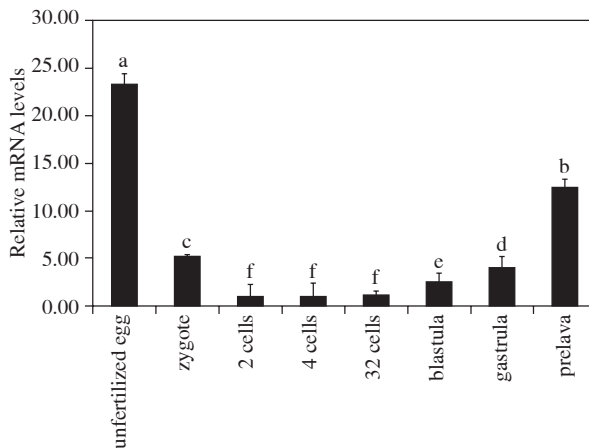


Fig. 3. qRT-PCR analysis of the level of mIgD mRNA in early developmental stages of *M. amblycephala*. The Y-axis represented the mean \pm SE ($N = 3$). The X-axis represented the developmental stage. Different letters above bars represented a significant difference at the levels of $p < 0.05$, and same letters above bars indicated no significant difference

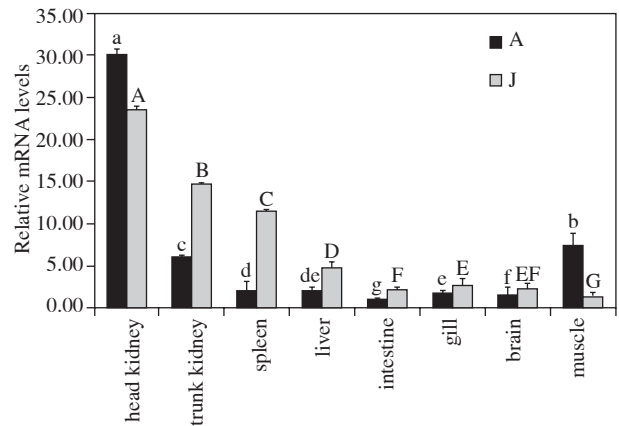


Fig. 4. qRT-PCR analysis of the level of mIgD mRNA in different tissues (head kidney, trunk kidney, liver, spleen, intestine, gill, brain, muscle) of juvenile (J) and adult (A) *M. amblycephala*. Different letters above pillars indicated a statistical significance at the level of $p < 0.05$

(C δ 3.1), bypassing the first two IgD exons [18]. In bony fishes, the Vn-Dn-Jn-IGHZ-Dn-Jn-IGHM-IGHD pattern is present on IGH loci [21]. Previous studies about fish IgH show that the structure of IgD is remarkably heterogeneous among fish species (with frequent C-domain duplications), while IgM and IgZ appear to be more conserved [8, 22]. The backbones of many bony fish delta chains are comprised of seven C δ domains, where a wide range of domain organization within fish lineages is observed. In the Japanese flounder (*Paralichthys olivaceus*) [23] and stickleback

(*Gasterosteus aculeatus*) [24], the IGHD locus consists of the C μ 1-C δ 1-C δ 2-C δ 3-C δ 4-C δ 5-C δ 6-C δ 7-TM1-TM2 exons, in which the homology of domains CH2-CH5, CH3-CH6 and CH4-CH7 suggests that C δ 2-C δ 3-C δ 4 duplicated to generate C δ 5-C δ 6-C δ 7 [8, 24]. In Atlantic salmon (*Salmo salar*) and catfish (*Ictalurus punctatus*), the duplications of C δ 2-C δ 3-C δ 4 have also been found [14, 25]. C δ 2-C δ 3-C δ 4 domains are repeated three times in Atlantic salmon IgHA [14] and catfish [25], but four times in Atlantic salmon IgHB [14]. However, the duplications of C δ 2-C δ 3-C δ 4

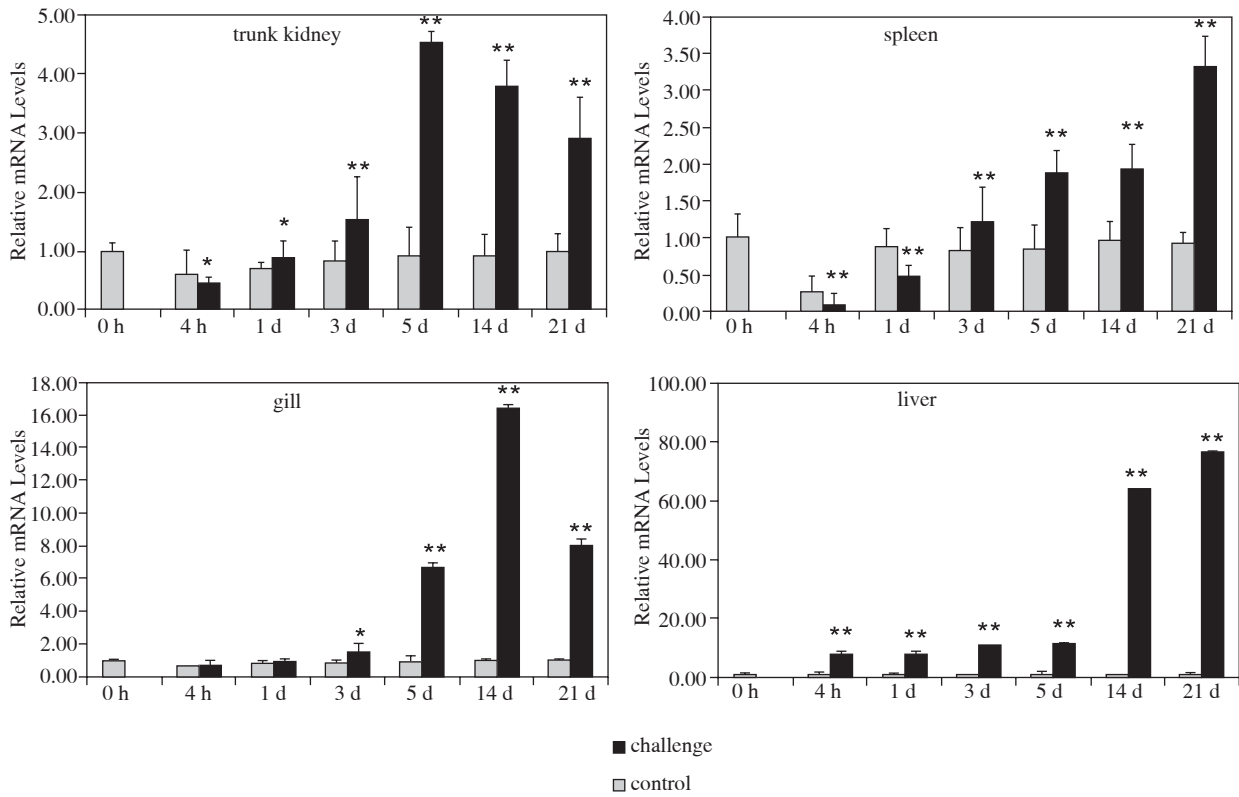


Fig. 5. The relative level of mIgD mRNA in different tissues (trunk kidney, spleen, gill, liver) of *M. amblycephala* after *A. hydrophila* infection in comparison with PBS control. Asterisks indicated statistical significant differences (* $p < 0.05$; ** $p < 0.01$)

have not been found in *M. amblycephala* mIgD. Multiple protein sequence alignment revealed that *M. amblycephala* mIgD showed high identity with the homologues of cyprinid fish, especially with *C. idella*, while the mIgD of *C. idella* has a structure of C δ 1-(C δ 2-C δ 3-C δ 4)₂-C δ 5-C δ 6-C δ 7-TM-UTR, with the repeat of C δ 2-C δ 3-C δ 4. It indicates that diversification of IgD may be due to germline changes that are species-specific, rather than due to different splicing patterns as described for IgM.

IgD was a new finding in teleosts, and it has seldom been studied with regard to its function [26]. The distribution of IgD transcripts in teleost organs has been examined in fugu (*Takifugu rubripes*), Atlantic cod and rainbow trout. In fugu, IgD was found to be expressed intensely in lymphoid tissues (i.e., PBL, spleen, head kidney and kidney) [27]. In Atlantic cod, IgD producing cells were evenly distributed throughout the hematopoietic tissues in head kidney and spleen, which suggested that cod IgD was mainly expressed as a B-cell receptor akin to IgD in mammals [28]. In rainbow trout, IgD-secreting plasma cells were found to be common in the kidney and spleen [29]. In this study, *M. amblycephala* mIgD was detected mainly in head kidney, trunk kidney and spleen in both juvenile and adult fish,

which was consistent with the above reports. Moreover, in both juvenile and adult fish, *M. amblycephala* sIgM was highly expressed in head kidney, trunk kidney and spleen in our previous study [30]. It indicates that head kidney, trunk kidney and spleen are predominant immune organs.

In adult *M. amblycephala*, the expression level of mIgD was the highest in head kidney, with the expression almost entirely localized in head kidney. The head kidney, having morphological and functional aspects similar to the mammalian bone marrow, is a major hematopoietic organ and site of production of antibodies and other immune cells in teleost fish [31, 32]. The highest expression level of mIgD in the head kidney may be attributed to the first appearance in the head kidney of mIgD positive B-cells, which may be transported to other lymphoid organs [33]. Compared with adult fish, the expression of mIgD in juvenile fish was higher in the trunk kidney, spleen, liver, intestine, gill and brain. Thus, expression and tissue distribution patterns of *M. amblycephala* mIgD exhibit considerable variation between juvenile and adult fish.

In the present ontogeny study, mIgD mRNA was detected in zygotes, suggesting the possibility of maternal mRNA transfer into oocytes. Indeed, it was found that

the level of mIgD mRNA was the highest in the unfertilized egg stage compared to other early developmental stages, which is similar to *M. amblycephala* sIgM [30]. In addition, the level of mIgD mRNA decreased dramatically from the unfertilized egg to zygote stage, reaching very low levels from the 2-cell to the 32-cell stages; by the prelarva stage, mIgD levels were much closer to those seen in the unfertilized egg. As such, it seems that the transcript of the mIgD gene is maternally transferred to eggs and degraded with embryonic development. Such phenomenon of maternal transfer has also been found in both sea bass (*Dicentrarchus labrax*) [34] and sea bream (*Sparus aurata*) [35]. Cell differentiation takes place in the blastula stage and generates the embryonic stem cell, which would proceed with immune organogenesis [36]. In the present study, the level of mIgD mRNA increased significantly from the blastula stage to prelarva, which might be attributed to the embryonic stem cell differentiation procedure.

The function of IgD in fish and mammals is not fully understood. Previous research indicated that fugu IgD may play an important role in the humoral immune system, as the expression pattern of IgD is similar to IgM [27]. In rainbow trout, the (mem)IgD(mem)(+)IgM(-) B lymphocyte subset expresses (mem)CCR7 and responds to viral hemorrhagic septicemia virus infections [37]. IgD is typically co-expressed with IgM by alternative splicing of a long primary mRNA transcript containing the rearranged VDJ exons and the C μ and C δ exons [38]. IgM knockout studies with mice have shown that IgD can largely substitute for IgM functions in B cells [39]. In this study, the expression patterns of mIgD in various tissues of *M. amblycephala* post *A. hydrophila* infection were in line with that of IgM [30, 40, 41]. Thus, mIgD may have an important role in the adaptive immune system, conferring protective functions against pathogens.

In *M. amblycephala*, mIgD mRNA in different organs exhibited various responses to *A. hydrophila* infection. It should be noted that, in the control group, the level of mIgD mRNA was down-regulated at 4 h in trunk kidney, spleen and gill of *M. amblycephala*; this down-regulation immediately after the injection procedure might be due to stress on the fish from having been handled and injected. On the other hand, in the treatment group, the level of mIgD mRNA reached a peak at 5 d and 14 d post infection with *A. hydrophila* in trunk kidney and gill, respectively, while mIgD mRNA expression reached a peak at 21 d in spleen and liver. Clearly, activation of immune response is a complicated and time-consuming process. For instance, in sea bass, the IgM level decreased during the first month post *Vibrio anguillarum* immunization, which was explained as the Ig being consumed through counteracting with antigen [42]. In our previous study, *M. amblycephala* MHC I transcripts were detected to decrease from 0 to 4 h after *A. hydrophila* injection in gill and liver [43]. More-

over, the level of sIgM mRNA was found significantly decreased at 4 h in trunk kidney, spleen and gill of *M. amblycephala* after *A. hydrophila* infection [30]. In this study, compared with the control group, the expression pattern of *M. amblycephala* mIgD gene was found down-regulated at 4 h after injection in trunk kidney and spleen. The early decrease is likely to be an adaptation rather than a deficiency of the immune system [42]. By comparison, the level of IgM mRNA significantly increased in blood cells of orange-spotted grouper after *Vibrio alginolyticus* challenge [44]. Further, in fugu (*Takifugu rubripes*), the expression of the IgD gene was intense in peripheral blood leucocytes (PBL) [27]. In the present study, after injection of *A. hydrophila*, the level of mIgD mRNA reached a peak at 14 d in gill (~16-fold), and then dropped back by 1/2 one week later, which is similar to the previous study [45]. After injection of *Flavobacterium columnare*, expression of IgD was significantly elevated at 1 and 2 weeks with 7.16 and 7.78-fold respectively in gill of mandarin fish, and then declined to the basal level one week later [45]. The different expression patterns of mIgD after the infection might be caused by different fish species or different bacteria. After stimulation with inactivated *A. hydrophila* strain T4, Chinese soft-shelled turtle IgD gene in peak expression relative to the control group were most dramatic in blood (~25-fold) [46]. The high expression levels of mIgD in the liver of challenged *M. amblycephala* is intriguing. The liver's lymphocyte population is selectively enriched in natural killer and natural killer T cells, which play critical roles in first-line immune defense against invading pathogens [47]. Further, in humans, about 30% of the total blood passes through the liver every minute [48], carrying about 10⁸ peripheral blood lymphocytes in 24 hours [49]. However, after *Aeromonas hydrophila* infection, the liver of treated *M. amblycephala* exhibited hepatorrhagia; thus, given the lack of identification for mIgD function, the significant increase in the level of mIgD mRNA may simply be a result of hepatorrhagia.

In conclusion, the full-length cDNA of *M. amblycephala* mIgD heavy chain has been cloned and characterized. Moreover, the spatio-temporal expression of mIgD was examined. Compared with adult *M. amblycephala*, the expression and tissue distribution patterns of mIgD in juvenile fish exhibit considerable variation. Finally, the immune response of the mIgD gene to *A. hydrophila* in *M. amblycephala* was also investigated; however, further research will be required to elucidate the function of mIgD and its mechanism in the immune response for teleosts.

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