

Original Research Articles

Cyclophilin A in *Megalobrama amblycephala*: Molecular characterization and expression analysis in response to *Aeromonas hydrophila* challenge

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Cyclophilin A (CypA) is a cell's primary receptor of Cyclosporin A (CsA). It is essential for biodiversity and plays an important role in the immune system. In this study, the full-length cDNA sequence (GenBank ID: PV256473) of *Megalobrama amblycephala* CypA was acquired by PCR and RACE technology. The complete cDNA sequence of *M. amblycephala* CypA is 901 bp. The lengths of the 5' untranslated region (UTR), the 3' untranslated region, and the open reading frame (ORF) are 77 bp, 329 bp, and 495 bp, respectively. It encodes a total of 164 amino acids. The theoretical isoelectric point of *M. amblycephala* CypA is 8.6, and the relative molecular weight is 17.43 KDa. According to homologous sequence retrieval in GenBank, the similarity between CypA amino acid sequence of *M. amblycephala* and other fish was *Cyprinus carpio* (94.51%), *Ctenopharyngodon idellus* (92.07%), *Carassius gibelio* (92.07%), *Labeo rohita* (92.07%), *Danio rerio* (91.46%), and *Onychostoma macrolepis* (91.46%). ESI-Pred3D predicted that the CypA of *M. amblycephala* contained 8 β -pleated sheets and double α -helices, which might form the active central region of CypA. MEGA 5.1 software was used for multiple sequence comparison analysis, and the adjacency method constructed the genetic phylogenetic tree. It was found that the CypA gene of *M. amblycephala* was clustered into one branch of Cyprinidae fish CypA, and CypA of *M. amblycephala* was closely related to CypA of *Cyprinus carpio*. Expressions of CypA in different tissues of *M. amblycephala* were detected through qPCR; it was found that the expression level of the CypA gene was highest in the head kidney, followed by liver, kidney, and spleen. The *M. amblycephala* was immersed in *Aeromonas hydrophila* for infection; it was found that expression levels of CypA in head kidney, liver, gills, and intestines first increased, then decreased in the first 72 h of infection. Specifically, the expression levels reached a peak at 6h in the head kidney, at 12 h in the liver and gills, and at 24h in the intestines. It indicated that the expression of *M. amblycephala* CypA gene could be significantly induced after *A. hydrophila* infection—expression levels of CypA in the head kidney peak earlier than in the liver and intestines. Moreover, the relative expression level of CypA in the head kidney at peak (15.02-fold) is significantly higher than that in the liver (6.89-fold), intestines (9.92-fold), and gills (7.42-fold). It is suggested that CypA might play an essential role in the head kidney of *M. amblycephala* in defense against bacterial infection.

INTRODUCTION

Cyclophilins (CyPs) are a multi-functional protein family with extensive natural distribution and highly conserved properties. They are intracellular receptors of the immuno-

suppressant Cyclophilin A (CsA), which is known as CSA-binding protein.¹ CypA research focuses on its distribution in different organisms, analysis of related protein sequences, structure, and critical biological functions.² Many studies have reported that CypA is closely related to the occurrence of tumors. The expression of CypA was inhib-

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ited in non-small cell lung cancer cells by RNA interference technology, and the down-regulation of CypA expression resulted in a significant decrease in the proliferation of non-small cell lung cancer cells.² Through proteomics and cytobiology, it was found that expression levels of CypA were also significantly up-regulated in esophageal squamous cell carcinoma, male breast cancer, prostatic cancer, head-neck tumor, tongue squamous cell carcinoma, and other tumors, and CypA participated in malignant biological behaviors.³⁻⁷ The molecular chaperone function of CypA played a vital role in the repair of misfolded and broken conditional complexes, which were caused by improper signal activation in tumor formation, and it could maintain the steady state of proteins, which might be essential to the survival of tumors.⁸ In addition, CypA participates in virus replication as an immunosuppressor and is involved in the body's immune response.⁹ Qiu et al. found that CypA played an important role in resistance to viral infection in mammals, indicating that CypA participated in the innate immune response.¹⁰ In recent years, it has been found that CypA may play an immune stress role in aquatic organisms, such as *Chlamys farreri*, *Hyriopsis schlegelii*, *Eriocheir sinensis*, *Penaeus monodon*, *Litopenaeus Vannamei*, *Pelteobagrus fulvidraco* and *Ictalurus punctatus*.¹¹⁻¹⁵

Megalobrama amblycephala, with delicious taste, high meat content, and a small head, is deeply loved by consumers. Since promoting culture in 1960, it has become the sixth large-scale freshwater cultured fish in China.¹⁶ In recent years, with the rapid development of *M. amblycephala* breeding industry, pathogenic factors such as bacteria, viruses and parasites have caused rampant breeding diseases, and high-density breeding has led to eutrophication of water bodies, which has seriously threatened *M. amblycephala* breeding industry. Therefore, studying the immune system and its regulatory mechanism is vital for the sustainable development of *M. amblycephala* culture.

A. hydrophila infection is one of the major causes of large-scale death in cultivation of *M. amblycephala*. Due to the short onset time and high mortality rate, *A. hydrophila* infection has become a constraint in the green development of the *M. amblycephala* aquaculture industry. Nevertheless, the immune response mechanism of CypA in different tissues of *M. amblycephala* after *A. hydrophila* infection remains unknown. Therefore, this study aimed to clone the full-length cDNA of *M. amblycephala* CypA for sequence analysis, and compare it with the homologous sequences of other species to construct the phylogenetic evolutionary tree. Expression levels of CypA in different tissues of *M. amblycephala* were detected by quantitative real-time PCR (qPCR). After *A. hydrophila* infection, expression levels of CypA were detected in various tissues of *M. amblycephala*. The results of this study will provide basic experimental data to study disease control and anti-infection mechanisms of *M. amblycephala*.

MATERIALS AND METHODS

EXPERIMENTAL M. AMBLYCEPHALA

M. amblycephala (50±2 g) were collected from the aquatic breeding base at the Hunan Applied and Technology University. *M. amblycephala* were cultured in the laboratory cyclic aquaculture system for 14 d before sampling, during which aeration with oxygen was supplied for 24 h per day to keep enough oxygen dissolved in the water. The water temperature was 26±2 °C. The 100 mg tissues of liver, spleen, head kidney, intestines, gills, kidneys, and heart were collected from healthy *M. amblycephala*, respectively. All tissues were frozen in liquid nitrogen and stored in a refrigerator at a temperature of -80 °C.

A. HYDROPHILA INFECTION

A. hydrophila was acquired from the Hunan Applied and Technology University microbiology laboratory. Monoclonal strains were selected, inoculated in the Luria-Broth (LB) liquid medium, and cultured for 18 h by shaking at 28 °C. The solution was then centrifuged at a rate of 4000 rpm for 10 min, and the supernatant was removed. Next, the remaining sample was diluted via sterile PBS solution, and the concentration of bacteria after dilution was determined by plate count. Meanwhile, the concentration of bacteria was diluted to 1.0×10⁹ CFU/mL for later use. Three parallel repeating groups were established with 30 *M. amblycephala* samples in each group. All *M. amblycephala* samples were immersed in aquaculture boxes with 1.0×10⁹ CFU/mL *A. hydrophila* for 30 min, and then transferred into breeding tanks with aeration. Samples were collected at 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h, respectively. Healthy *M. amblycephala* samples without infection were chosen as the control group. In each sampling process, 5 *M. amblycephala* samples were collected from the experimental and control groups. 100 mg each of gill, intestine, liver, head kidney, and spleen tissues were collected, respectively.

TOTAL RNA EXTRACTION AND REVERSE TRANSCRIPTION

Utilizing the Simgen animal tissue total RNA extraction kit, total RNA of *M. amblycephala* liver was extracted. Concentration and purity of RNA (OD₂₆₀/OD₂₈₀) were tested using the nucleic acid concentration tester. The integrity of RNA was tested by electrophoresis and an RNA sample with clear bands and good integrity was chosen as the template. Reverse transcription was carried out using the Simgen reverse transcription kit. The acquired cDNA of *M. amblycephala* liver was stored at a temperature of -20 °C for later use.

AMPLIFICATION OF FULL-LENGTH CDNA SEQUENCES OF M. AMBLYCEPHALA CYP A

According to the cDNA sequences of Cyprinidae fish CypA found in the NCBI database, primers (Table 1) were designed using Premier 5. Amplification of the middle se-

Table 1 Primers used to amplify the *Megalobrama amblycephala* CypA gene

Primer name	Primer sequence (5'– 3')
Primers for intermediate cDNA sequence	
CypA-F	TAAGTTCATGTGCCAGGGTGG
CypA-R	CCCGAACACAACGTGCTTGCC
RACE Primers	
Primer for 3' RACE (3' GSP)	GATTACGCCAAGCTTGGAAAGAGTGTCTGGGAGGATTG
Primer for 5' RACE (5' GSP)	GATTACGCCAAGCTTGGGATGGGAAGGAGGTGTAAGC
Long primer	TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
Short primer	CTAATACGACTCACTATAGGGC
Primers for ORF	
ORF- F	CTTCCCGATGAGCTGCGGC
ORF- R	CAGGACCATGACACATGG
qPCR Primers	
CypA-qF	CGATGGAAAGAGTGTCTGGGAGG
CypA-qR	CGCAAACCTTGTCTGCCGTAGATG
ACTB-F	ACCCACACCGTGCCCATCTA
ACTB-R	CGGACAATTCTCTTCGGCTG

quences of CypA used cDNA extracted from liver tissues as the template. The PCR reaction conditions were as follows: 94 °C, 5 min; 94 °C, 30 s; 54 °C, 30 s; 72 °C, 1 min; 30 cycles; 72 °C, 10 min; and finally, terminated at 4 °C. PCR products were recycled and purified. After linking, transformation, and PCR identification, the positive bacterial liquid were sequenced.

According to the middle sequence of the CypA of *M. amblycephala* gained from sequencing, 5' and 3' RACE primers (Table 1) were designed. Using the total RNA of the liver as a template and RACE kit (Takara, Japan), the two ends of the CypA of *M. amblycephala* were amplified. The PCR conditions were as follows: 95 °C, 3 min; 95 °C, 30 s; 72 °C, 2 min; 8 cycles; 95 °C, 30 s; 68 °C, 30 s; 72 °C, 2 min; 6 cycles; 95 °C, 30 s; 64 °C, 30 s; 72 °C, 2 min; 30 cycles; extension at 72 °C for 5 min; and finally, terminated at 4°C. After recycling and purification, PCR products were connected to a linear pRACE vector and then transformed into the competent *E. coli* DH5 α cells. The positive bacterial liquid was sequenced after screening by culture medium, followed by PCR identification. The acquired cDNA segments were spliced for sequence analysis and comparison. The ORF primers were designed further to verify the cDNA sequences of *M. amblycephala* CypA.

SEQUENCE ANALYSIS

Using DNASTAR software, the completed cDNA sequence of CypA of *M. amblycephala* was acquired through splicing. In NCBI database (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>), utilizing the online ORF finder, the amino acid sequence of CypA of *M. amblycephala* was predicted. In GenBank, using the Blastn tool, the homologous sequences of CypA were searched. The amino acid structural domains of CypA were annotated using UniProt and SMART. Using the SWISS-MODEL, the tertiary structure of *M. ambly-*

cephala CypA was predicted. Multi-sequence comparison was analysed with Clustal X 1.83, and the phylogenetic tree of the genetic system was established with MEGA 5.1.

RELATIVE EXPRESSION OF *M. AMBLYCEPHALA* CYP A

Different tissues of *M. amblycephala* were collected. RNA was extracted using the Simgen animal tissue total RNA extraction kit. Utilizing the degenerate reverse transcription kit (TaKaRa), the cDNA template was obtained by reverse transcription. According to *M. amblycephala* CypA sequenc, qPCR primers (Table 1) were designed, and the reference gene was β -actin gene. Expressions of CypA in different tissues of *M. amblycephala* were detected using a qPCR kit (TaKaRa). The qPCR amplification conditions were as follows: 95 °C, 5 min; 95 °C, 20 s; 56 °C, 20 s; 72 °C, 20 s; 40 cycles; 72 °C, 8 min. The relative expression level of *M. amblycephala* CypA in different tissues and at various time after immersion infection were calculated via the $\Delta\Delta CT$ method.

DATA ANALYSIS

Experimental results are expressed as mean $\pm$ standard error. Different expression levels of CypA in different tissues of *M. amblycephala* and at different time points after infection were examined through one-way analysis of variance (ANOVA) using SPSS statistical software. The significance level is $P < 0.05$.

RESULTS

FULL-LENGTH CDNA SEQUENCES OF *M. AMBLYCEPHALA* CYP A

The complete cDNA sequence of *M. amblycephala* CypA is 901 bp (GenBank ID: PV256473). The 5' noncoding region

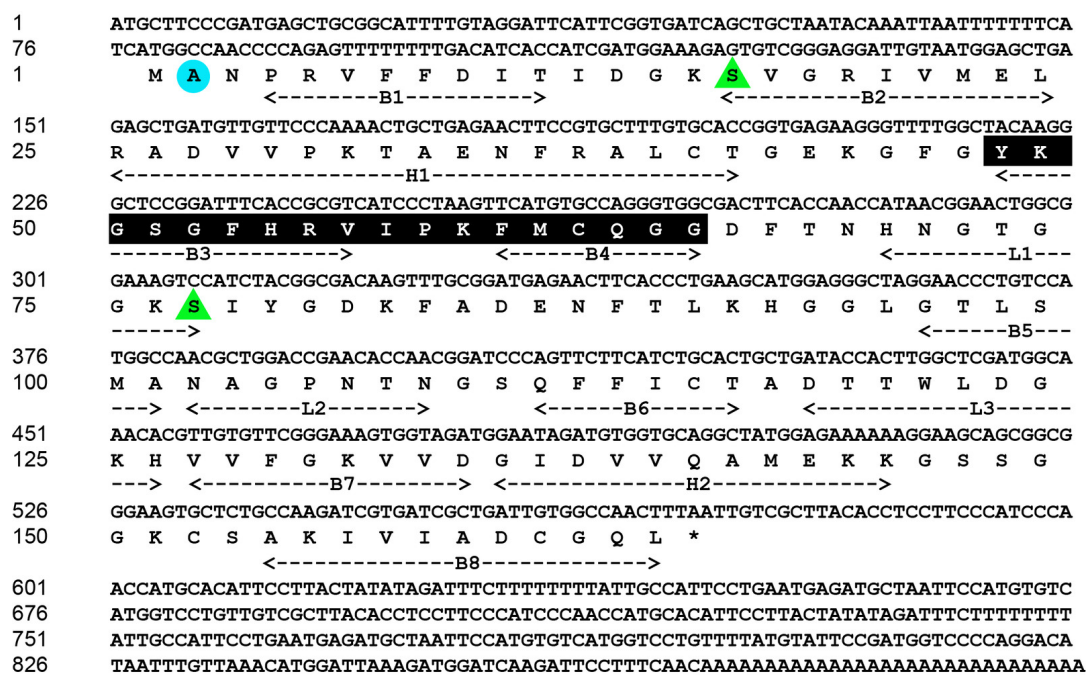


Figure 1. The complete cDNA sequence and deduced amino acid sequence of the .CypA gene in *Megalobrama amblycephala* (GenBank Accession No: PV256473).

* denotes a termination codon; A double underscore indicates a poly (A) sequence; Dark shadows show the characteristic divergent ring structure of the CypA. The green triangle shadow shows possible phosphorylation sites. The blue circle shadow shows possible acetylation sites. "B" indicates β -pleated sheet; "H" indicates α -helix; "L" indicates loop.

is 77 bp. The 3' noncoding region is 329 bp, which includes a termination codon and a poly-A tail. The open reading frame is 495 bp and it encodes 164 amino acids. The molecular weight of CypA is 17.43 KDa. The theoretical isoelectric point is 8.6. The molecular formula of *M. amblycephala* CypA amino acid sequence is $C_{768}H_{1202}N_{214}O_{230}S_{10}$, which contains 16 acidic amino acids (Asp+Glu) and 19 alkaline amino acids (Arg+Lys). The extinction coefficient is 8730 and the absorption coefficient is 0.501. The instability coefficient is 0.57, indicating that CypA is a very stable protein (Figure 1).

HOMOLOGY ANALYSIS OF CYPA IN *M. AMBLYCEPHALA*

According to comparative analysis of CypA with other fish, the similarity was as follows: *Cyprinus carpio* (94.51%), *Ctenopharyngodon idellus* (92.07%), *Carassius gibelio* (92.07%), *Labeo rohita* (92.07%), *Danio rerio* (91.46%), and *Onychostoma macrolepis* (91.46%) (Table 2). Multiple sequence comparisons between the amino acids of CypA in *M. amblycephala* and those of seven other species was carried out. Results showed that the amino acid sequence of CypA in *M. amblycephala* was similar to that of other species, and all had a peptidyl prolyl cis-trans isomerase (PPIase) family tag sequence (FKGSFHRIPGFMCGG). Moreover, they also contain essential amino acid sequences (Arg⁵⁵, His⁵⁴, Phe⁶⁰, Phe¹¹³, His¹²⁶) for PPIase activity and 13 conservative CSA binding residues in the exact location (Figure 2).

Table 2. Similarity between CypA amino acid sequences of *Megalobrama amblycephala* and other species

species	Similarity values	accession number
<i>Cyprinus carpio</i>	94.51%	XP_042621474.1
<i>Ctenopharyngodon idellus</i>	92.07%	XP_051763799.1
<i>Carassius gibelio</i>	92.07%	XP_052463475.1
<i>Labeo rohita</i>	92.07%	XP_050977433.1
<i>Danio rerio</i>	91.46%	NP_001315353.1
<i>Onychostoma macrolepis</i>	91.46%	XP_058646356.1

TERTIARY STRUCTURE OF *M. AMBLYCEPHALA* CYPA

The tertiary structure (PDB id: 2z6wB) of CypA reported by Kallen et al.¹⁷ was used as a template, and the tertiary structure of CypA in *M. amblycephala* was gained through ESyPred3D prediction (Figure 3). The tertiary structure was analyzed according to prediction results. It was found that CypA in *M. amblycephala* contained 8 β -pleated sheets and 2 α -helices, which formed a cylinder structure (Figure 3). It was speculated to form an active center region. Additionally, the tertiary structure of CypA in *M. amblycephala* shows that the Phe48-Gly65-bit amino acid sequence (FKGSFHRIPGFMCGG) forms a divergent loop structure, which is the characteristic sequence of CypA.

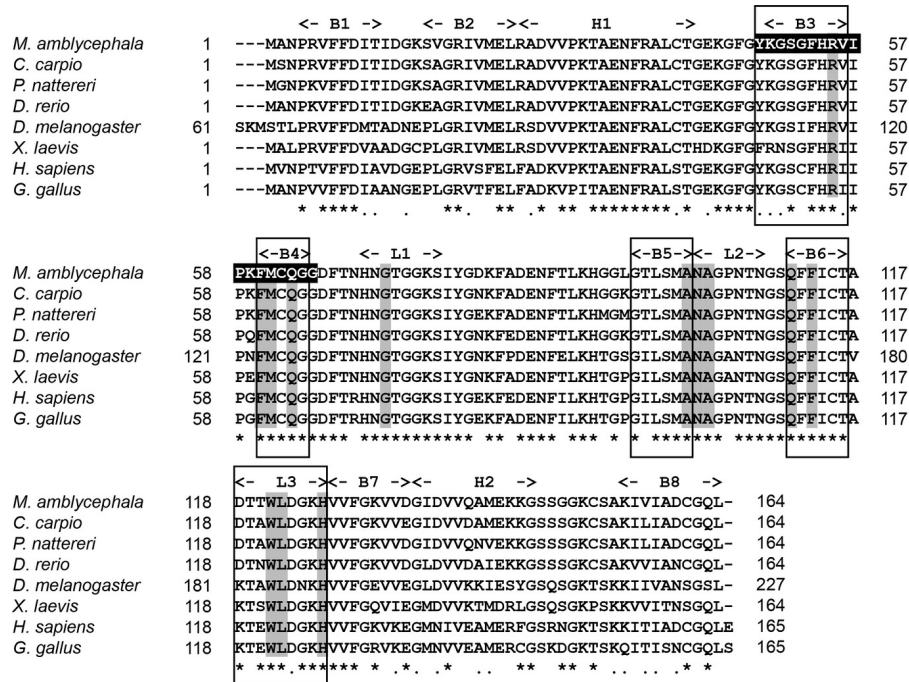


Figure 2. Multiple sequence alignment analysis of CypA amino acid sequences of *Megalobrama amblycephala* and other fishes.

The black box indicates essential amino acids Sequence for PPLase activity; the shadow indicate conserved residues which constitute the CsA-binding site; the deep shadow shows the peptidyl prolyl cis-trans isomerase family tag sequence.

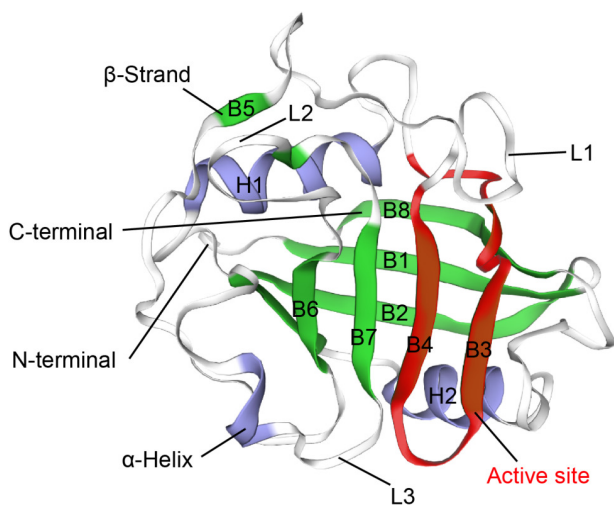


Figure 3. The tertiary structure of *Megalobrama amblycephala* CypA.

"B" indicates β-pleated sheet; "H" indicates α-helix; "L" indicates loop.

EVOLUTIONARY TREE OF THE GENETIC SYSTEM OF CYP A

The predicted amino acid sequence of *M. amblycephala* CypA was compared with those of other species, and the phylogenetic tree was constructed by the adjacency method. *M. amblycephala* CypA is clustered with CypA of Cyprinidae fish, and CypA of *M. amblycephala* is most closely related to CypA of *Cyprinus carpio* (Figure 4).

EXPRESSION OF CYP A IN DIFFERENT TISSUES OF *M. AMBLYCEPHALA*

The mRNA expression level of *M. amblycephala* CypA was highest in the head kidney, followed by liver, kidneys, and spleen. There is no significant difference between the mRNA expression levels in the gills and intestines. The relative expression level of CypA in the heart of *M. amblycephala* was the lowest (Figure 5).

IMMUNE RESPONSE OF *M. AMBLYCEPHALA* CYP A TO *A. HYDROPHILA*

M. amblycephala was infected by immersion with *A. hydrophila*. The CypA expression first increased and then decreased in the first 72 h of infection in different tissues (Figure 6). The mRNA level of CypA in the liver increased significantly at 3 h (1.73-fold), reached its peak at 12 h (6.89-fold), and then declined gradually. By 72 h, it had decreased to 2.25-fold that of the control group. The relative expression of CypA in head kidney increased significantly at 3 h (5.79-fold), reached its peak at 6 h (15.02-fold), and then decreased gradually at 12 h. The expression of CypA in gills peaked at 12 h (7.42-fold) and then decreased gradually. The expression level in gills decreased to 1.52-fold that of the control group at 72 h. The relative expression of CypA in intestines reached its maximum at 24 h, which was 9.92-fold that of the control group, and then decreased to 3.69-fold that of the control group at 72 h (Figure 6).

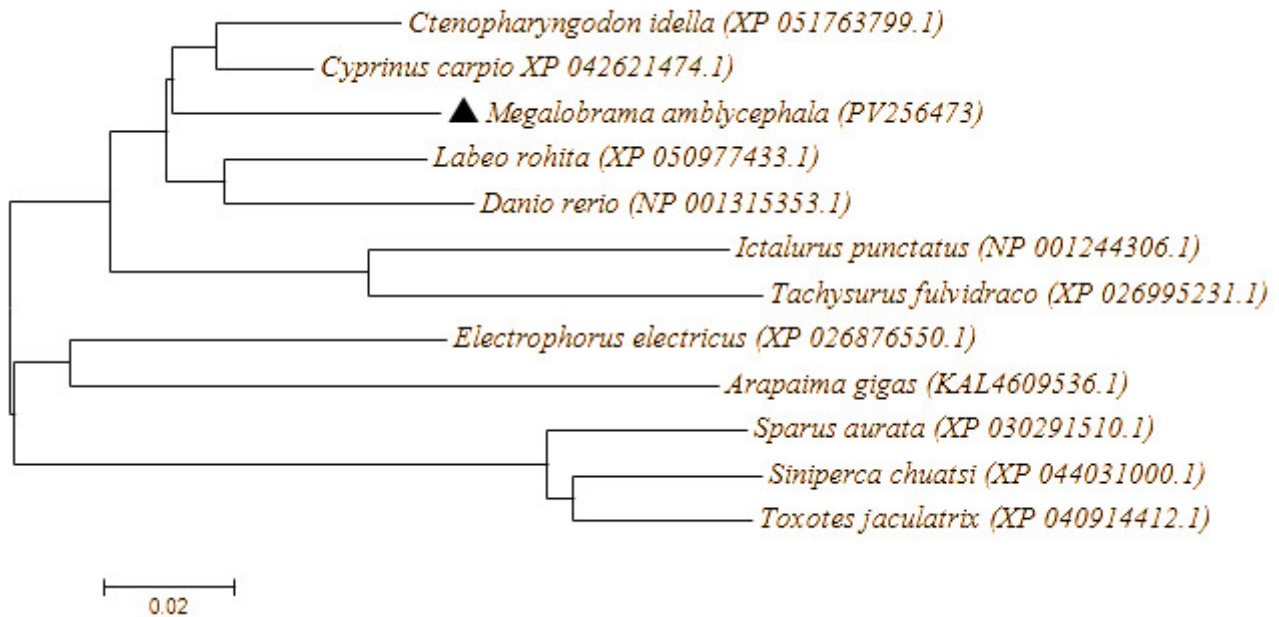


Figure 4. A phylogenetic tree of *Megalobrama amblycephala* CypA and other species was constructed using MEGA 7.0 software with the neighbour-joining method.

The bootstrap values are presented as numbers next to the branches. The evolutionary distances are given as scale bars.

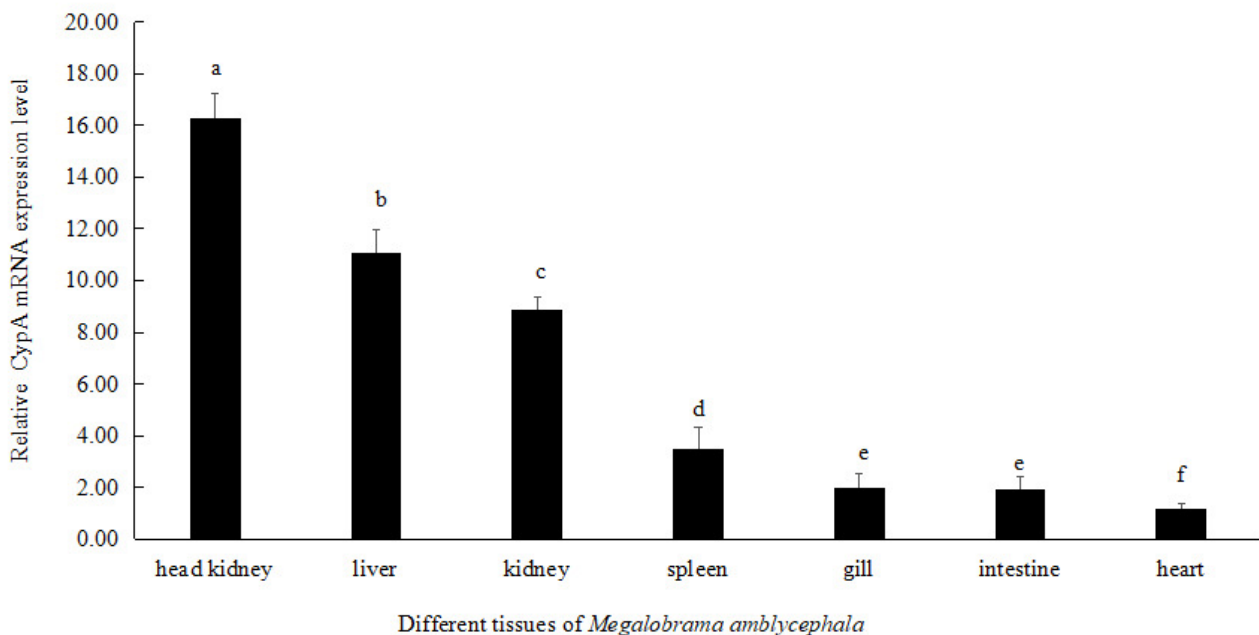


Figure 5. The level of CypA mRNA in various tissues of *Megalobrama amblycephala*.

Various letters above pillars indicated statistical significance (p < 0.05).

DISCUSSION

Cyclophilins (Cyps) are a type of multi-functional protein family with extensive natural distribution and highly conserved properties. This study obtained CypA from *M. amblycephala* through cloning for the first time. The completed cDNA sequence of *M. amblycephala* CypA was 901 bp with a 495 bp-long open reading frame at 78-572 bits,

which encodes 164 amino acids. The sequence also includes a 77 bp 5' noncoding region, a 329 bp 3' noncoding region, and a 30 bp poly(A) tail. However, no tailing signal has been found yet. Through multi-sequence comparative analysis, *M. amblycephala* CypA contained a PPIase active site. In both vertebrates and invertebrates, this site is highly conserved, and is located at the 48th to 65th amino acids (FKGSAFHRIIPGFMCGG). The conservative amino acid

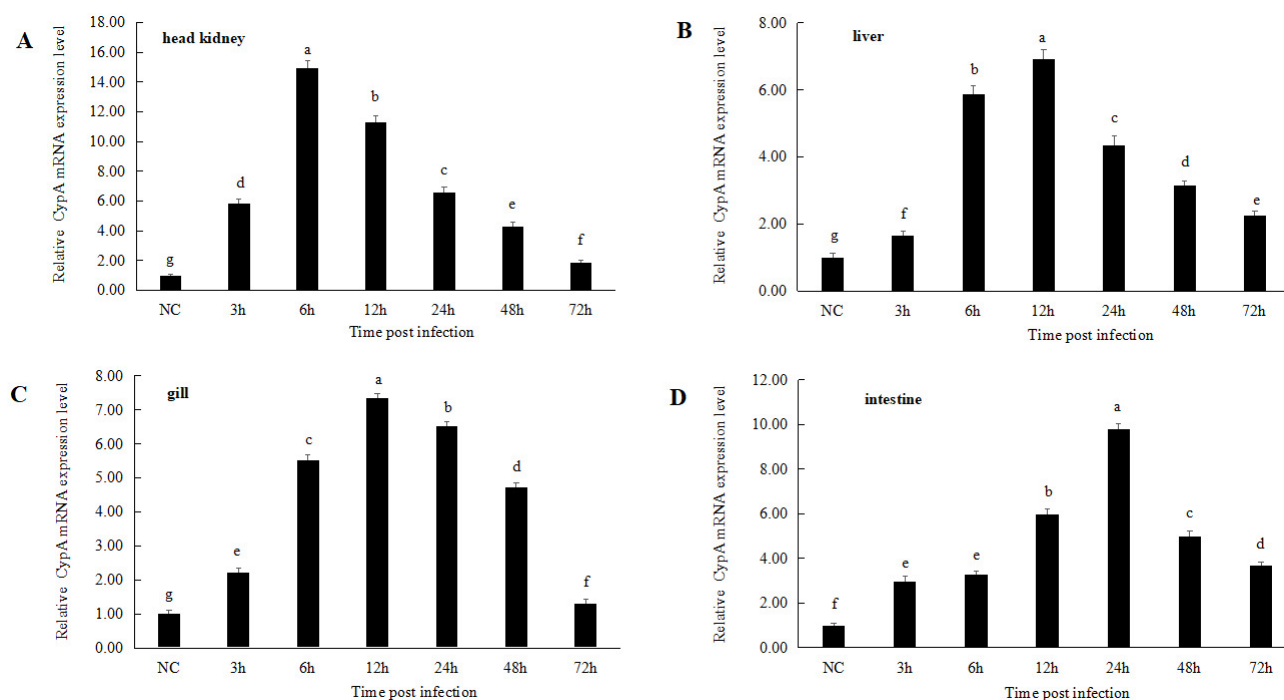


Figure 6. The changes of CypA gene expression in various tissues of *Megalobrama amblycephala* after infection *Aeromonas hydrophila* by immersion.

NC is a control group without infection. Various letters above pillars indicated statistical significance ($p < 0.05$).

residues in *M. amblycephala* CypA are crucial to the level-1 structure of CypA sequences and PPIase activity, which are related to other biological functions of CypA.^{18,19} CypA of *M. amblycephala* is a kind of PPIase and it binds with immunosuppressor CSA as an intracellular receptor.

According to the prediction of tertiary structure, it was found that *M. amblycephala* CypA is composed of 8 β -pleated sheets and 2 α -helices. Moreover, the CypA structure of *M. amblycephala* showed that the amino acid sequence (FKGSAFHRIIPGFMCQGG) at the 48-65th amino acids formed a divergent loop structure and thereby developed a characteristic sequence of cyclophilin. The evolutionary tree of the genetic system demonstrated that CypA in *Megalobrama amblycephala* is clustered with CypA in *Cyprinus carpio*, indicating the closest parentage between them.

Studies on CypA of vertebrates demonstrated that these proteins participate in many biological processes. CypA is involved in immune defense in mammals after viral infection and play a key role in pathogenesis of some inflammatory diseases in humans.²⁰ Nevertheless, in innate immune systems of aquatic animal, there exists little knowledge concerning the function of CypA. Xu Ling detected expression distributions of CypA in various tissues by qPCR and found that expressions of CypA in the liver and gonads of *Hyriopsis Schlegelii* were significantly higher compared to those in other tissues.²¹ Moreover, mRNA expression levels of *H. Schlegelii* CypA in heart and kidney are relatively higher than those in other tissues.²¹ The mRNA level of *H. Schlegelii* CypA was lowest in intestines, and there had no significant difference in the gills, axon foot, intestines, and hemolymph.²¹ CypA is expressed in the hepatopancreas,

ovaries, muscles, heart, brain, and hemolymph of *Penaeus monodon*.¹⁰ Similar results have been observed in *Chlamys farreri*; the mRNA level of *Chlamys farreri* CypA is highest in gonads, but its expression in muscles and hemolymph is the lowest.⁹ In *M. amblycephala*, CypA is expressed in the head kidney, liver, intestines, gills, spleen, kidneys, and heart, and its expression in head kidney is the highest, followed by liver, kidneys, spleen, gill and intestines. The relative expression of CypA in the heart of *M. amblycephala* is the lowest. It is suggested that *M. amblycephala* CypA might play a key role in the head kidney.

Infected by *A. hydrophila*, no significant changes were found in terms of mRNA expression of CypA in the hemolymph of *Hyriopsis Schlegelii*.²¹ However, mRNA expression level of *H. Schlegelii* CypA in the gonads increased significantly at 4 h after infection ($P < 0.05$) and then reached a peak.²¹ Expression of CypA in the gonads of *H. Schlegelii* begins to increase 2 h after infection. Still, it decreases after 8 h and reaches a valley at 24 h, after which it increases partially at 48 h.²¹ A similar law has been found in the liver of *H. Schlegelii*, after infection by *A. hydrophila*, the expression of CypA increased significantly at 4 h and then reached a peak.²¹ The mRNA expression level of *H. Schlegelii* CypA in the liver increased at 2 h after infection, then decreased at 8 h and reached a valley at 48 h.²¹ This indicates that expression of CypA might be induced by *A. hydrophila* in the gonads and liver of *H. Schlegelii*, which was in line with expression of CypA in the gonads of *Chlamys farreri* after infection by *Vibrio anguillarum*.^{9,21} After infection by *Edwardsiella tarda*, the expression level of *Ictalurus punctatus* CypA in egg cell lines was upregulated, which indicated that CypA might play a key immune role in the early devel-

opment stage of *Ictalurus punctatus* infected by *Edwardsiella tarda*.¹⁵ After LPS stimuli, expression of *Penaeus monodon* CypA in the hepatopancreas was upregulated.¹⁰ To further explore the potential functions of CypA in *M. amblycephala*, *M. amblycephala* was immersed in *A. hydrophila* for infection. The expression level of CypA in the head kidney, liver, gills, and intestines first increased and then decreased in the first 72 h after infection. Moreover, the expression level of *Megalobrama amblycephala* CypA in the head kidney peaked at 6 h. The mRNA level of CypA peaked at 12h in gills and liver and 24h in intestines. This demonstrates that *A. hydrophila* can stimulate CypA expression in *M. amblycephala*, and CypA might participate in the immune defense of fish against exogenous microbiological stimuli.

After infection by *A. hydrophila*, the mRNA level of CypA in the head kidney of *M. amblycephala* reached the peak (6 h) earlier than in the liver (12 h) and intestines (24 h). Moreover, the relative expression level of *Megalobrama amblycephala* CypA mRNA at peak was also higher in the head kidney than in liver, intestines, and gills. It was suggested that CypA might play a key role in resisting bacterial infection in the head kidney. Nevertheless, the special effect of CypA in the head kidney is still unknown, and further studies are needed to obtain a more comprehensive understanding. Further learning on CypA is conducive to exploring its functions in fish and understanding the mechanism of fish immune responses. This is also beneficial for a better understanding of the immune response mechanism of fish.

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AUTHORS' CONTRIBUTION

Conceptualization: Juanjuan Lu (Equal), Hu Xia (Equal). Methodology: Juanjuan Lu (Equal), Hu Xia (Equal), Yanan Gong (Equal). Writing – original draft: Juanjuan Lu (Equal), Hu Xia (Equal). Formal Analysis: Hu Xia (Equal), Fuyan Chen (Equal), Jianchao Bu (Equal), Pinhong Yang (Equal). Investigation: Hu Xia (Equal), Fuyan Chen (Equal), Jianchao Bu (Equal), Pinhong Yang (Equal). Writing – review & editing: Hu Xia (Equal). Funding acquisition: Hu Xia (Lead). Resources: Hu Xia (Lead). Supervision: Hu Xia (Lead).

COMPETING INTERESTS – COPE

The authors of this study declare no conflict of interest.

ETHICAL CONDUCT APPROVAL – IACUC

The experiment was conducted with approval from the IACUC (Institutional Animal Care and Use Committee) of Hunan Applied and Technology University, Changde, China.

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All are available upon reasonable request.

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