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Global characterization and expression analysis of interferon regulatory factors in response to *Aeromonas hydrophila* challenge in Chinese soft-shelled turtle (*Pelodiscus sinensis*)

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ABSTRACT

Interferon regulatory factors (IRFs) were originally identified as transcriptional regulators of type I interferon (IFN) expression. Recent studies have widely identified the roles of IRFs as central mediators in immune defence against pathogen infection. However, the functional roles and expression profiles of IRFs are still unclear in Chinese soft-shelled turtle (*Pelodiscus sinensis*). In this study, eight members of the *PsIRF* family were identified in *P. sinensis* through a genome-wide search. These PsIRF genes contained the conserved domains of this group of proteins, including the N-terminal DNA-binding domain and C-terminal IRF-associated domain. Phylogenetic analyses among IRF homologs showed that the PsIRFs shared the closest phylogenetic relationships with IRFs of other turtle species. Further molecular evolutionary analyses revealed evolutionary conservation of the PsIRF genes. Moreover, expression profiling demonstrated that eight *PsIRF* genes exhibited constitutive expression in different tissues of *P. sinensis*. Several genes, such as *PsIRF1*, *PsIRF2* and *PsIRF4*, showed predominant expression in the spleen and were significantly upregulated upon *Aeromonas hydrophila* infection. Remarkably, PsIRF1, PsIRF2 and PsIRF4 exhibited rapid increases in their protein expression levels post-infection and were mainly expressed in the splenic red pulp according to immunohistochemistry analysis. These results provide rich resources for further exploration of the roles of PsIRFs in immune regulation in *P. sinensis* and other turtles.

1. Introduction

Interferon regulatory factors (IRFs) act as transcriptional regulators of type I interferon (IFN) expression and consist of multiple transcription factors [1]. Studies suggest that IRFs exhibit various functions in immune responses, antimicrobial defence, haematopoietic differentiation and apoptosis regulation [2,3]. Generally, IRF proteins possess a conserved N-terminal DNA-binding domain (DBD) that is characterized by tryptophan-rich repeats and forms a helix-loop-helix motif [4]. The DBD domain recognizes the IFN-stimulated response element and is responsible for binding to the DNA sequence in the promoters of IFN and many other genes by various IRFs [1]. The C-terminal region of IRFs is a less well conserved IRF-associated domain (IAD), which is critical for specific protein-protein interactions and confers the diverse regulatory roles of IRF family proteins [1,4]. Both the DBD and IAD direct the functional roles of IRFs. Different numbers of IRF members are found in different species. To date, a total of 11 members of the IRF family have been identified in vertebrates [5]. Based on their evolutionary molecular relationships, IRF family members are classified into four subfamilies: IRF1 (IRF1, 2 and 11), IRF3 (IRF3 and 7), IRF4 (IRF4, 8, 9 and 10) and IRF5 (IRF5 and 6) [6,7].

Different IRF family members perform different non-redundant cellular functions and exhibit specific expression to trigger transcriptional regulation or post-translational modification [8]. An increasing number of studies are focussing on the antimicrobial immune defence roles of IRFs as well as their immunological functions [2,3]. IRF1 and IRF2, the first identified IRF family members, function not only as regulators of the IFN system but also as key transcription factors in the regulation of the cell cycle and apoptosis [4]. Despite the high sequence identity of the N-terminal region between IRF1 and IRF2, both of which act as activators [1,4], the divergent C-terminal sequences may suggest opposite functions [3,7]. IRF1 suppresses oncogene-induced

Abbreviations: Interferon regulatory factor, IRF; interferon, IFN; DNA-binding domain, DBD; IRF-associated domain, IAD; maximum likelihood, ML; nonsynonymous, *Ka*; synonymous, *Ks*; immunohistochemistry, IHC; quantitative real-time PCR, qRT-PCR

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transformation [9,10], while IRF2 inhibits the transcriptional activity of IRF1 and promotes oncogenesis by antagonizing the other protein [11]. Moreover, IRF4 and IRF8 share high sequence homology, exhibit specific expression mainly in immune-related myeloid and lymphoid cells [12] and function equivalently in determining the differentiation of pre-B-cells [13,14]. IRF4 plays a unique role in regulating lymphocyte activation and the generation of immunoglobulin-secreting plasma cells in the immune response [15], whereas IRF8 primarily regulates the proliferation of myeloid progenitor cells and their differentiation into macrophages [14,16]. In addition, it has been reported that IRF6 is not directly involved in innate immunity but plays an important role in keratinocyte differentiation [4,17]. The functions of individual IRFs in immune system regulation have largely been studied in mammals, especially in humans and mice [1]. More recently, IRF family genes have been identified and described in aquaculture species such as *Megalobrama amblycephala* [6], *Cynoglossus semilaevis* [7], *Danio rerio* [18], *Ctenopharyngodon idella* [19] and *Gadus morhua* [20]. However, there are few studies involving the genome-wide discovery and functional analysis of IRF family genes in turtles.

Chinese soft-shelled turtle (*Pelodiscus sinensis*), belonging to the ectothermic amniotic reptiles, is one of the most important commercially cultured aquaculture species in Asian countries and presents high economic value [21]. *P. sinensis* provides a specific evolutionary link between ectothermic anamniotic fishes and amphibians and endothermic amniotic birds and mammals [22]. Thus, using *P. sinensis* as a model animal, numerous recent studies involving the characterization and functional analysis of critical genes involved in various biological processes and stress responses have been performed [23–25]. Furthermore, the public genomic sequences of *P. sinensis* [26] and reported RNA-seq data [27,28] provide useful resources for gene discovery and description in *P. sinensis*. Notably, as *P. sinensis* is a high-value freshwater turtle, the intensive farming of this species is rapidly expanding and intensifying. Infectious diseases are also steadily increasing in the aquaculture of *P. sinensis*, leading to serious economic losses [23,29]. *Aeromonas hydrophila*, a Gram-negative bacterium, is one of the most frequent lethal pathogens in aquatic species. Recently, infection with *A. hydrophila* has been repeatedly reported to cause various diseases in *P. sinensis*, such as septicaemia, furunculosis and red neck diseases [23,24,29]. Therefore, understanding the immune responses to bacterial infections and the regulatory roles of immunity-related IRFs is necessary for the sustainable farming of *P. sinensis*. In this study, the major aims were to analyse the evolutionary conservation and structural features of PsIRF family genes and to examine the expression profile of PsIRFs in different tissues. Moreover, the dynamic expression of PsIRF at the mRNA and protein levels in response to *A. hydrophila* challenge was detected. The results of this study will provide insights for in-depth functional studies of PsIRF genes in the immune response of *P. sinensis* and other turtles.

2. Materials and methods

2.1. Ethics statement

All experimental procedures and animal care were approved by the Animal Research Institute Committee of Northwest A&F University, Shaanxi, China. The protocol was approved by the Science and Technology Agency of Shaanxi Province under permit NO. SYXK (SN) 2018–0003. The field studies did not involve any endangered or protected species. All efforts were made to minimize animal suffering.

2.2. Animals and bacterial challenge

Eighteen three-year-old healthy *P. sinensis* with an average weight of 1.0 ± 0.15 kg were obtained from Yangcheng Lake in Suzhou (31°N, 120°E), Jiangsu, China. Turtles showed no clinical signs (such as septicaemia, furunculosis, soft and deformed dorsal shell, oncotic neck, red neck, white abdominal shell, cankered leg and facial deformity) or laboratory evidence of *A. hydrophila* or other infections and were acclimated in the laboratory for one week before the experiments. During the acclimation period, turtles were breeding in a recirculating water tank with fresh water at 25 °C and were fed with commercial standard diets. After acclimation, these turtles were divided into different groups and subjected to different treatment. Prior to collecting the tissues, turtles were euthanized and killed by cervical dislocation. All the tissue samples were immediately harvested after euthanasia within 15 min.

For gene expression analysis, samples of different tissue, including the heart, spleen, blood, liver, intestine and kidney, were collected from three turtles without infection and immediately stored in liquid nitrogen. For the bacterial challenge experiment, twelve turtles were intraperitoneally challenged with live cells of *A. hydrophila* strain AS 1.927 (10^6 CFU; China General Microbiological Culture Collection Center, Beijing, China) according to a previous report [24]. The spleen was separately collected at 6 h, 12 h, 24 h and 48 h post-challenge with three replicates. Additionally, spleen samples from three turtles that were intraperitoneally injected with an equal volume of PBS were obtained and considered as the control samples at 0 h post-challenge. A portion of the spleen samples was immediately fixed for immunohistochemistry (IHC) analysis. The other portion of the spleen samples was immediately placed in liquid nitrogen and stored for total RNA extraction.

2.3. Identification of PsIRF genes and sequence analysis

The human and mouse IRF protein sequences were obtained from the NCBI database and were used as BLASTP query sequences to search against the genomic sequences of *P. sinensis* [26]. Furthermore, the proteins with an IRF domain (pfam00605) were searched against the genomic sequences of *P. sinensis* using the HMMER (Hidden Markov Model, HMM, version 3.2.1) search tool with an *E*-value cut-off of 1.0 [30]. The candidate IRF proteins were further confirmed through domain analysis against public databases including the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/cdd), Pfam (http://pfam.xfam.org) and SMART (http://smart.embl-heidelberg.de) and were referred to as PsIRFs based on their homologous genes and annotation descriptions. Both the nucleotide and amino acid sequences of the PsIRF genes were obtained and used for sequence analysis. The conserved domains of the PsIRF proteins were predicted by SMART searching with the default parameters. The physical and chemical characteristics of the PsIRFs were analysed using the ProtParam tool of ExPASy (http://web.expasy.org/protparam).

2.4. Phylogenetic analysis and syntenic analysis of IRF homologs

Protein sequences of IRF family genes from eight species, including humans (*Homo sapiens*), house mouse (*Mus musculus*), chicken (*Gallus gallus*), green sea turtle (*Chelonia mydas*), western painted turtle (*Chrysemys picta*), three-toed box turtle (*Terrapene mexicana*), African clawed frog (*Xenopus laevis*) and zebrafish (*Danio rerio*), were downloaded from the NCBI database and used to analyse the phylogenetic relationships among IRF-homologous proteins. Detailed information on the IRF sequences used for phylogenetic analysis is provided in Supplementary Table S1. Multiple sequence alignment was performed using MUSCLE software. The phylogenetic tree was constructed by using MEGA 7.0 software based on the maximum likelihood (ML) method and bootstrap values with 1000 replications. The subfamilies classified by phylogenetic analysis were given names according to previous studies [6,7]. Based on the information in the NCBI database, the neighbouring genes surrounding the IRFs in syntenic regions were searched and compared among *P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana*. Syntenic analysis was performed according to these neighbouring genes.

Table 1
Identification of *PsIRF* family genes in *Pelodiscus sinensis*.

Name	Location	Nucleotide ID	Protein ID	Nucleotide length	Amino acid length	CDS status
PsIRF1	NW_005856644.1	XM_006128514.3	XP_006128576.1	951	316	Complete
PsIRF2	NW_005857027.1	XM_006130171.3	XP_006130233.1	1050	349	Complete
PsIRF4	NW_005871026.1	XM_014581444.2	XP_014436930.1	1341	446	Complete
PsIRF4L	NW_005857009.1	XM_014576956.2	XP_014432442.1	1218	405	Complete
PsIRF5L	NW_005851206.1	XM_025180754.1	XP_025036539.1	541	180	Partial
PsIRF6	NW_005853904.1	XM_025182446.1	XP_025038231.1	1383	460	Complete
PsIRF7	NW_005855646.1	XM_006124885.3	XP_006124947.2	1614	537	Complete
PsIRF8	NW_005871002.1	XM_006137362.3	XP_006137424.1	1290	429	Complete

2.5. Detection of positive selection

Pairwise comparisons of IRF homologs were performed by calculating the *Ka* (nonsynonymous) and *Ks* (synonymous) substitution rates. The amino acid sequences of the IRFs were aligned using ClustalX software. The alignment results and the corresponding coding sequences were submitted to PAL2NAL (http://www.bork.embl.de/pal2nal) for conversion of the file format. Then, KaKs Calculator 2.0 software was used to estimate *Ka* and *Ks* values and the *Ka/Ks* ratio (ω value) with the ML method and default parameters. $\omega > 1$ indicates positive selection; $\omega < 1$ indicates negative/purifying selection; and $\omega = 1$ represents neutral evolution [31]. A boxplot was generated by using the R program.

2.6. Protein structure prediction

The protein 3D structure of IRFs was constructed via the homology model method. The Phyre2 server was used to predict protein structures on the basis of known templates. The DBD domains of IRF1-, IRF2- and IRF4-homologous proteins were analysed using the c2dllA template, while the IRF6 DBD domain was analysed using the c2o61A template. The IAD domains of IRF4- and IRF6-homologous proteins were analysed using c5bviB and c3dshA templates, respectively. PyMOL Viewer software was used to view 3D structures and to perform structural comparisons.

2.7. Expression analysis of PsIRF genes

Quantitative real-time PCR (qRT-PCR) was performed to detect the expression patterns of *PsIRF* genes. Total RNA from each sample was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Samples from the heart, spleen, blood, liver, intestine and kidney were used for tissue-specific expression analysis. Samples from *A. hydrophila*-infected spleens were used for dynamic expression analysis. The qRT-PCR analysis was performed according to a previous report [24], with three biological replicates and three technological replicates. Gene-specific primers (Supplementary Table S2) were designed using Beacon Designer software (Premier Biosoft International, USA). The *P. sinensis β-Actin* (EU727174.1) gene was used as an internal control. Relative expression levels of *PsIRF* genes were calculated using the $2^{-\Delta\Delta CT}$ method.

2.8. Immunohistochemistry (IHC) analysis

IHC analysis was performed to assess the PsIRF protein expression according to previously described methods [28]. Briefly, spleen sections were deparaffinized in xylene, dehydrated in an ethanol series and blocked with 3% H_2O_2 in distilled water. The primary anti-IRF1 (ab186384, Abcam Inc., Cambridge, MA, USA; 1:200), anti-IRF2 (ab124744, Abcam Inc., Cambridge, MA, USA; 1:50) and anti-MUM1/IRF4 (ab104803, Abcam Inc., Cambridge, MA, USA; 1:100) antibodies were incubated with the sections overnight at 4 °C. The negative controls were reacted with PBS instead of the specific antibodies. A biotinylated secondary antibody and Vector ABC reagent (Vector Laboratories, Burlingame, CA) were subsequently added according to the manufacturer's instructions, followed by staining using the FAST DAB Peroxidase Substrate (Sigma, St. Louis, MO, USA) and counterstaining with haematoxylin for 10 s. The slides were dehydrated and observed under a light microscope. The validity of the antibody in *P. sinensis* was determined through Western blot analysis.

2.9. Statistical analysis

Statistical analysis was performed with SPSS 16.0 software (Inc., Chicago, IL, USA). The significance of differences was assessed by one-way ANOVA. A *P*-value < 0.05 was considered statistically significant. All data are presented as the means ± SEM (standard error of the mean).

3. Results

3.1. Identification and analysis of PsIRF family genes

In this study, genome-wide searches were performed against the genome sequences of *P. sinensis*, resulting in the identification of eight candidate *PsIRF* sequences (Table 1). After verification in the public NCBI, Pfam and SMART databases, these candidate sequences were considered to be *PsIRF* family genes in *P. sinensis*. Both the nucleotide and amino acid sequences of eight *PsIRF* genes were obtained and are presented in Supplementary Table S3. These *PsIRF* genes, except for *PsIRF5L*, included the complete open reading frame (ORF). Among these full-length *PsIRF* sequences, *PsIRF1* exhibited the minimum length of its nucleotide (951) and amino acid (316) sequences (Table 1). Further physiological and biochemical property analyses showed that *PsIRF1* presented a minimum molecular weight of 36.3 kDa (Supplementary Table S3). More information on the physiological and biochemical characteristics of *PsIRF* family genes, including their molecular weights, theoretical pI values, instability indexes, aliphatic indexes and grand average of hydropathicity (GRAVY), are also provided. Phylogenetic analysis showed that these PsIRF proteins were obviously classified into three subgroups (Fig. 1A). Conserved domain analyses of the PsIRF proteins revealed that all the PsIRFs contained the conserved IRF motif in the N-terminal region (Fig. 1B). Most of these PsIRFs, except PsIRF1, PsIRF2 and PsIRF5L, also exhibited the IRF-3 motif in the C-terminal region. These results showed that the PsIRFs sharing close phylogenetic relationships presented similar protein domains and structures (Fig. 1), suggesting possible similar biological functions in *P. sinensis*.

3.2. Phylogenetic relationship and classification of IRF homologs

The IRF family genes from eight other species, including *H. sapiens*, *M. musculus*, *G. gallus*, *C. mydas*, *C. picta*, *T. mexicana*, *X. laevis* and *D. rerio*, were searched against their genome sequences, and the sequences

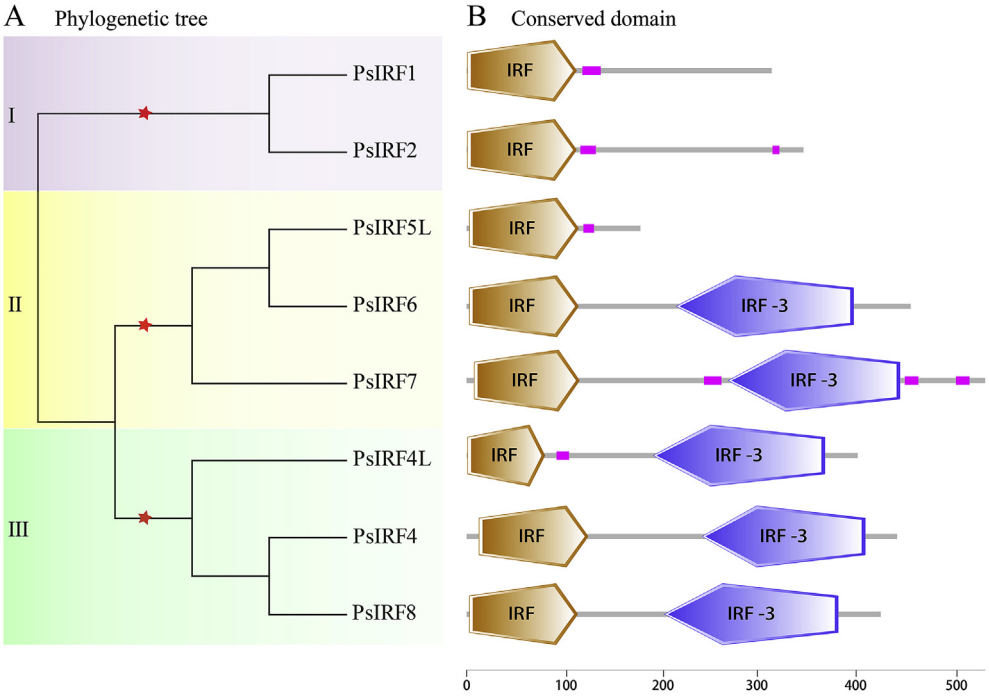

Fig. 1. Identification of PsIRF family genes in *Pelodiscus sinensis*. A: Phylogenetic analysis and classification of PsIRF genes. I, II, III and asterisks indicate the different subgroups. B: The conserved domains of PsIRF genes.

were obtained in the NCBI database. Evolutionary and comparative analysis revealed that *P. sinensis* exhibited the closest evolutionary relationships with *C. mydas*, *C. picta* and *T. mexicana* and harboured similar members of the IRF family (Fig. 2A). All four turtle species (*P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana*) belong to the cryptodira branch. Most of selected species presented no IRF10 gene, except *D. rerio*, which exhibited one IRF10 member. The same IRF family members were found between *H. sapiens* and *M. musculus*. The amino acid sequences of IRF family genes from *P. sinensis* and eight other species were used for phylogenetic analysis. A phylogenetic tree was constructed and showed that these IRF genes were classified into four major subfamilies, which were referred to as the IRF1, IRF3, IRF4 and IRF5 subfamilies (Fig. 2B). The largest subfamily was the IRF4 subfamily, which consisted of IRF4 and IRF8 homologs. The IRF1 subfamily was the second largest subfamily and consisted of IRF1 and IRF2 homologs. Notably, PsIRF genes presented closer phylogenetic relationships with their homologous genes from *C. mydas*, *C. picta* and *T. mexicana*, which is in accord with the evolutionary relationships among these turtle species. Importantly, four genes (IRF1, IRF2, IRF4 and IRF6) were commonly found in *P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana*, suggesting evolutionary conservation of these genes.

3.3. Comparison and syntenic analysis of IRF genes

To investigate the syntenic relationships of IRF1, IRF2, IRF4 and IRF6 homologs among *P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana*, the genomic localization and neighbouring genes of the IRFs were analysed separately. The genomic distribution showed that eight *PsIRF* family genes were located on different scaffolds (Supplementary Table S3). The organization of the surrounding genes of the IRFs was compared among *P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana* (Fig. 3). Syntenic analysis revealed that the up- and down-stream genes surrounding the IRFs were highly conserved in these turtles, further suggesting the evolutionary conservation of IRF family genes. In addition, the same gene order and orientation of tandemly organized genes were found in the neighbouring regions of IRFs among the four species.

3.4. Molecular evolutionary analysis of IRFs

To further assess the evolution of the IRF1, IRF2, IRF4 and IRF6 genes among the four turtle species (*P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana*), the ω value (*Ka/Ks* ratio) was used to estimate evolution rates and selective pressure. The average ω values of the IRF1, IRF2, IRF4 and IRF6 homologs were less than 1 (Fig. 4A), suggesting that the evolutionary pattern of IRFs was conserved among the four turtle species. Moreover, the average ω values displayed the pattern IRF1 > IRF2 > IRF4 > IRF6 in the four groups of tested IRF genes. In addition, pairwise comparisons of *PsIRF* family members in *P. sinensis* were analysed, and the ω values were calculated (Fig. 5). The results showed that the ω values of pairwise *PsIRFs* were less than 1, indicating that the evolution of the *PsIRF* family occurred under the influence of strong purifying selection.

Moreover, the protein 3D structures of IRF1-, IRF2-, IRF4- and IRF6-homologous proteins were predicted and compared among *P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana*. The alignment results for IRF protein structures are shown in Fig. 4B–G. The IRFs from *P. sinensis* (green), *C. mydas* (blue), *C. picta* (red) and *T. mexicana* (yellow) are indicated in different colours. The structures of the IRF1 and IRF2 homologs only exhibited the DBD domains and showed high similarity between the four species (Fig. 4B and C). Both the IRF4 and IRF6 protein homologs contained the DBD and IAD domains. The structures of the two major domains overlapped between IRF4 homologs (Fig. 4D and E). For the IRF6 homologs, the proteins were highly similar between *P. sinensis* and *C. mydas* but were slightly different from those of *C. picta* and *T. mexicana* (Fig. 4F and G).

3.5. Tissue-specific expression of PsIRF genes

The relative expression levels of *PsIRF* genes in the heart, spleen, blood, liver, intestine and kidney of *P. sinensis* were validated by qRT-PCR analysis. Expression profiling revealed that eight *PsIRF* genes were constitutively expressed in the six healthy tissues, although these genes presented different expression patterns in different tissues (Fig. 6). Both the *PsIRF1* and *PsIRF2* genes exhibited higher expression levels in the

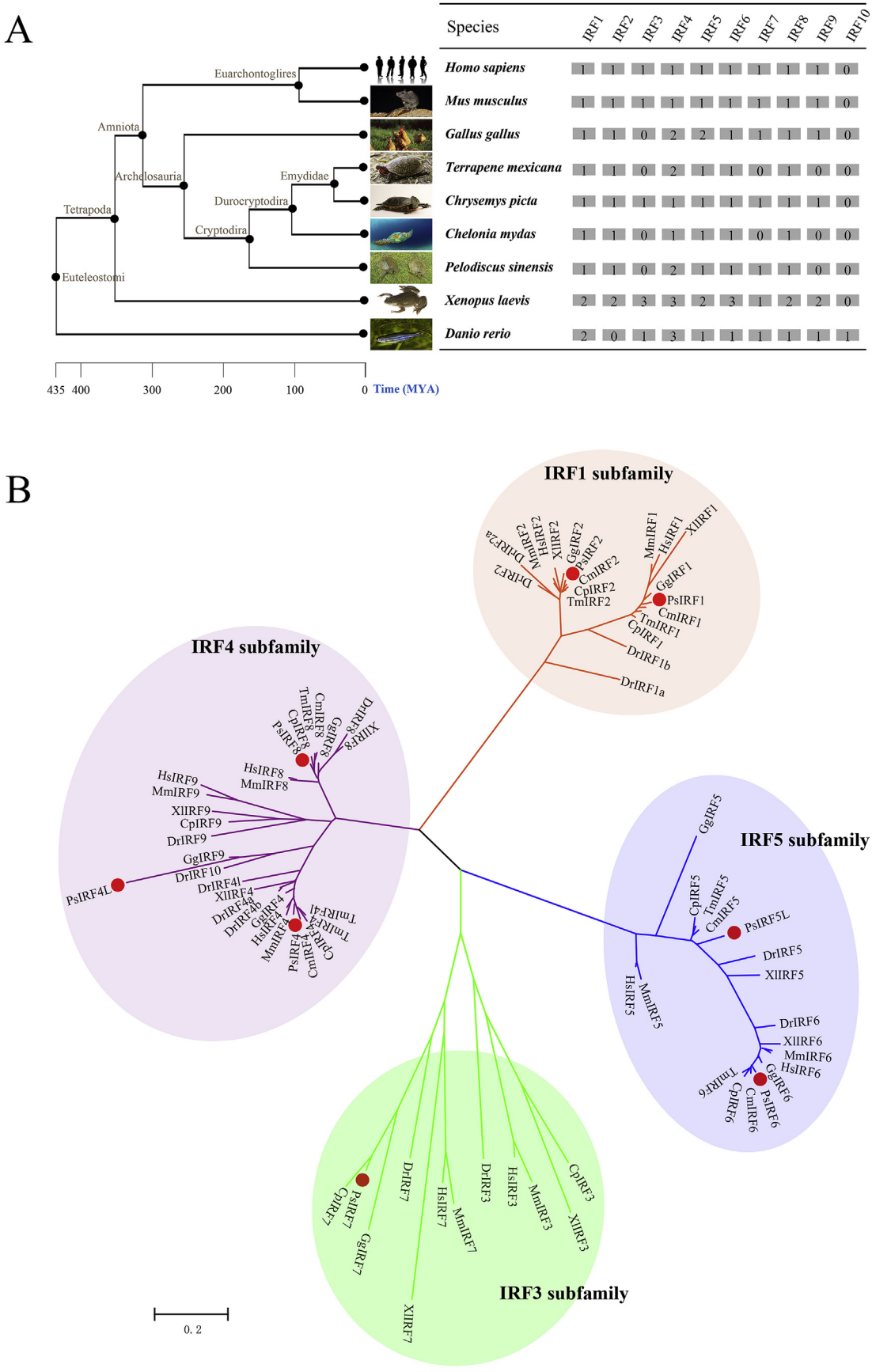

Fig. 2. Overview of IRF family genes in different species. A: Statistics for the numbers of IRF family genes. MYA: million years ago. B: Phylogenetic analysis and classification of IRF-homologous genes. Red circles indicate the PsIRFs of *Pelodiscus sinensis*. Hs: *Homo sapiens*, Mm: *Mus musculus*, Gg: *Gallus gallus*, Tm: *Terrapene mexicana*, Cp: *Chrysemys picta*, Cm: *Chelonia mydas*, Xl: *Xenopus laevis*, Dr: *Danio rerio*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

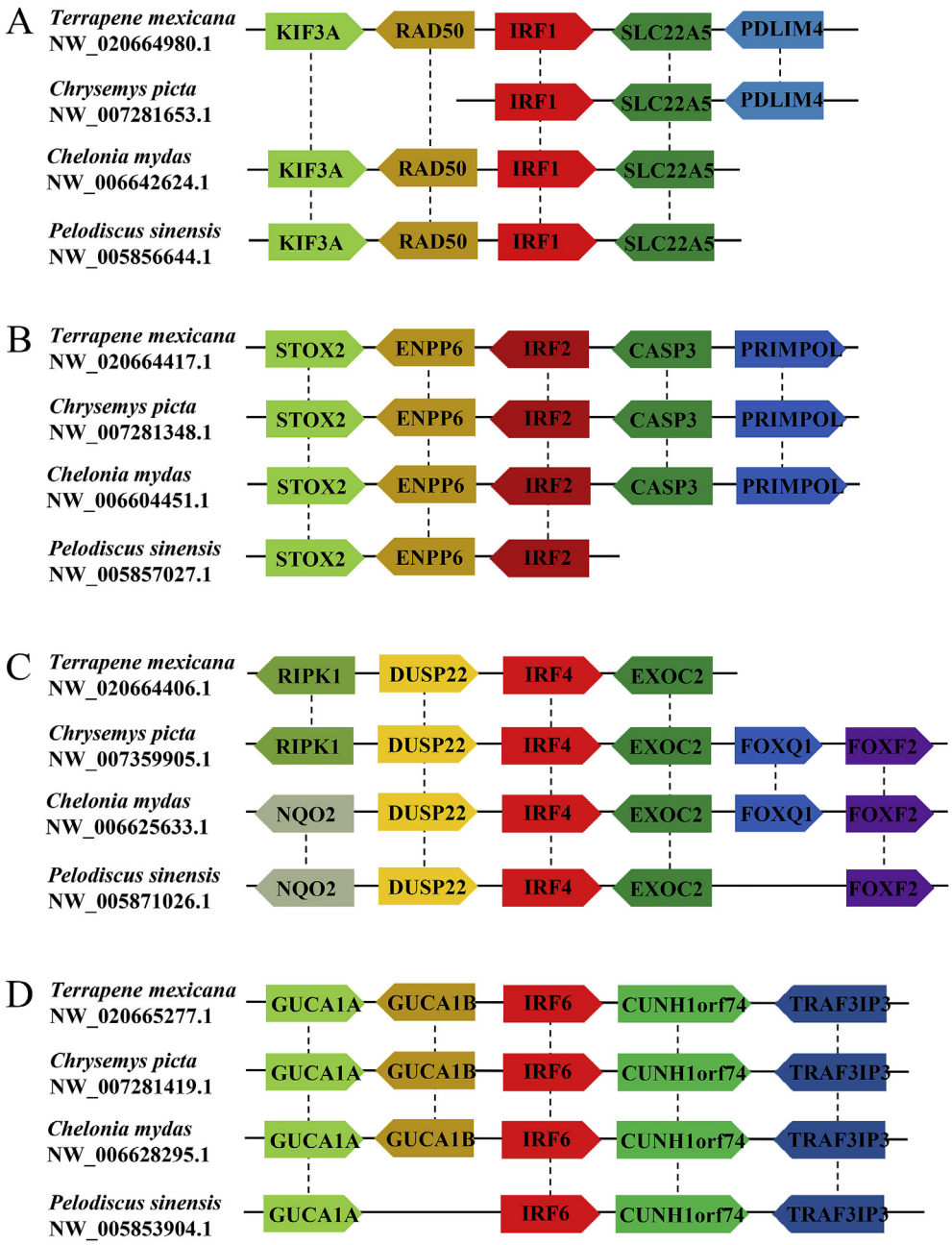

Fig. 3. Syntenic analyses of IRF1 (A), IRF2 (B), IRF4 (C) and IRF6 (D) homologs among *Terrapene mexicana*, *Chrysemys picta*, *Chelonia mydas* and *Pelodiscus sinensis*.

spleen and kidney, whereas they presented lower expression levels in the liver. Similar expression patterns were detected between the *PsIRF4* and *PsIRF4L* genes, which exhibited the highest expression levels in the spleen and blood. The *PsIRF5L* gene showed the highest expression in the heart, while *PsIRF6* and *PsIRF7* genes showed the highest expression in the liver. In addition, *PsIRF8* was highly expressed in the intestine. The tissue-specific expression patterns of *PsIRF* genes provide rich resources for their functional exploration.

3.6. Dynamic expression of PsIRFs in response to A. hydrophila infection

To examine the dynamic expression of *PsIRF* genes in response to bacterial infection, the expression patterns of *PsIRFs* were analysed at 0 h, 6 h, 12 h, 24 h and 48 h post-*A. hydrophila* challenge. QRT-PCR analysis was performed to detect the relative expression levels of *PsIRFs* in the spleen, which is one of the main immune organs in *P. sinensis*. These *PsIRF* genes were significantly differentially expressed and showed time-dependent expression patterns upon *A. hydrophila* infection (Fig. 7). The expression of most *PsIRF* genes was upregulated post-infection. Notably, the expression levels of several genes, such as *PsIRF4*, *PsIRF4L*, *PsIRF7* and *PsIRF8*, were immediately increased at 6 h. Thereafter, the *PsIRF4* and *PsIRF4L* genes were downregulated at 24 h, while *PsIRF8* expression was decreased at 12 h and then increased until 48 h. The expression of *PsIRF1* and *PsIRF2* was increased after infection and peaked at 24 h. The highest expression of *PsIRF6* was observed at 12 h and then slightly decreased until 48 h. In addition, the expression of the *PsIRF5L* gene was slightly decreased at 6 h and then gradually increased until 48 h.

3.7. Localization and expression of PsIRF proteins in the spleen after A. hydrophila infection

Expression analysis of *PsIRF* mRNA revealed that the *PsIRF1*, *PsIRF2* and *PsIRF4* genes were strongly highly expressed in the spleen (Fig. 6),

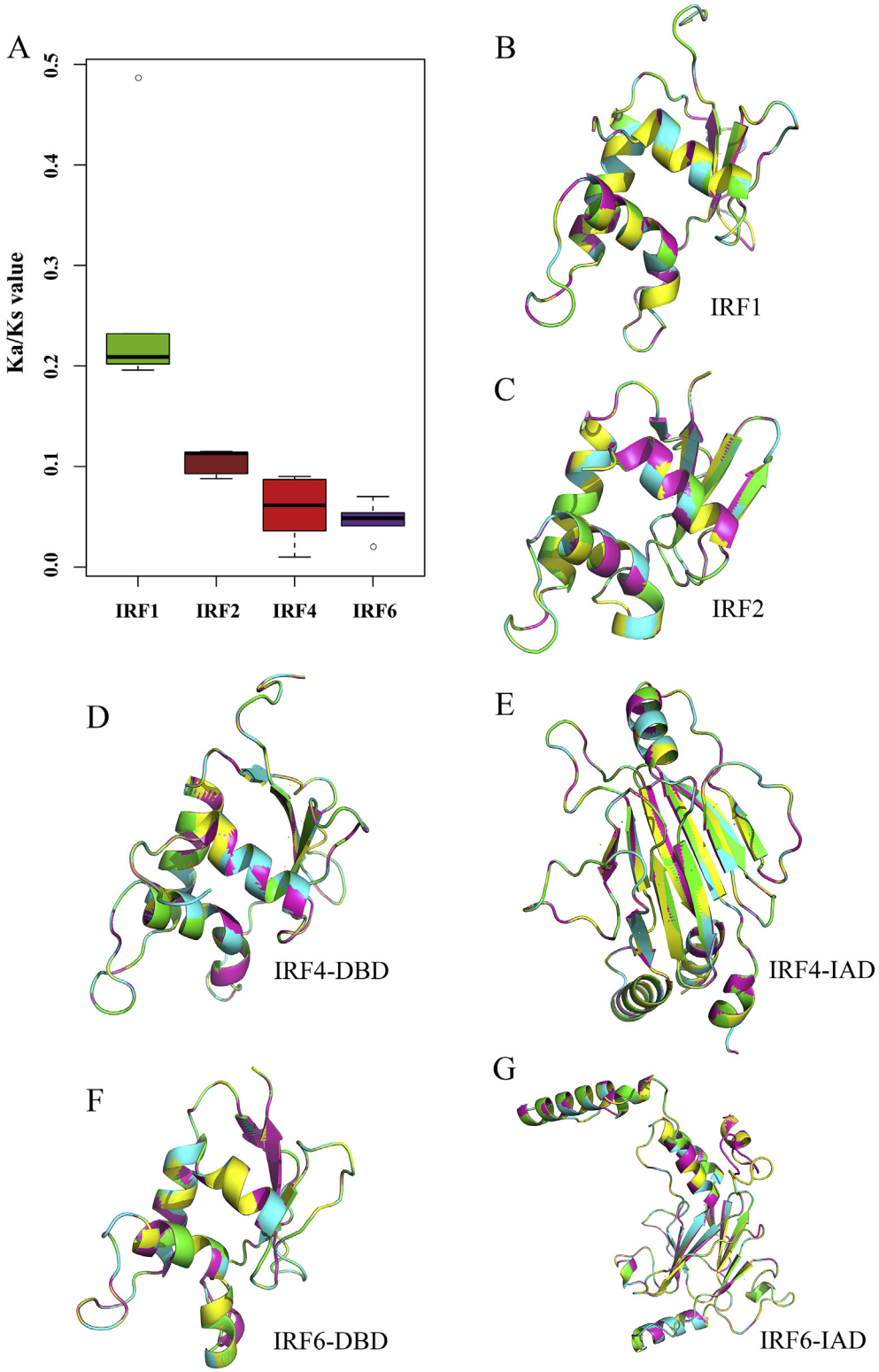

Fig. 4. Evolutionary analyses of IRF1, IRF2, IRF4 and IRF6 homologs among *Terrapene mexicana*, *Chrysemys picta*, *Chelonia mydas* and *Pelodiscus sinensis*. A: Comparison of *Ka/Ks* values of IRF1, IRF2, IRF4 and IRF6 homologs. B, C: Structural comparison of IRF1- and IRF2-homologous proteins. D, E: Structural comparison of the DNA-binding domain (DBD) and IRF-associated domain (IAD) of IRF4 homologs. F, G: Structural comparison of the DBD and IAD of IRF6 homologs. The predicted protein structures in *T. mexicana* (yellow), *C. picta* (red), *C. mydas* (blue) and *P. sinensis* (green) are indicated in different colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and their expression levels were increased at 6 h after *A. hydrophila* infection (Fig. 7). Furthermore, IHC analysis was performed to detect the localization and expression variations of the PsIRF1, PsIRF2 and PsIRF4 proteins in the spleen of *P. sinensis* at 0 h, 6 h and 12 h after *A. hydrophila* infection. Positive reactions for the PsIRF1, PsIRF2 and PsIRF4 proteins were observed in similar spleen sections, and the PsIRF1, PsIRF2 and PsIRF4 proteins were expressed at three time points post-infection (Fig. 8). Immunostaining was primarily detected in the splenic red pulp, whereas little immunostaining was detected in the splenic white pulp, consisting of the periarteriolar lymphatic sheath (PALS) and periellipsoidial lymphatic sheath (PELS). Moreover, the variation in protein expression was similar between the PsIRF1, PsIRF2 and PsIRF4 proteins in response to *A. hydrophila* infection. Weak immunostaining of the three proteins was observed at 0 h, whereas intense immunostaining was observed at 6 h. In addition, moderate immunostaining was observed at 12 h, with the exception of sustained

	PsIRF1	PsIRF2	PsIRF4	PsIRF4L	PsIRF5L	PsIRF6	PsIRF7	PsIRF8
PsIRF1	NA							
PsIRF2	0.135	NA						
PsIRF4	0.612	0.347	NA					
PsIRF4L	0.485	0.457	0.136	NA				
PsIRF5L	0.351	0.345	0.251	0.244	NA			
PsIRF6	0.517	0.527	0.407	0.381	0.102	NA		
PsIRF7	0.507	0.514	0.345	0.332	0.276	0.381	NA	
PsIRF8	0.427	0.416	0.112	0.135	0.237	0.316	0.394	NA

Fig. 5. Estimation of *Ka/Ks* values among PsIRF family genes using the maximum likelihood (ML) method.

intense immunostaining of the PsIRF4 protein at this time point. No staining was detected in the negative control sections. Upon *A. hydrophila* challenge, the protein expression changes in PsIRF1, PsIRF2 and PsIRF4 were consistent with their mRNA expression patterns according to qRT-PCR analysis. The rapid responses of the PsIRF1, PsIRF2 and PsIRF4 genes to bacterial infection indicated their putative roles in immune defence.

4. Discussion

Innate immunity is the first line of defence, provides crucial signals for activating immune responses against pathogen infections and results in the production of lists of inflammatory cytokines [32], such as type I IFN, interleukin (IL) and tumour necrosis factor (TNF). In the IFN signalling pathway, IRFs are key regulators of type I IFN and are vital for directing immune responses to pathogens [1]. In view of the important roles of IRFs in regulating gene expression in the immune system [2,3], many studies have focused on the functional discovery of IRF family members. Moreover, genome-wide identification and analyses of IRF family genes have been reported in many species [6,7,18–20]. Nevertheless, information on the characteristics and expression profiles of IRF family genes in *P. sinensis* is still deficient. *P. sinensis* is an important aquaculture reptile in China and holds a special evolutionary position and significance as a potential novel animal model. In this study, global searches against the genome sequences of *P. sinensis* identified a total of eight *PsIRF* family genes, and these PsIRFs were systematically characterized at the genome-wide level for the first time in *P. sinensis*.

4.1. Evolutionary conservation of PsIRF family genes

The basic structural features of IRF transcription factors are essential for their intrinsic roles in manipulating target IFN expression and immune responses [1,4]. A conserved N-terminal DBD domain is typical of IRF genes and is necessary for recognizing and binding to specific elements in target promoter sequences [1]. In the present study, structural analyses revealed that an IRF domain was well conserved among all PsIRFs, matching the specific characteristics of IRF transcription factors [1,6,7,20]. The amino acid sequences in the C-terminal region showed more diversity within these PsIRF members. The diversification of IRF C-terminal sequences favours associations with other transcription factors [1,4]. PsIRF1 and PsIRF2 shared structural similarity in the DBD domain and exhibited the closest phylogenetic relationships, indicating that they may play similar roles. The sequence differences between PsIRF genes are indispensable for their distinctive functions.

The expansion of gene family members accompanies the evolution of species [33]. Most vertebrates generally exhibit nine IRF members [5], while fishes harbour more potential IRF members [18]. The number of *PsIRF* family genes in *P. sinensis* is comparable to the number of *IRFs* in other species, including other turtles, humans, mice and chickens, whereas the number is lower than that in zebrafish, as a representative of fish species [18]. Analysis of the phylogenetic relationships between *P. sinensis* and other species was further performed and provided a solid foundation for better understanding the function of *PsIRF* genes [6,7,20]. The majority of PsIRF genes were closer to the corresponding homologous genes from *C. mydas*, *C. picta* and *T. mexicana* in terms of their phylogenetic relationships. Species evolution analysis showed that *P. sinensis* and three other turtle species, *C. mydas*, *C. picta* and *T. mexicana*, belong to the cryptodira and diverged approximately 138–185 million years ago (MYA). The similarity of *IRF* family members among *P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana* is consistent with the closer evolutionary relationships between these species, which demonstrates the evolutionary similarity of *IRF* genes in the four turtles.

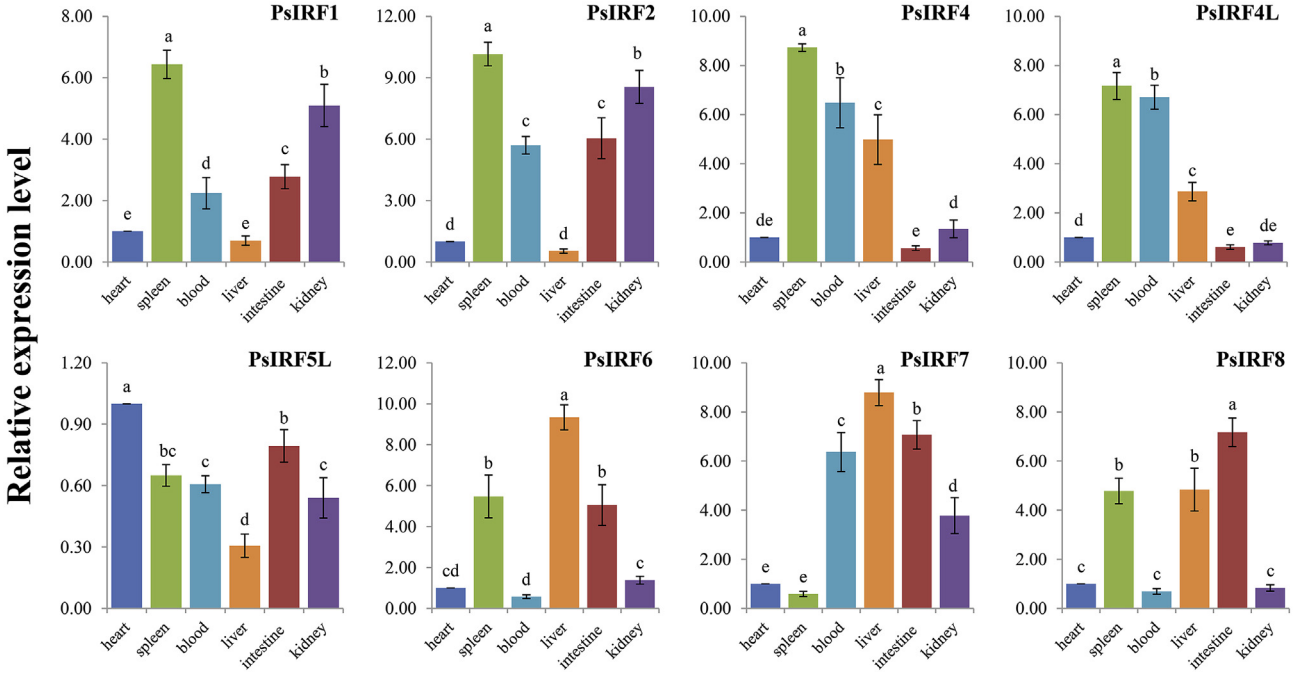

Fig. 6. Expression pattern of *PsIRF* genes in different tissues (heart, spleen, blood, liver, intestine and kidney) of *Pelodiscus sinensis*. The values with different letters are significantly different at $P < 0.05$. Each bar shows the mean ± SEM of triplicate assays.

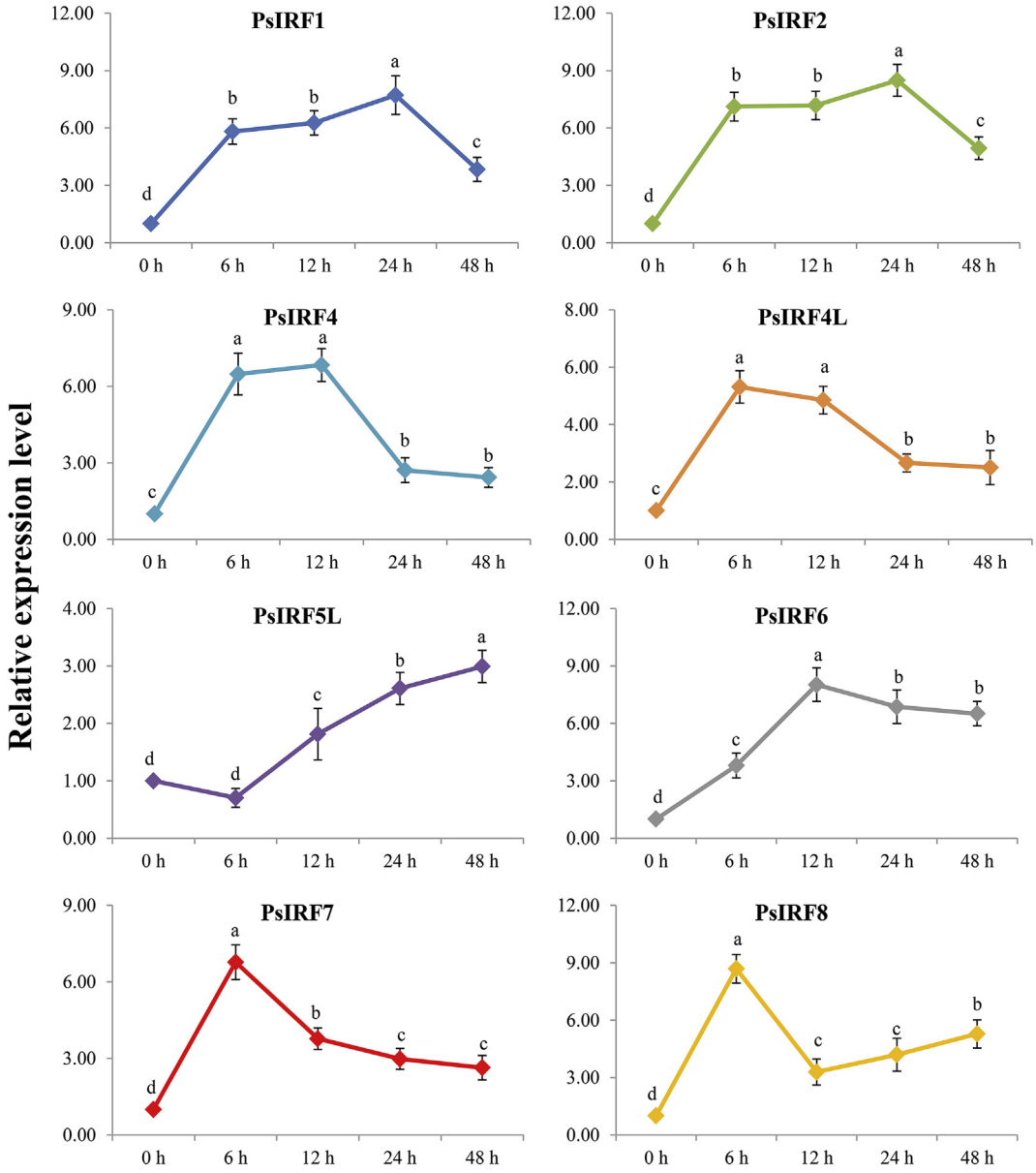

Fig. 7. Dynamic expression of *PsIRF* genes in the spleen of *Pelodiscus sinensis* in response to *Aeromonas hydrophila* infection. The values with different letters are significantly different at $P < 0.05$. Each bar shows the mean ± SEM of triplicate assays.

Accumulating evidence has demonstrated that the whole genomes of vertebrates underwent two rounds of duplication in their long evolutionary history [34]. Genome duplication provides the possibility of generating more members of a gene family with various biological functions as well as duplicated genes [33,35]. Two IRF4 genes were found in the genome of *P. sinensis* and *T. mexicana*, while more duplicated IRF4 genes exist in *D. rerio*. The variation in the number of IRF family members between different species may be due to gene duplication and gene loss events [33]. Gene duplication is usually followed by gene loss, which is regarded as the major source of adaptive functional novelty in eukaryotes [33,35]. In the present study, comparative analysis showed that IRF3, IRF9 and IRF10 members are absent in the *P. sinensis* genome, which is in accord with previous reports that humans and mice lack IRF10, and chickens lack IRF3 [7,36]. Syntenic analysis also revealed that several genes surrounding the PsIRFs in other turtles were not found in *P. sinensis*. It has been speculated that some individual genes might be directly lost from the genome during the evolution of species [34]. Additionally, the chromosomal distribution and organization of IRF genes and the neighbouring genes in syntenic regions were parallel and conserved among *P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana*. Remarkably, the pairwise ω values, which are an important measure of genetic differentiation [31], were less than 1 between critical IRF homologs, indicating a slow evolutionary rate and purifying selection during the evolution of PsIRF genes in *P. sinensis*. Further analyses revealed that the selected IRF protein homologs shared nearly overlapping 3D structures among *P. sinensis*, *C. mydas*, *C. picta* and *T. mexicana*. The conserved protein structures and functional domains may determine the evolved gene functions. The high evolutionary conservation of IRFs among the four turtle species might be associated with the original functions of IRFs.

4.2. Characterization of PsIRF genes in immune responses and in response to bacterial infection

The tissue expression pattern is considered a potential critical index indicating gene functional roles. The expression of IRF family members has been extensively validated in various tissues of many aquatic species [6,7,18–20]. In this study, the expression patterns of *PsIRF* family

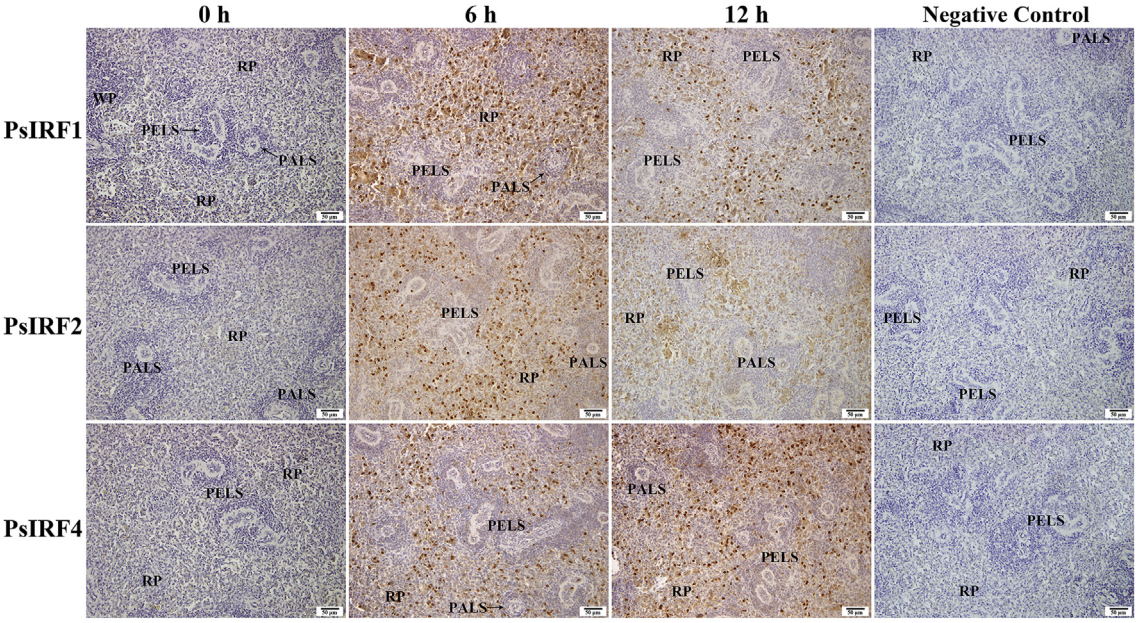

Fig. 8. Immunohistochemical localization and expression of the PsIRF1, PsIRF2 and PsIRF4 proteins in the spleen of *Pelodiscus sinensis*. RP: red pulp. WP: white pulp. PALS: periarteriolar lymphatic sheath. PELS: periellipsoidial lymphatic sheath. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

genes in six tissues (heart, spleen, blood, liver, intestine and kidney) of *P. sinensis* were systematically analysed by qRT-PCR. Eight *PsIRF* genes showed constitutive expression in six tissues, although their expression levels differed between different tissues. The constitutive expression of *PsIRFs* was similar to findings in some fishes, such as *M. amblycephala* [6], *C. semilaevis* [7] and *G. morhua* [20]. Studies in large yellow croaker (*Pseudosciaena crocea*) reported that the expression of *IRF1* was predominantly detected in the gills and spleen [37]. Expression analysis in rainbow trout (*Oncorhynchus mykiss*) demonstrated that *IRF1* and *IRF2* were constitutively expressed in the head, kidney, gill and spleen but not in the liver [38]. As expected, low expression levels of *PsIRF1* and *PsIRF2* were also detected in the liver of *P. sinensis*. Several *PsIRF* genes, including *PsIRF1*, *PsIRF2*, *PsIRF4* and *PsIRF4L*, exhibited relatively high levels of expression in the *P. sinensis* spleen, an important immune organ, which is consistent with the results in fishes [12,20,37,38]. Tissue-specific expression of *PsIRF* genes, especially genes with spleen-specific expression, implies their functions in immune defence.

In response to endogenous and external microbial stimuli, IRFs are activated and modulate the expression of key cytokines and transcription factors triggering immune responses [39,40]. Recently, with the rapid development of *P. sinensis* aquaculture, widely occurring infectious diseases are threatening the aquaculture industry, especially in operations challenged by *A. hydrophila*, causing great economic losses [23,29,41]. In addition to the presence of virulence factors, the host immune response influences the severity of pathogen infection [23,42]. The expression of immune-relevant IRF genes under infection is significant for the antibacterial immune response. Previous studies have revealed that the transcript expression of *IRF* family genes responds to experimental bacterial or virual challenge [6,7,37]. In *M. amblycephala*, the transcriptional level of the *IRF2* gene in the spleen was upregulated after stimulation by *A. hydrophila* [43]. In this study, eight *PsIRF* genes were found to be differentially expressed in the spleen after *A. hydrophila* infection. Most *PsIRF* genes, especially *PsIRF1*, *PsIRF2* and *PsIRF4*, were significantly upregulated post-infection. Moreover, the protein expression levels of PsIRF1, PsIRF2 and PsIRF4 were rapidly increased upon *A. hydrophila* infection, suggesting the putative roles of PsIRF1, PsIRF2 and PsIRF4 in defence against pathogen infection and the regulation of cellular immune responses [7,37,43,44].

IRF1, whose role in the regulation of IFN-inducible genes is well established, has been demonstrated to be vital for providing early defence against pathogen infection [4]. Caipang et al. [45] reported that IRF1 could regulate the early immune response in Japanese flounder and seemed to mediate an important defence mechanism against virus infections by modulating the production of certain antiviral substances. Although IRF1 and IRF2 exhibit a significant level of homology, IRF2 plays an antagonistic role to that of IRF1 in the transcriptional regulation of IFN genes through binding to the same recognition site of IRF1 and preventing IFN production in normally growing cells [7,46,47]. Moreover, IRF2 could function to interfere with NF-κB activation [48]. It has also been reported that the functional interplay between IRF1 and IRF2 serves as a mechanism for regulating IL-7 production in humans [49]. In the present study, parallel expression patterns of PsIRF1 and PsIRF2 at the mRNA and protein levels were found in *P. sinensis* after *A. hydrophila* infection, implying that PsIRF1 and PsIRF2 may concurrently participate in immune responses through their respective functions. IRF4 is a lymphoid- and myeloid-restricted member of the IRF transcription factors and mediates the control of the development and functions of immune cells, such as lymphoid, myeloma and B/T cell differentiation [4,15]. IRF4 is required for lymphocyte activation during immune responses and may act as a master regulator of autoimmunity [15]. Additionally, the expression variation of IRF4 in response to viral and bacterial challenge has been recently characterized in fishes such as *O. mykiss* [12], *Oplegnathus fasciatus* [50] and *Monopterus albus* [51]. Induction of IRF4 mRNA expression was detected upon infection. Both the protein and mRNA levels of PsIRF4 in the *P. sinensis* spleen were immediately increased at 6 h upon *A. hydrophila* infection, indicating the putative antimicrobial roles of PsIRF4 in the innate immune response [50,51]. Different IRF4 homologous genes exhibit diverse expression patterns upon exposure to external bacterial and viral pathogens [6,7,20]. However, similar spatio-temporal characteristics of *PsIRF4* and *PsIRF4L* gene expression were found in *P. sinensis*. In addition, IHC localization showed that the PsIRF4 protein as well as the PsIRF1 and PsIRF2 proteins were mainly expressed in the splenic red pulp, further implying that these genes may play particular roles in immune cell signalling. The exact functions of PsIRFs in immune responses clearly require further investigation, which is crucial for developing strategies for infectious disease control and

prevention in the aquaculture of *P. sinensis*.

5. Conclusion

In this study, eight PsIRF family genes were identified in *P. sinensis*. These PsIRFs presented conserved protein domains and shared close phylogenetic relationships with corresponding homologous genes in other turtle species. Further analyses of syntenic neighbouring genes, evolutionary selective pressure and protein structural comparisons demonstrated the evolutionary conservation of PsIRF family genes. Expression profiling by qRT-PCR revealed that PsIRF genes were constitutively expressed in different tissues of *P. sinensis* and were significantly differentially expressed in the spleen after *A. hydrophila* infection. Moreover, IHC analysis showed that the protein levels of PsIRF1, PsIRF2 and PsIRF4 in the spleen were upregulated upon *A. hydrophila* infection. These findings provide rich information for the exploration of the roles of PsIRFs in immune defence against pathogen infection.

Author contributions

T.L. and H.Z. conceived and designed the study. T.L. and S.C. were responsible for data analysis. T.L. and Y.H. carried out the gene expression analysis and IHC analysis. T.L. wrote the manuscript. T.L., S.C. and H.Z. revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.013.

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