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Molecular cloning and characterization of interferon regulatory factor 1 (IRF-1), IRF-2 and IRF-5 in the chondrostean paddlefish *Polyodon spathula* and their phylogenetic importance in the Osteichthyes

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ABSTRACT

The interferon regulatory factor (IRF) with its 10 members is a very important gene family related to innate immunity. Currently, most fish IRFs reported are from bony fish (teleosts). Cloning and sequencing of IRFs from chondrosteans, the so-called "ancient fish" including sturgeon, paddlefish, bichir and gar, are absent from the literature. In this study, three IRF genes PsIRF-1, PsIRF-2 and PsIRF-5, were cloned and characterized from the paddlefish (*Polyodon spathula*). PsIRF-1 includes an open reading frame (ORF) of 972 bp that encodes a putative protein of 324 amino acids; PsIRF-2 includes an ORF of 1023 bp encoding 341 amino acids and PsIRF-5 includes an ORF of 1491 bp that encodes 497 amino acids. The PsIRF-5 gene structure is similar to those in mammals but differs from those in teleosts in the first and second exons. Phylogenetic studies of the putative amino acid sequences of PsIRF-1, PsIRF-2 and PsIRF-5 based on the neighbor-joining and Bayesian inference method for Osteichthyes found widely accepted inter-relationships among actinopterygians and tetrapods. Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis of PsIRF-1, PsIRF-2 and PsIRF-5 in different paddlefish tissues shows higher levels of expression in gill, spleen and head kidney. Poly (I: C) (polyinosinic-polycytidylic acid) stimulation *in vivo* up-regulated PsIRF-1 and PsIRF-2 expression, while PsIRF-5 gene expression did not respond to the challenge of Poly (I: C).

1. Introduction

Interferon (IFN), a kind of multifunctional cytokine, has a very important role in the process of innate immunity in animals. Interferon regulatory factors (IRFs) essential for the generation of interferon were discovered as transcriptional factors regulating the transcription of IFN and IFN-induced genes (Mamane et al., 1999). IRFs have been confirmed to have some other important functions, including antiviral defense, immune regulation and growth control (Barnes et al., 2002). In fact, the 10 members of the IRF family play many different roles in the cell. Structurally, all IRFs have extensive homology in the DNA-binding domain (DBD), which covers the first ~115 amino acids where a cluster of 5 or 6 tryptophan residues is responsible for binding to the promoters of target genes. IRF-3–10, possess an IRF association domain (IAD), whereas IRF-1 and IRF-2 contain IAD2. IRF-1 was originally identified as the transcriptional activator of the type I IFN gene and IFN-induced genes (Kroger et al., 2002). IRF-2 has usually been regarded as a transcriptional repressor that acts in an antagonistic manner with IRF-1, but its transcriptional activating function was found in several other genes, including histone H4, VCAM-1, gp91phox, interleukin-7 and the class II transactivator (Luo and Skalnik, 1996; Oshima et al., 2004; Vaughan et al., 1995; Xi and Blanck, 2003). In addition, recent research has revealed other functions of IRF-2, including the production of interleukin-7 in human intestinal epithelial cells and generation of peripheral NK cells (Oshima et al., 2004; Taki et al., 2005). The function of IRF-5 is not very clear but its structure is similar to that of IRF-6. Interestingly, the expression of IRF-5 is induced by IFN-α/β stimulation, suggesting its participation in the IFN system. In mammals, IRF-5 regulates the expression of IFN-α and IFN-β and the immune reaction induced by IRF-5 is virus-specific (Barnes et al., 2002, 2001). Some reports have indicated that IRF-5 is involved in the RIG-I and Toll-like receptors immune pathways (Honda and Taniguchi, 2006) and plays a crucial role in the differentiation of lymphocyte and apoptosis (Tamura et al., 2008). At present, the cloning and characterization of IRF genes in fish has been limited to bony fish (teleosts), such as zebrafish, pufferfish, Atlantic salmon and channel catfish. Especially, the IRF-1 and

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IRF-2 genes are reported for teleosts (Jia and Guo, 2008; Shi et al., 2008; Sun et al., 2006; Zhang et al., 2003) but not for chondrosteans. The Osteichthyes are an important group of vertebrates that has two main branches, the sarcopterygians and the actinopterygians, both of which include almost all extant fish species. There are several other primitive groups on the basal clad of actinopterygians; namely, polypterus (bichirs and ropefish), sturgeon, paddlefish (*Polyodon spathula*), gar (needlefish) and amia (bowfish or dog fish). These ancient fish species originated far earlier than teleosts and possess very important evolutionary information. We need more information for lower vertebrates to understand the mechanism of innate immunity and to help us understand its roles in a comparative way. Little is known about IRFs in paddlefish, an important and widely cultured species. The aim of this work was to enrich our knowledge of the paddlefish IFN system by studies of the PsIRF-1, PsIRF-2 and PsIRF-5 genes. Here, we report the full-length cDNA sequences of IRF-1, PsIRF-2 and PsIRF-5 and the genomic structure of IRF-5 from the paddlefish. Phlylogenetic analysis for Osteichthys by the neighbor-joining (NJ) and Bayesian inference (BI) method was based on the IRF-1, PsIRF-2 and PsIRF-5 gene sequences. Finally, their expression levels were examined in different tissues and organs, and the expression profiles in different tissues was analyzed after injection of poly (I: C).

2. Materials and methods

2.1. Fish and immune challenge

Six adult paddlefish (body weight ~700 g) were purchased from a fish farm in Jingzhou, Hubei Province, China and kept in aerated tanks at room temperature for 7 days before the experiments. Three fish were injected intraperitoneally (*i.p.*) with 4.0 mg of poly (I: C) (Sigma, USA) dissolved in phosphate-buffered saline (PBS), whereas the three control fish were injected with same volume of PBS alone. After 24 h, the liver, head kidney, trunk kidney, gill and spleen were collected for real time PCR analysis.

2.2. RNA and genomic DNA extraction

Total RNA was extracted from liver, brain, heart, gill, head kidney, trunk kidney, intestine and spleen using TRIzol® Reagent (Takara, Japan) according to the manufacturer's instructions. RNA samples were treated with DNase (Promega, USA) to remove genomic DNA contamination. The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}), and its quality was monitored by calculation of the A_{260}/A_{280} ratio >1.8. Genomic DNA was purified from mixed samples of liver and spleen using extraction with phenol/chloroform.

2.3. cDNA cloning

To obtain the full-length cDNA of PsIRF-1, PsIRF-2 and PsIRF-5, total RNA extracted from different tissues was mixed, and reverse-transcribed into cDNA by Powerscript II reverse transcriptase (RT) with a coding sequence primer (SMART™ rapid amplification of cDNA ends (RACE) cDNA Amplification Kit, Clontech, USA). Five pairs of degenerate primers IRF1-hF1, 2/IRF1-hR1, 2; IRF2-hF1, 2/IRF2-hR1, 2 and IRF5-hF/IRF5-hR (Table 1) were designed on the basis of the known sequence of the IRF-1, IRF-2 and IRF-5 genes sequences download from NCBI data base (Table 2). A partial conserved fragment for each gene was obtained by PCR with the following protocol: 94 °C for 5 min; then 32 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s; and finally, 72 °C for 6 min. The full-length cDNA was obtained by rapid amplification of cDNA ends (Clontech, USA) using the primers based on the previous amplified conserved fragments. For 5′ RACE, the gene specific primer IRF1-5R1, IRF2-5R1, and IRF5-5R1(Table 1) and UPM (supplied by the kit) were used for the first round RACE-PCR, and primer IRF1-5R2, IRF2-5R2, and IRF5-5R2 (Table 1) and NUP (supplied by the kit) were used in the nested RACE-PCR,. For 3′ RACE, the amplification was carried out with the primer IRF1-3F1, IRF2-3F1 and IRF5-3F1(Table 1) and UPM for first round 3′ RACE-PCR. Primers IRF1-3F2, IRF2-3F2 and IRF5-3F2 (Table 1) and NUP were used in the nested RACE-PCR. The resulting fragments of 5′ and 3′ RACE were cloned into the pMD18-T vector(Takara, Japan) and sequenced, respectively. Assembly of the full-length gene was performed with the SeqMan program from Lasergene package (DNASTAR).

Table 1
Sequences of primers used in the present study.

Primer	Sequence (5′–3′)	Usage
IRF1-hF1	CARATHCCNTGGATGCAYGC	First round homology PCR
IRF1-hR1	GCYTTCCAKGTYTTNGGRTC	First round homology PCR
IRF1-hF2	ATGCCNGTVGARMGRATGMG	Second round homology PCR
IRF1-hR2	CTGGCTTGTTACAGTCTTG	Second round homology PCR
IRF1-5R1	ACTAAATTCTAAATGATTATCTG	First round 5′-RACE PCR
IRF1-5R2	TTGGACTGTCATGTGGGGCTGTG	Nested 5′-RACE PCR
IRF1-3F1	AAGACGCATGCCTGTTCAAAC	First round 3′-RACE PCR
IRF1-3F2	GTGGGCCCTACACACAGGGAA	Nested 3′-RACE PCR
IRF2-hF1	CARATHCCNTGGATGCAYGC	First round homology PCR
IRF2-hR1	GCYTTCCAKGTYTTNGGRTC	First round homology PCR
IRF2-hF2	ATGCCNGTVGARMGRATGMG	Second round homology PCR
IRF2-h R2	TCC TGT ATG GAT TGC CCA GT	Second round homology PCR
IRF2-5R1	GTCAGTGTCAGCATTCTGTAGAC	First round 5′-RACE PCR
IRF2-5R2	AGCGGAAATTGGCTTTCCATGTCT	Nested 5′-RACE PCR
IRF2-3F1	TGTGGAAAAAGATGCTCCTTT	First round 3′-RACE PCR
IRF2-3F2	CTGGGCAATCCATACAGGAAA	Nested 3′-RACE PCR
IRF5-hF	TGGGCTHWGGARACNGGSAA	Homology PCR
IRF5-hR	ACCTCRTAKATYTTRWARGGCT	Homology PCR
IRF5-5R1	CAAAGGGATGTTATTAGCAGTCA	First round 5′-RACE PCR
IRF5-5R2	ATTGCCATTGGGGTTGGTGGTGA	Nested 5′-RACE PCR
IRF5-3F1	AGCCCGATCCAGCCAAGTGGAA	First round 3′-RACE
IRF5-3F2	TGCCCTCAACAAGAGTCGCGA	Nested 3′-RACE PCR
IRF5-gF	ATGAGTCTCCAGCCACGGCGGATGC	Gene amplification
IRF5-gR	AAATCAAGCTTGTGCGGACTTAG	Gene amplification
β-actinF	ACTGCTGCTTCCTCTTCC	Real-time PCR
β-actinR	GGCATCAGGTCTTTACGG	Real-time PCR
IRF1-rF	AACAAGGTGACGAGCACGAA	Real-time PCR
IRF1-rR	TCTGAGGGCACAGTAATGAT	Real-time PCR
IRF2-rF	ATCGCCAGTGTCTTCATATG	Real-time PCR
IRF2-rR	TGAAGTTAGTTTTGCCCGAC	Real-time PCR
IRF5-rF	CATCACCACCAACCCCAATG	Real-time PCR
IRF5-rR	TGTTCCATATCTACATGACC	Real-time PCR
IRF1-sF	CCCGTTGCCTGCCTGCAGTAGTTG	Southern blotting probe
IRF1-sR	AGAGCTTTTACCAAAGGGATAGC	Southern blotting probe
IRF2-sF	CGAATGACAAGAAACCCTCATCA	Southern blotting probe
IRF2-sR	AGAAGCAAGATGCAATTAAGCC	Southern blotting probe
IRF5-sF	CCTCATTCTGGAGATCCGCG	Southern blotting probe
IRF5-sR	TGCAATTCTATGTCAGGGAAGCG	Southern blotting probe

Note: N represents all four nucleotides; H, A, C or T; R, G or A; Y, C or T.

2.4. Amplification of genomic sequence of PsIRF-5

Owing to the different numbers of exons and introns in IRF-5 of mammals and bony fish (Xu et al., 2010), the genomic sequence of PsIRF-5 was amplified and characterized for the purpose of exploring the structure of the chondrostean IRF-5 gene. Primers IRF5-gF and IRF-gR (Table 1) used to obtain the full-length genomic sequence were designed on the basis of the cDNA full-length sequence. PCR amplification was done with LA TaqTM polymerase (Takara, Japan) using the following protocol: 94 °C for 6 min then 34 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 6 min and finally, extension at 72 °C for 10 min. The intron/exon structure of the identified genomic sequence was determined by alignment of the full-length cDNA with the genomic sequence using BLAST2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

Table 2
GenBank accession numbers of IRFs used in this study.

Name	Species	Gene	Accession number
Cattle	*Bos taurus*	IRF-5	NM_001035465
Chick	*Gallus gallus*	IRF-5	NP_001026758
Zebrafish	*Danio rerio*	IRF-5	NM_212875
Claw frog	*Xenopus tropicalis*	IRF-5	NP_001039206
Human	*Homo sapiens*	IRF-5	NM_001098630
Mouse	*Mus muscullus*	IRF-5	NM_012057
Atlantic Salmon	*Salmo salar*	IRF-5	BT044767
Paddlefish	*Polyodon spathala*	IRF-5	JF511658
Paddlefish	*Polyodon spathala*	IRF-1	JF511656
Paddlefish	*Polyodon spathala*	IRF-2	JF511657
Rainbow trout	*Oncorhynchus mykiss*	IRF-2	AY034055
Snakehead	*Channa argus*	IRF-2	EF067850
Mouse	*Mus musculus*	IRF-2	NM_008391
Human	*Homo sapiens*	IRF-2	NM_002199
Atlantic salmon	*Salmo solar*	IRF-2	NM_001123615
Human	*Homo sapiens*	IRF-1	NM_002198
Mouse	*Mus musculus*	IRF-1	NP_032416
Claw frog	*Xenopus tropicalis*	IRF-1	NM_001006694
Chick	*Gallus_gallus*	IRF-1	NM_205415
Common quail	*Coturnix_coturnix*	IRF1	AJ271052
Chick	*Gallus gallus*	IRF2	NM_205196.1
Claw Frog	*Xenopus laevis*	IRF-2	NM_001095257.1
Sheep	*Ovis aries*	IRF-2	AF228445.1
Grass carp	*Ctenopharyngodon idella*	IRF-1	GU997098.1
Atlantic Salmon	*Salmo salar*	IRF-1	BT048538.1
Turbot	*Scophthalmus maximus*	IRF-1	AAY68278
Snakehead	*Channa argus*	IRF-1	EF067849
Sea squirt	*Ciona intestinalis*	IRF-5	XM_002120496
Shark	*Chiloscyllium plagiosum*	IRF-1	HM044308

2.5. Sequence analysis

All sequences generated were used to search for similarity using BLAST at the NCBI web servers (http://blast.genome.ad.jp). The multiple sequence alignments were built using Clustal W (ver.2.0.11) with the default option. The sequences were assembled using SEAVIEW and BioEdit and the nucleotide sequence was translated into the protein sequence using Translate Tool (http://www.expasy.org/tools/dna.html). Bootstrap values with 1000 replications were calculated to estimate the robustness of each internal node.

2.6. Phylogenetic analysis and molecular evolution

Multiple sequence alignment was done with Clustal W (Thompson et al., 1997) with the default settings. The genetic distance of each species was calculated using the Kimura 2 parameter. The phylogenetic trees were constructed using the neighbor-joining (NJ) and Bayesian inference (BI) method. Most of the parameters in program for phylogenetic reconstruction were set at default values. And the model selection for BI calculation was estimated using Model-test (Posada and Crandall, 1998). The trees were edited and modified using TreeView (ver. 1.6.6).

The BI phylogenetic analysis was performed with MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003) based on the nucleotide sequence alignment. A posterior sample of trees was obtained by Markov chain Monte Carlo simulation with 5×10^6 generations from a random starting tree, four Markov chains (three heated and one cold) sampled every 1×10^3 generations using the substitution model selected by Model-test 3.7 (Posada and Crandall, 1998) with the BI criterion. The K80 + G and HKY + G models were selected for IRF-1 and IRF-2 data sets, respectively. Bayesian analysis was done twice for each data set using different random numbers to confirm consistency between runs. The samples obtained during the first 2.5×10^6 generations were discarded as 'burn-in' and a 50% majority rule consensus tree that summarizes topology and branch-length information was calculated from the remaining 5002 sampled trees.

2.7. Reverse transcription PCR (RT-PCR)

RT-PCR was used to analyze the tissue distribution of IRF-1, IRF-2 and IRF-5 mRNA in healthy individuals. A total RNA sample of 0.2 μg from brain, gill, intestine, heart, head kidney, trunk kidney, liver and spleen was reverse-transcribed with M-MLV reverse transcriptase using oligo(dT) (Promega, USA) to prime the reaction. The first strand cDNA was used to amplify the specific fragment by PCR with the gene-specific primers. PCR was done in a 25 μl reaction system with 1 μl of reverse-transcribed product. Primer pairs IRF1-rF/IRF1-rR, IRF2-rF/IRF2-rR and IRF5-rF/IRF5-rR (Table 1) were used for amplification of the PsIRF-1, PsIRF-2 and PsIRF-5. The RT-PCR products were separated by electrophoresis in 2% agarose gel containing ethidium bromide (EtBr) and visualized under UV light. The β-actin cDNA fragment was amplified by PCR with β-actin F and β-actin R primers (Table 1) as an internal control.

2.8. Real time quantitative PCR experiments

A quantitative real time PCR experiment was done to study the inducible expression profile of PsIRF-1, PsIRF-2, PsIRF-5 and β-actin genes upon challenge with poly (I: C). A 1.0 μg sample of total RNA from each tissue from three individuals was reverse-transcribed into cDNA by random primers using the first strand synthesis system (Promega, USA). RNA samples were treated with DNase (Promega, USA) to remove genomic DNA contamination before reverse transcription. Real time PCR was done with a Bio-RAD system. The PCR mixture in a volume of 20 μl contained SYBR® Green Real Time PCR Master Mix (Toyobo, Osaka, Japan), 0.2 μM each specific forward and reverse primers (Table 1) and 1.0 μl of diluted cDNA (50 ng/μl). The PCR protocol was: 94 °C for 4 min; then 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. Paddlefish β-actin was used as the internal control. The levels of target gene expression were normalized to β-actin and expressed as the fold change relative to the level of expression in the control (Livak and Schmittgen, 2001).

2.9. Southern blotting analysis

Genomic DNA was extracted from paddlefish kidney and liver using the phenol/chloroform method and digested at 37 °C for 6 h with *Dra*I, *Pst*I and *Bgl* II (1 unit each/1 μg DNA; Takara). DNA (10 μg/lane) was separated by electrophoresis in 0.7% agarose gel then transferred to a nylon membrane (Amersham) using upward capillary transfer. Transferred DNA was hybridized overnight with a probe labeled by the digoxigenin High Prime Labeling System (Roche). The blotted membrane was washed twice (5 min each time) at room temperature with 2 × SSC containing 0.1% SDS and then twice (15 min each time) at 65 °C with 0.1 × SSC containing 0.1% SDS. The 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system was used to visualize the hybridized bands.

3. Results

3.1. Cloning and identification of PsIRF-1, PsIRF-2 and PsIRF-5 full-length cDNA

The RACE method was used to obtain the IRF-1, IRF-2 and IRF-5 full-length cDNA. The PsIRF-1 full-length cDNA is 1858 bp (GenBank accession no: JF511656), including an open reading frame

(ORF) of 972 bp, a 5′-untranslated region (UTR) of 122 bp and a 3′-UTR of 764 bp. The PsIRF-2 full-length cDNA is 1767 bp (GenBank accession no: JF511657), including an ORF of 1023 bp, a 5′-UTR of 193 bp, and a 3′-UTR of 551 bp; The PsIRF-5 full-length cDNA is 2178 bp (GenBank accession no:JF511658), including an ORF of 1491 bp encoding 497 amino acids, a 5′-UTR of 335 bp and a 3′-UTR of 352 bp.

The putative protein sequences of PsIRF-1 and PsIRF-2 are very similar to those of teleosts and mammals. Multiple alignments (Fig. 1) showed that there is a winged helix DBD (Leu10–Cys114). The first 115 residues of PsRF-1 and PsIRF-2 sequences match those of mammals and teleosts. The DBD of IRF-1 and IRF-2 that contains the tryptophan motif of Trp11, Trp26, Trp38, Trp 46, Trp58 and Trp77 also is the characteristic of the two members of IRF family. Just like the other IRF-1 and IRF-2, as well as the N-terminal DBD, we identified the transcriptional activation domain (TAD), IRF association domain 2 (IAD2) and the C-terminal transcriptional repression domain (TRD) in C-terminal region. The result of southern blotting indicated that the paddlefish IRF-1, IRF-2 and IRF-5 are single copy gene (Fig. 3). As in teleosts such

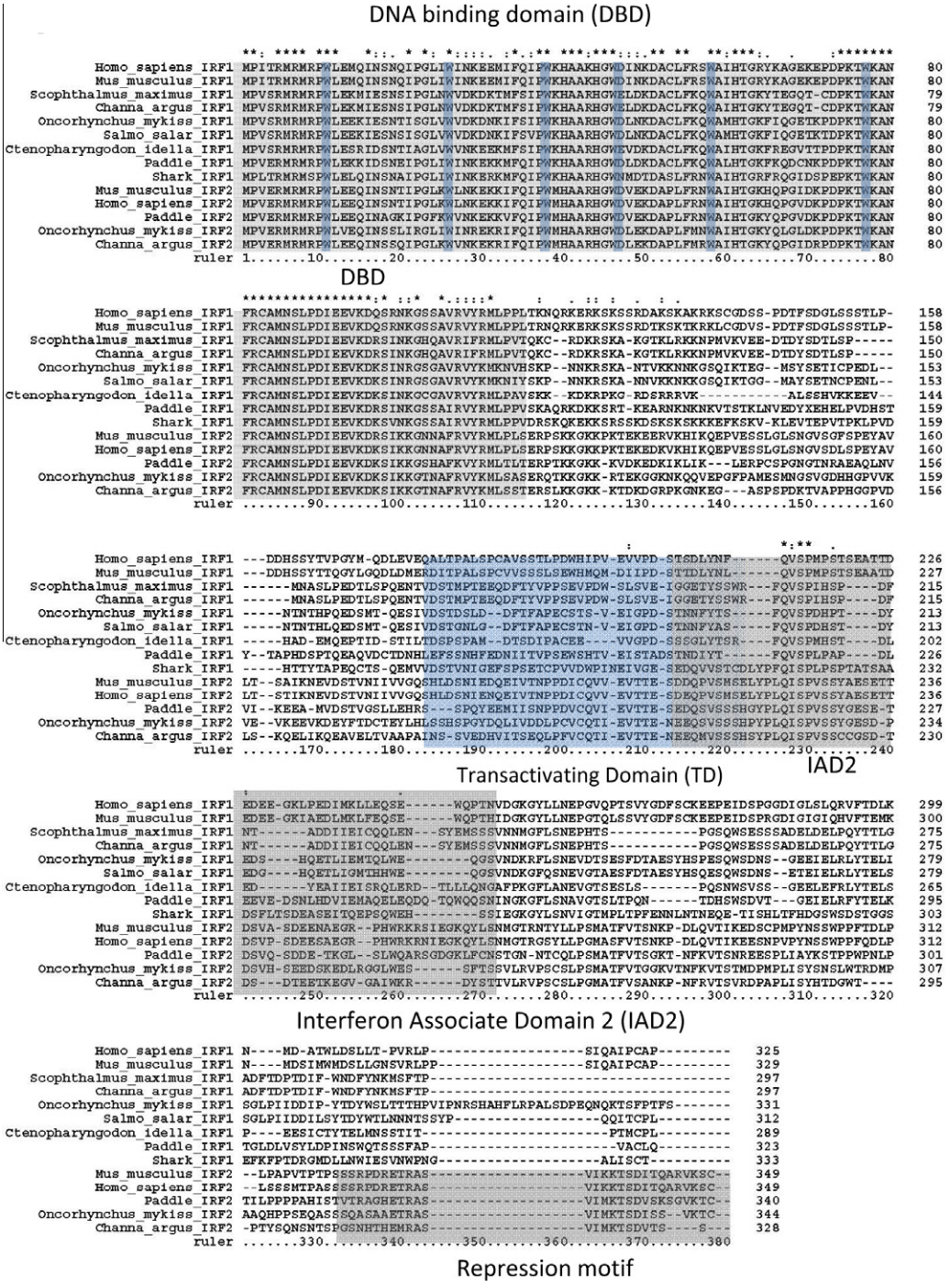

Fig. 1. Alignment of the putative protein sequence of the PsIRF-1 and PsIRF-2 and in comparison to the IRF-1 and IRF-2 sequences of vertebrates using the Clustal W program. The abbreviated names and GenBank accession numbers of the genes are given in Table 2. Each conserved residue is indicated with an asterisk (∗) and similar residues are indicated with a period (.). The conserved tryptophan residues, IAD2, regression motif and transactivating domain are shown in gray.

as zebrafish, grass carp and salmon and other vertebrate IRF-5, the putative amino acid sequence of PsIRF-5 (Fig. 2) had a winged-helix DBD (Leu10–Cys114) and an IRF association domain (IAD). There are only five tryptophan residues (Trp13, Trp28, Trp40, Trp60 and Trp79) in the DBD. An alignment of PsIRF-5 with other IRF-5 sequence indicated that the predicted PsIRF-5 IAD was located between residues 232 and 437. A C-terminal region homolog search showed that PsIRF-5 has a serine/threonine-rich domain located between residues 467 and 507 (Fig. 2). The alignment of PsIRF-5 amino acid sequences with those of other vertebrates showed that the DBD region is rather well conserved (Fig. 2), with the highest degree of identity when comparing with other two domains. The degree of identity of the proline-rich domain of the middle region (MR) was low between PsIRF-5 and other vertebrate IRF-5 sequences, owing to the number of amino acids in the MR.

3.2. PsIRF-5 genomic sequence and gene structure

The PsIRF-5 gene has a length of 5095 bp (GenBank accession no. JF511655). As shown in Fig. 4, the PsIRF-5 gene is composed of eight exons and seven introns and all exon/intron boundaries follow the rule of the splice acceptor-AG/GT-splice donor for splicing (Breathnach et al., 1978). The size of 1st exon of the PsIRF-5 gene is 174 bp, which break into two exons (the 1st and 2nd exon)

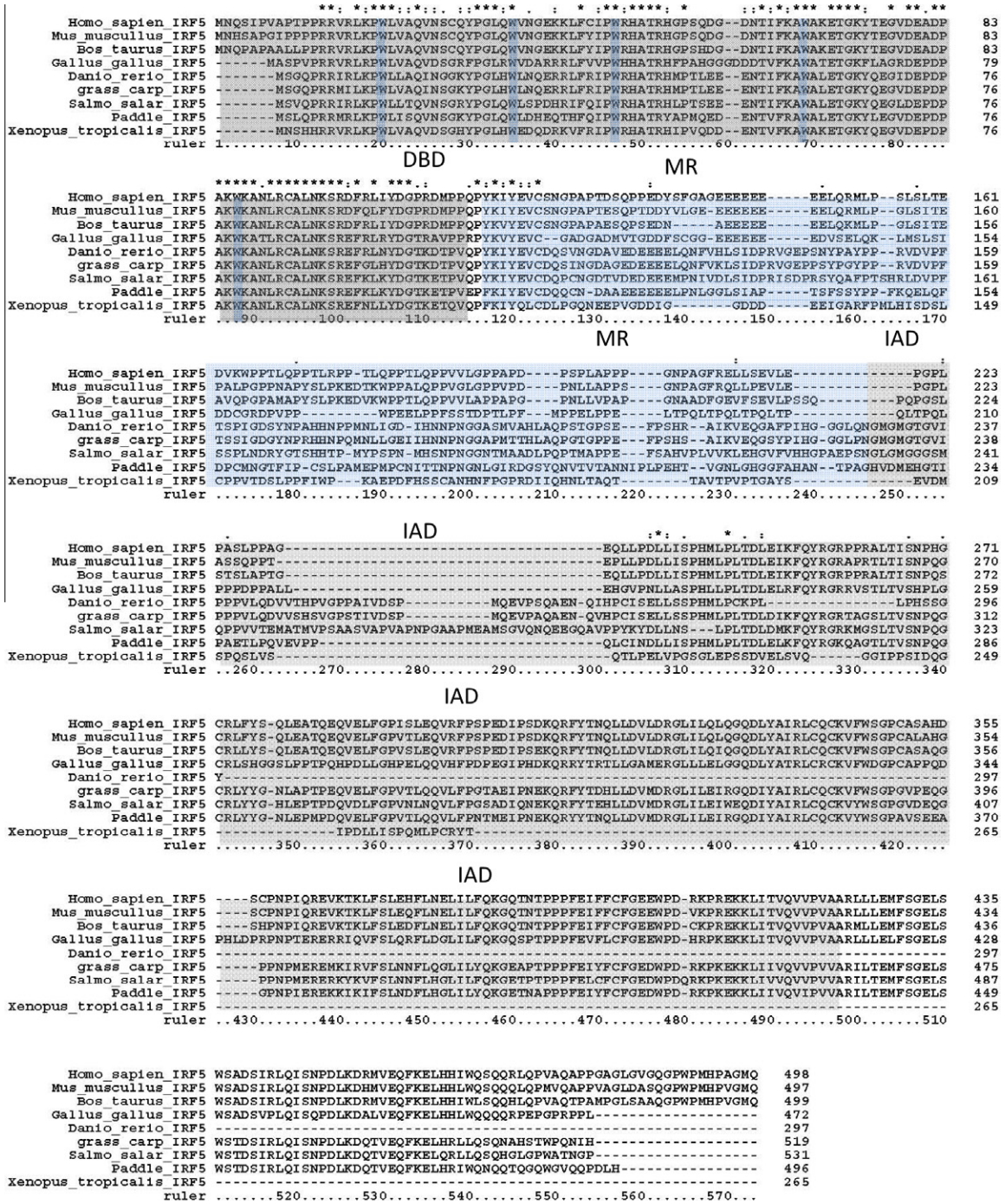

Fig. 2. The alignment of the putative protein sequence of the PsIRF-5 with IRF-5 sequences of vertebrates using the Clustal W program. The abbreviated names and GenBank accession numbers of the genes are given in Table 2. Each conserved residue is indicated with an asterisk (∗) and similar residues are indicated with a period (.). The conserved tryptophan residues IAD and MR that possess a proline-rich domain are shown in gray.

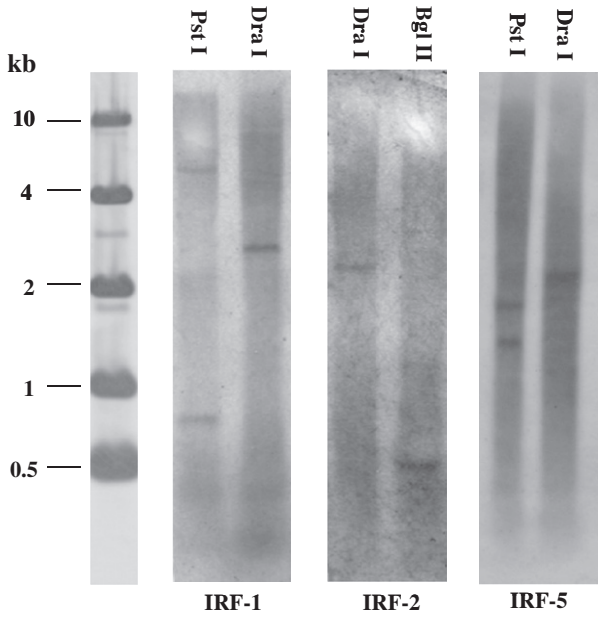

Fig. 3. Southern blotting analysis for paddlefish IRF-1, IRF-2 and IRF-5. The restriction enzymes *Pst*I, *Dra*I and *BgL*II were used for IRF-1, −2 and −5 analysis. Ten micrograms DNA each lane was electrophoresed on 0.7% agarose gel.

in zebrafish and grass carp IRF-5 gene, while all other exons have to same size to the corresponding exons in zebrafish and grass carp IRF-5 gene. The intervening seven introns are 519, 410, 684, 253, 494, 549 and 343 bp. The DBD exists only in the first exon in paddlefish and mammals, indicating that an additional intron exists in grass carp and zebrafish as the result of a division of the first exon observed in mammals into two exons in teleosts.

3.3. Phylogenetic analysis and molecular evolution

PsIRF-1 and PsIRF-2 and PsIRF-5 belong to different IRF gene subfamilies, so the sequence alignment and phylogenetic analysis were done separately.

Alignment of the amino acid sequence of PsIRF-1 and PsIRF-2 with those of teleosts and mammals revealed a high level of conservation, especially in the DBD (Fig. 5A). The greatest variation occurs in IAD2 and the C-terminal region. IRF-1 and IRF-2 are the two main branches of the tree based on this data set (Fig. 5A) and PsIRF-1 and PsIRF-2 are clustered with their counterparts. Generally, the IRF-1 and IRF-2 gene trees infer an inter-relationship among the Osteichthyes. The two main branches include actinopterygians and sarcopterygians with high bootstrap values and the tetrapod and teleost species, also with high bootstrap values, are clustered together. There are some differences between PsIRF-1 and PsIRF-2; the teleost IRF-1 genes are clustered together at the base of the tree, which is in accord with the widely accepted inter-relationship among vertebrate Osteichthyes but tetrapod IRF-2 genes with high bootstrap values are clustered together.

In order to explore the phylogenetic implications of the IRF-5 gene in vertebrates, we aligned the amino acid sequence of paddlefish with those of mammalian, avian, amphibians and teleost species. The inter-relationship of Osteichthyes in vertebrates elucidated by the phylogenetic tree based on the putative amino acid sequence of PsIRF-5 is the same as that widely accepted. In the IRF-5 gene tree, teleosts such as salmon, zebrafish and grass carp form a monophyly, tetrapods, including human, cattle, mouse, chicken and frog, form another monophyly and the paddlefish, an ancient species before the emergence of teleosts, is located on the basal clade. We found the same topological structure in the unrooted tree based on the same data set (Fig. 5B).

The IRF-1, IRF-2 and IRF-5 gene trees reconstructed using Bayesian methods are shown in Fig. 6A and 6B, respectively. In the IRF-1 and IRF-2 gene tree, the Osteichthyes are split into two lineages with strong posterior probability (PP): A (PP = 0.97) and B (PP = 0.98). IRF-1 and IRF-2 form two separate lineages, both with strong support (PP = 1.00). All Osteichthyes IRF-5 sequences form two lineages with moderate support: A (PP = 0.75) and B (PP = 0.77). The PP values of the nodes mentioned above are high, suggesting strongly that the genes of each clade are orthologous. Actually, the topological results based on Bayesian methods are very similar and the important difference is the support values on each node. Generally, the nodes on the Bayesian tree have higher confidence values than bootstrap based on NJ or parsimony.

3.4. Tissue distribution of PsIRF-1, PsIRF-2 and PsIRF-5

The expression of PsIRF-1, PsIRF-2 and PsIRF-5 mRNA in various tissues was examined by RT-PCR (Fig. 7). The results showed that PsIRF-1, PsIRF-2 and PsIRF-5 are expressed constitutively in the brain, gill, intestine, heart, head kidney, trunk kidney, liver and spleen of healthy paddlefish. High levels of expression were found in the gill, spleen and head kidney, and the other tissues and organs have low levels.

3.5. Induction of PsIRF-1, PsIRF-2 and - PsIRF-5 transcripts in organs by poly (I: C)

Expression of the PsIRF-1, PsIRF-2 and PsIRF-5 and β-actin genes was monitored by quantitative real time PCR in tissues such as

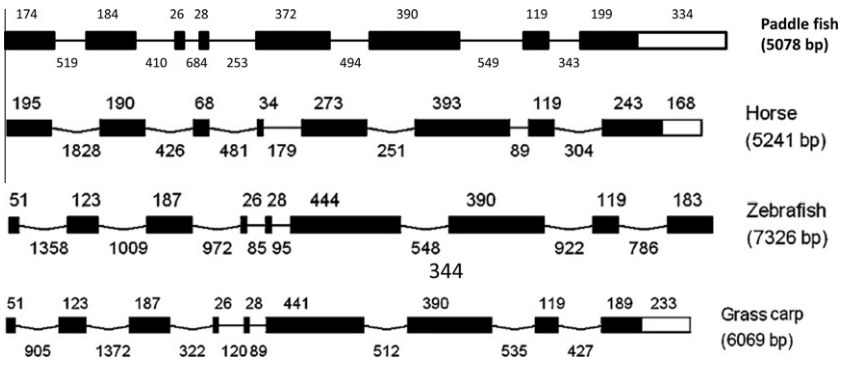

Fig. 4. Diagrams of the exon–intron arrangement of IRF-5 genes from paddlefish (GenBank accession no JF511655), zebrafish (Gene ID 405811), horse (Gene ID 100071786) and grass carp (GenBank accession no. FJ556995). Exons and introns are indicated by boxes and lines, respectively. The filled boxes and lines show the coding region, the open boxes and lines show the UTR. The number of nucleotides in each exon and intron is shown above and below the corresponding element, respectively.

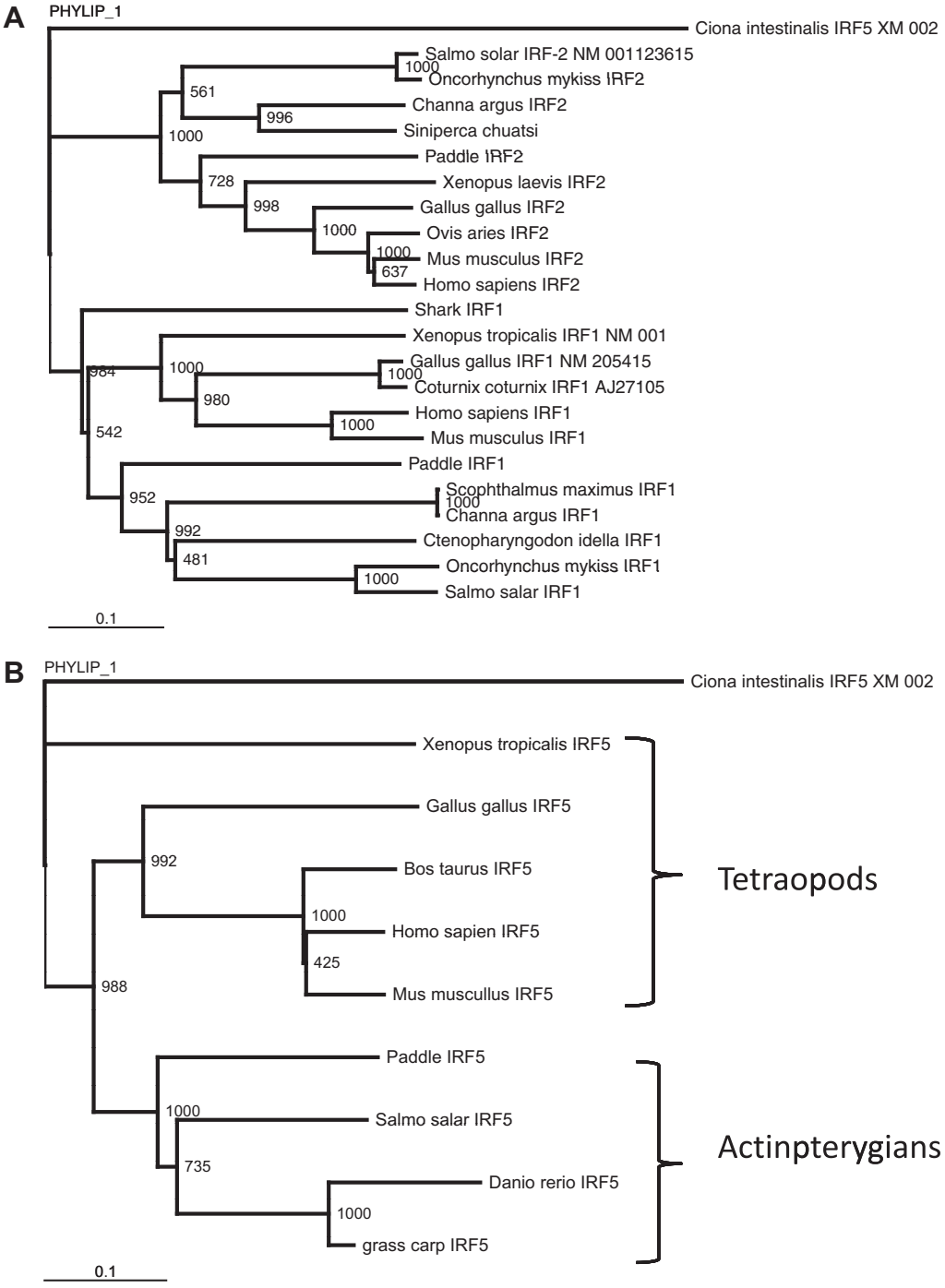

Fig. 5. Phylogenetic tree of IRFs in vertebrates. A neighbor-joining phylogenetic tree of IRF proteins was constructed based on the analysis of amino acid sequences by program CLUSTER-W and MEGA 3.1. The numbers at nodes are bootstrap values (1000 replicates) in percent. The sequences of the IRFs used for the analysis are derived from the GenBank and SwissProt: A. Phylogeny tree based on IRF-1, IRF-2; B. Phylogeny based on IRF-5; C. unrooted tree based on IRF-1, −2 and −5 for vertebrate.

liver, head kidney, trunk kidney, gill and spleen challenged with poly (I: C) for 24 h. We found that the PsIRF-1 and PsIRF-2 but not PsIRF-5 gene expression is up-regulated *in vivo* in response to stimulation with poly (I: C). At 24 h after injection *i.p.,* PsIRF-1 gene expression was significantly up-regulated in all the tissues tested (4–8.8-folds increase, respectively, $P < 0.05$) (Fig. 8). Expression of the PsIRF-2 gene was also significantly up-regulated with 2–2.8-folds increase. However, expression of the PsIRF-5 gene was not affected or increased less than 2-folds by treatment with poly (I: C) (Fig. 8).

4. Discussion

In this study, the IRF-1, IRF-2 and IRF-5 genes were cloned completely for the first time in a chondrostean fish, the paddlefish. With its 10 members, the IRF gene family plays an essential role in innate immunity and most of our knowledge of IRFs is for those in humans and other mammals owing to their medical and veterinarian applications. Along with the development of commercial fisheries, increasing numbers of IRF genes are being identified and cloned from teleosts (bony fish) but no IRF gene has been

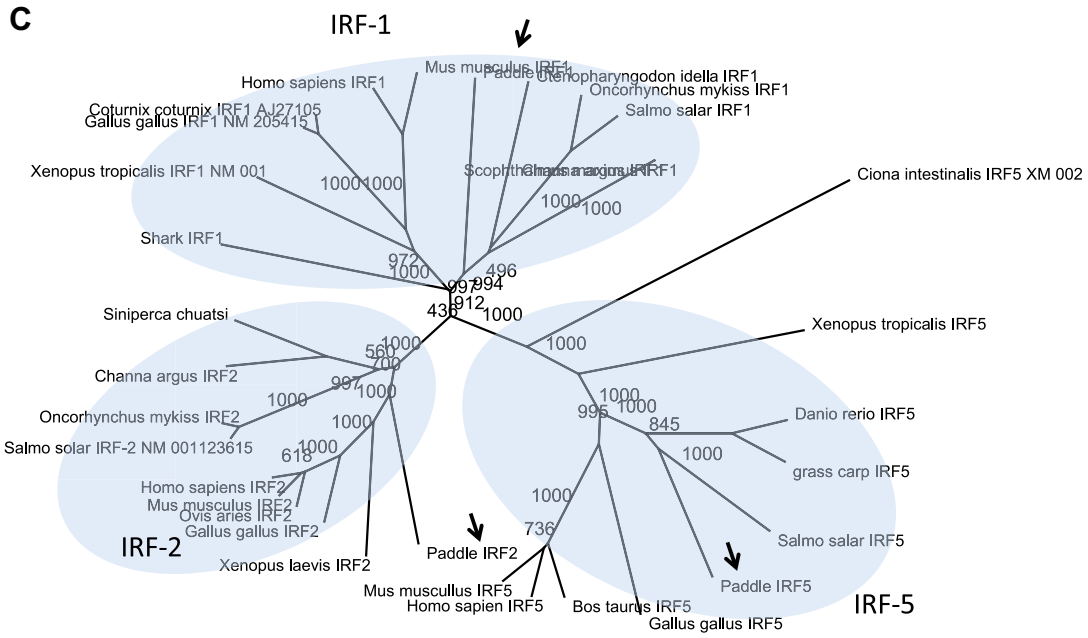

Fig. 5 (*continued*)

cloned and characterized for chondrosteans such as sturgeon, bichir, gar and amia. Chondrostean fish form a group that is important both in fishery and evolution. Evolutionarily, the paddlefish is located just before the teleosts and is much more primitive than bony fish. The chondrostean group is a combination of tetrapods and teleosts but we had no knowledge of the IRFs within it. The present study provides knowledge of chondrostean IRFs. The cloning and characterization of chondrostean IRF genes is essential to understanding the mechanism of innate immunity. The paddlefish is endemic to North America and was introduced to China more than 25 years ago where it has become an important species in the aquaculture industry. It is important that we gain more knowledge to take advantage of innate immunity in fish farming. The cloning and characterization of IRF-1, IRF-2 and IRF-5 genes from chondrostean species extend our understanding of the evolution and mechanism of the IRF family. In the present study, the IRF-1, IRF-2 and IRF-5 genes were cloned completely for the first time in the chondrostean paddlefish. The three PsIRF cDNAs, on the basis of the overall amino acid sequence, identity and phylogenetic analysis, are homologous to IRF-1, IRF-2 and IRF-5 in teleosts, amphibia, birds and mammals deposited in public databases, especially NCBI.

4.1. Characterization of PsIRF-1, PsIRF-2 and PsIRF-5

The amino acid sequences of PsIRF-1 and PsIRF-2 are similar to those in mammals and bony fish (Jia and Guo, 2008; Sun et al., 2006); especially in the DBD region, where >95% of sites have a high degree of identity among IRF-1 and IRF-2 both separately and together. An IRF associate domain 2 (IAD2) was identified for all species examined. We identified a repression motif for IRF-2, but not for IRF-1, in mammals and bony fish. We identified a transactivating domain in the MR just before IAD2 in IRF-2. IRF-1 and IRF-2 have the same gene structure in vertebrates, especially the teleosts (Jia and Guo, 2008; Shi et al., 2008; Sun et al., 2006).

Similar to IRF-1 and IRF-2 in other vertebrates such as mammals and teleosts, PsIRF-1 and PsIRF-2 contain six tryptophan residues (at positions 11, 26, 38, 46, 58 and 77) that are highly conserved in the IRF family: NH2-X10-W-X14-W-X11-W-X7-W-X11-W-18-W, as in many teleost and mammal species. Similar to IRF-5 in other vertebrates, the grass carp IRF-5 contains only five tryptophan residues (at positions 13, 28, 40, 60 and 79), which are highly conserved among the IRF family (Eroshkin and Mushegian, 1999; Nehyba et al., 2002). PsIRF-5 matches perfectly the IRF family signature located in the first 79 N-terminal amino acids, with five tryptophan residues spaced by 11–19 amino acids (NH_2-X12-W-X14-W-X11-W-X19-W-X18-W), as in grass carp (GenBank accession no. FJ556994), zebrafish (GenBank accession no. CAQ13397) and other teleost species (Swissprot Database no. Q13568).

In zebrafish and grass carp, the IRF-5 gene has nine exon and eight introns but in paddlefish it has eight exons and seven introns, with the size of the first paddlefish exon equal to the size of the first plus the second exons in grass carp and zebrafish, which is the same as in the horse and other mammals (Fig. 4). However, the size of the first plus the second exons in the IRF-5 of grass carp and zebrafish is similar to the size of the first exon in paddlefish and mammals, and other exons are very similar in size to those in fish and mammals. It is interesting to see that the DBD spans the first and second exons in the teleosts grass carp and zebrafish, but it exists only in the first exon in paddlefish and mammals, indicating that an additional intron exists in grass carp and zebrafish, which is a division of the first exon in paddlefish and mammals into two exons in fish. The first and second exons of PsIRF-5 are merged into a single exon, whereas the other PsIRF-5 exon and intron structures are similar to those in teleosts (Fig. 4).

4.2. PsIRF-1, PsIRF-2 and PsIRF-5 gene transcripts in healthy individuals and those challenged with poly (I: C)

The expression of IRF-1 and IRF-2 mRNA in teleost fish has been reported in all organs examined, despite differences at the level of expression (Sun et al., 2006). The much higher expression of IRF-5 in the head kidney of grass carp might imply that cells secreting

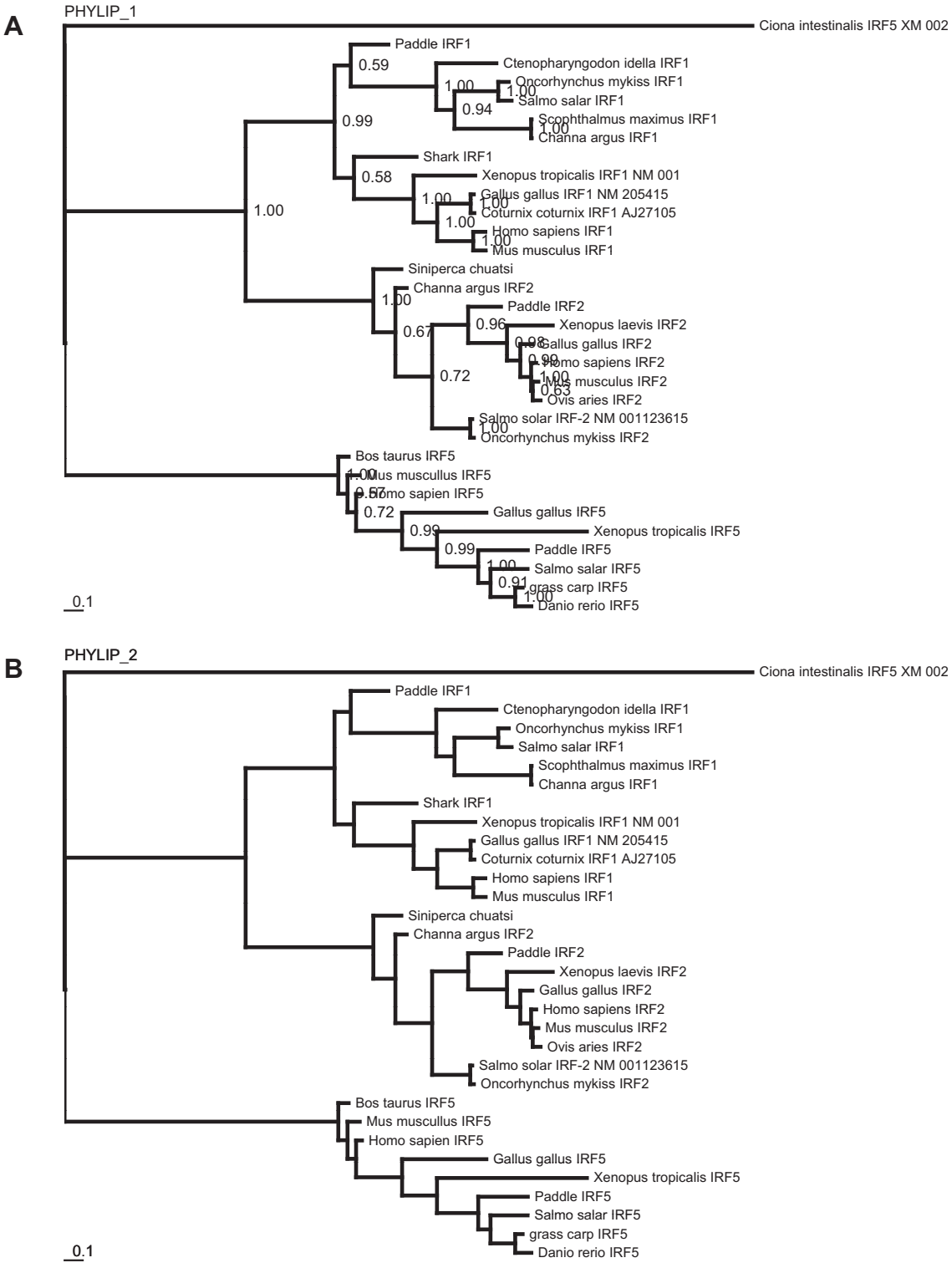

Fig. 6. Two bayesian trees for vertebrate based on IRF-1, IRF-2 and IRF-5.

IRF-5 are much more abundant in this lymphoid organ than they are in spleen (Xu et al., 2010) and our study shows the same pattern.

It is well known that IRF-5 plays a crucial role in the expression of IFN-α and -β in mammals, and the mediated immune response is virus-specific (Barnes et al., 2001; Tamura et al., 2008). In our study, unlike PsIRF-1 and PsIRF-2, expression of the PsIRF-5 gene was not up-regulated at 24 h after poly (I: C) injection (Fig. 7). Also, the PsIRF-1 gene has a much higher level of expression than that of the IRF-2 gene under the same conditions.

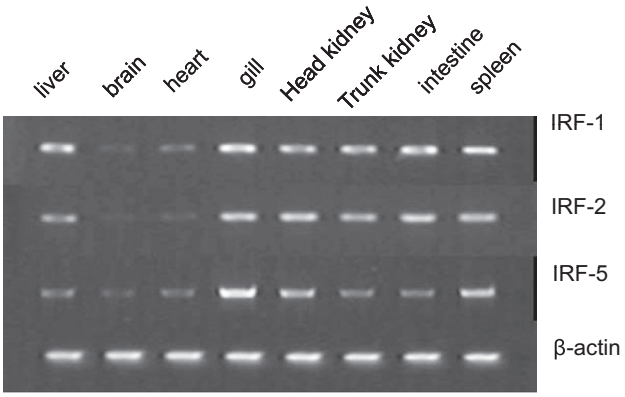

Fig. 7. Expression of PsIRF-1, PsIRF-2 and PsIRF-5 in different tissues of paddlefish. Semi-quantitative PCR was performed with total RNA isolated from head kidney, middle kidney, liver, brain, spleen, intestine, heart and gill. The first strand cDNA was used to amplify the specific fragment by PCR with the gene-specific primers and same volume cDNA as β-actin cDNA fragment amplification. The RT-PCR products were analyzed by electrophoresis on a 2% agarose gel and documented with a Gel Documentation System.

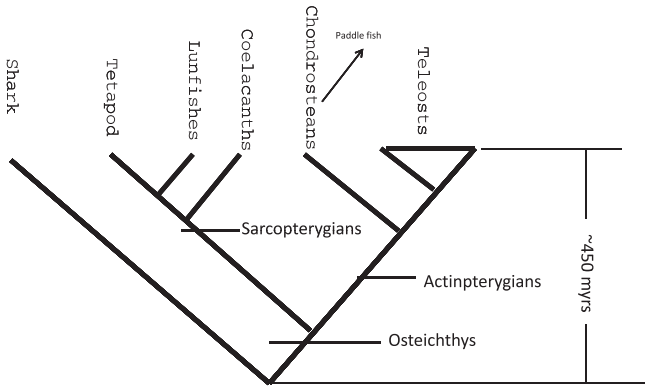

Fig. 9. Inter-relationship among Osteichthys modified from Yu et al., 2010.

4.3. The phylogeny of Osteichthyes based on IRF-1, IRF-2 and IRF-5 genes and their molecular evolution

The phylogenetic studies in the present work had two objectives: one was to verify the sequence of the cDNA cloned. We compared it with the known sequence of identified genes and looked to see if it clustered together with the same or similar genes. The sequences named PsIRF-1, PsIRF-2 and PsIRF-5 all clustered with their counterparts in different species. The results allowed us to confirm the target genes. The second objective was to reconstruct the phylogeny of Osteichthyes using the IRF genes as markers.

According to the fossil record, morphological characters and molecular phylogeny, Osteichthyes are phylogenetically separated into two branches, the actinopterygians and the sarcopterygians, and their inter-relationship is illustrated by Fig. 9. The actinopterygian branch includes the bony fish (teleosts) and the chondrostean fish (soft bony with hard scale). Almost all known ~27,000 fish species are included in the teleosts and belong to >40 orders, whereas only four orders with few species are included in the chondrostean branch. The chondrostean fish are much older than teleosts in geological time (~400 million years) and have a very rich fossil record; the only extant species are polypterus, paddlefish, sturgeon, gar and amia. The sarcopterygian branch includes crossopterygians (lungfish and coelacanth) and tetrapods, which are limbed animals such as amphibians, reptiles, birds and mammals. The available dataset is based mainly on mitochondrial and a few chromosomal gene sequences (Bininda-Emonds et al., 2007; Hedges et al., 1990; Inoue et al., 2003; Xia et al., 2003). In this study, we used the immunity-related gene data to explore the phylogeny in Osteichthyes. As shown in Fig. 5, the phylogenetic tree based on the putative PsIRF-5 amino acid sequence has the same topological structure as that described above. Phylogenetic analysis of PsIRF-1 and PsIRF-2 export shows results similar to those found for PsIRF-5. In contrast, in the tree generated from PsIRF-2, the paddlefish is clustered with the tetrapods and not with the teleosts. In conclusion, the IRF-1and IRF-5 genes have proved a rich source of information and potential for phylogenetic reconstruction. However, the IRF-2 gene, perhaps due to selective pressure or purification, is not suitable for reconstruction of the phylogeny on a long timescale. According to the fossil record, actinopterygians and sarcoptyerygians separated >400 millions ago (Yu et al., 2010).

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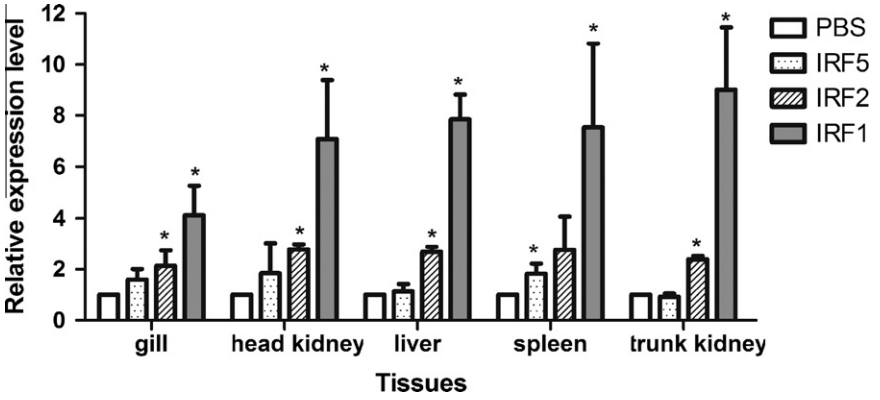

Fig. 8. Expression patterns of IRF-1, IRF-2 and IRF-5 gene induced by poly (I: C) in gill, head kidney, middle kidney, liver and spleen. The fish weight about ~700 g each were injected *i.p.* with 4.0 mg of poly (I: C) (Sigma, USA) dissolved in phosphate-buffered saline (PBS), whereas the three control fish were injected with same volume of PBS alone. Three individuals were sampled at 24 h post-injection on each occasion, with gill, head kidney, middle kidney, liver and spleen dissected out. Each data point is expressed as the mean of three replicates ± standard error (S.E.).The level of significance of the comparison to the control is indicated by $^{*}P < 0.05$.

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