

Gene structure and transcription of IRF-2 in the mandarin fish *Siniperca chuatsi* with the finding of alternative transcripts and microsatellite in the coding region

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Abstract The gene of interferon regulatory factor-2 (IRF-2) has been cloned from the mandarin fish (*Siniperca chuatsi*). The IRF-2 gene has 6,418 nucleotides (nt) and contains eight exons and seven introns, encoding two mRNAs. The two IRF-2 mRNAs each contained an open reading frame of 873 nt, which both translate into the same 291 amino acids but differed in their 5' untranslated region: one mRNA was transcribed initially from the exon 1 bypassing exon 2, while the other was transcribed from the exon 2. The microsatellites (CA repeats) could be found in the carboxyl terminal region of mandarin fish IRF-2, which result in the truncated form molecules. The microsatellites' polymorphism was investigated, and eight alleles were found in 16 individuals. The microsatellites were also examined in IRF-2 of several freshwater perciform fishes. The transcription of the IRF-2 in different tissues with or without poly inosine–cytidine stimulation was analyzed by real-time PCR, and the constitutive transcription of both molecules could be detected in all the tissues examined.

Keywords IRF-2 · Gene organization · Microsatellite · Mandarin fish · Chinese perch · *Siniperca chuatsi*

Abbreviations

IRF	interferon regulatory factor
ORF	open reading frame
nt	nucleotides
aa	amino acid(s)
UTR	untranslated region
Poly I: C	poly inosine-cytidine

Introduction

Interferon regulatory factors (IRFs) were first discovered as transcriptional factors regulating the transcription of interferons (IFNs) and IFN-induced genes (Mamane et al. 1999). So far, nine members in the IRF family have been reported in mammals, and they have been confirmed with some important functions, such as antiviral defense, immuno-regulation, and growth control (Barnes et al. 2002). 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 was usually regarded as a transcriptional repressor, acting in antagonistic manners with IRF-1, but its transcriptional activating function was also found in several other genes including *histone H4*, *VCAM-1*, *gp91phox*, *interleukin-7*, and *class II transactivator* (Vaughan et al. 1995; Luo and Skalnik 1996; Jesse et al. 1998; Xi and Blanck 2003; Oshima et al. 2004). Recently, IRF-2 deficiency revealed a novel checkpoint critical for the generation of peripheral NK cells (Taki et al. 2005). IRF-4 and IRF-8 are usually expressed at lymphoid tissues (Mamane et al. 1999). IRF-3, IRF-5, and IRF-7 have

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been recognized as direct transducers in virus-mediating signaling (Barnes et al. 2002), and IRF-7 is regarded as the master regulator of type I IFN-dependent immune responses (Kawai et al. 2004; Honda et al. 2005a,b). Structurally, all members of the IRF family share homology in their first 115 amino acids (aa), encompassing the DNA binding domain that contains a characteristic repeat of tryptophans; the carboxyl-terminal regions of these proteins are more diverse. All IRFs, except IRF-1 and IRF-2, have an IRF association domain (IAD) that is responsible for interaction with other family members or transcription factors, whereas IRF-1 and IRF-2 contain another association domain (IAD2) (aa 210–265 in human IRF-2) that is essential for their interaction with IRF-8 (Schaper et al. 1998; Meraro et al. 1999). The IRF-2 also contains the acidic region (aa 182–218) functioned as a transactivating domain (Jesse et al. 1998). Although research indicates the carboxyl terminus of IRF-2 (aa 325–349) rich in basic aa is a repressor motif (Yamamoto et al. 1994), some research did not support this view (Jesse et al. 1998; Chung and Kawamoto 2004).

In teleost fish, however, IRF genes have been sequenced only from a few species of fish, although the fish are a large heterogeneous group of lower vertebrates: IRF-1 and IRF-2 in rainbow trout *Oncorhynchus mykiss* (Collet et al. 2003a), IRF-7 similar sequence in crucian carp *Carassius auratus* (Zhang et al. 2003), and IRFs in pufferfish *Fugu rubripes* and Japanese flounder *Paralichthys olivaceus* which actually are IRF-1 molecules (Yabu et al. 1998; Richardson et al. 2001). In comparison to mammals, the knowledge of fish IRFs is rather limited, and much more research is needed to understand the diversity of the IRF members and their functions in fish immune responses. In China, the mandarin fish or the so-called Chinese perch, *Siniperca chuatsi* (Basilewsky), which belongs to Perciformes of the most diversified group of fish (Nelson 1994), is an important species for aquaculture. However, the immune gene information of perciform fish is scarce when compared with fish in other taxa, such as cyprinid and salmonid fish of high economical importance. In addition, the recent research revealed more important functions of IRF-2 (Oshima et al. 2004; Taki et al. 2005), and more information in lower vertebrates will help understanding its roles in a comparative way. Thus, the gene structure and transcription of IRF-2 from mandarin fish are reported in this paper.

Materials and Methods

Cloning the IRF-2 cDNA sequences by rapid amplification of cDNA ends–polymerase chain reaction (RACE-PCR)

Degenerate primers to obtain IRF-2 were designed by comparing all known IRF-2 sequences. All primers used in

this paper are listed in Table 1. Two mandarin fish, weighing about 230 g each, were injected with 400 µg poly inosine–cytidine (Poly I: C) (Sigma, USA), and 36 h later, the RNA was isolated using Trizol (Invitrogen, USA) from the spleen and reverse transcribed into cDNA by Powerscript II reverse transcriptase (RT) with coding sequence primer [SMART rapid amplification of cDNA ends (RACE) cDNA Amplification Kit, Clontech, USA]. The PCR cycling conditions were 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min, and then a final elongation step at 72°C for 5 min. PCR products were cloned into pGEM-T vector (Promega, USA) and sequenced.

To recover the full-length cDNA sequence, 3' and 5' RACE were performed by using the gene specific primers and adaptor primers. The PCR cycling conditions were 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 2 min, and then a final elongation step at 72°C for 5 min. The basic local alignment search tool program from the National Center for Biotechnology Information was used to identify similar sequences. Multiple sequence alignments were performed using the CLUSTAL W 1.8 program. The phylogenetic tree based on the results of alignments was obtained by using the Mega 2.1 program (Kumar et al. 2001).

Cloning genomic sequences of the mandarin fish IRF-2

Two IRF-2 mRNA sequences isolated by RACE-PCR differed only in the 5' untranslated region (UTR) and were named as IRF-2a and IRF-2b. To determine whether the two mRNAs were transcribed from the same or different IRF genes, primers were designed at the beginning and end of each cDNA and long-distance PCR was used to obtain the full sequence of the corresponding gene (Takara LA Taq, Takara, Japan) (Table 1). The 5' flanking region of IRF-2 gene was obtained by using Universal Genome Walker™ kit (Clontech). The sequence of the 5' flanking region was analyzed by using P-Match and TRANSFAC database for potential transcriptional factor binding sites (Chekmenev et al. 2005).

Southern blotting analysis

Genomic DNA was extracted from mandarin fish kidney using a Wizard Genomic DNA Purification Kit (Promega) and digested at 37°C for 6 h with one unit *Dra* I, *Pst* I, *EcoR* V (Takara), each per 1 µg DNA. DNA (10 µg/lane) was electrophoresed on 0.7% agarose gel and transferred to nylon membrane (Amersham, UK) using upward capillary transfer. Transferred DNA was hybridized with a probe labeled using the digoxigenin High Prime Labeling System (Boehringer, Mannheim, Germany). The hybridized bands

Table 1 Primers used for the mandarin fish IRF-2 cloning and expression analyses

Name	Sequence (5'–3')	Application
D1	GGATG(C/A)G(G/C)ATG(C/A)G(A/T/G/C)CC(A/T/G/C)TGG	IRF-2 conserved region cloning
D2	GC(C/T)TTCCA(T/G)GT(C/T)TT(A/T/G/C)GG(A/G)TC	IRF-2 conserved region cloning
2-5p1	GCCCATCTCATGAAGAGCGGAGCATC	IRF-2 cDNA 5' RACE
2-5p2	CTGGACGGTCTATGCCTGGCTGG	IRF-2 cDNA 5' RACE
2-3p1	TGGCTGGGACCTGGAGAAAGATG	IRF-2 cDNA 3' RACE
Actin F	GAC ATC AAG GAG AAG CTG TGC T	Actin real-time PCR
Actin R	ATG CTG TTG TAG GTG GTC TCG T	Actin real-time PCR
IRF2S1	TCGTAGAAGCAGTAGGTGTTG	IRF-2 Southern blotting probe
IRF2S1	GCCATTCTGCTGTCCAACACT	IRF-2 Southern blotting probe
IRF2aF	GTCCAAACTGAGCAGGACACA	IRF-2a real-time PCR
IRF2bF	AGACTTCTACAGGACGACATGA	IRF-2b real-time PCR
IRF2R	ATGAAGAGCGGAGCATCTTC	IRF-2a and IRF-2b real-time PCR
MF	GTCTGTGACAG(C)AT(TC)GA(AG)GTGACCAC	IRF-2 microsatellites region
MR	TGTGGTCAAACTGTGGCTCTG	IRF-2 microsatellites region

were visualized by using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system.

Transcription of IRF-2a and IRF-2b in different tissues analyzed by real-time PCR

To examine the temporal changes of IRF-2 transcripts in different tissues, a group of three mandarin fish, each weighing about 300 g, were each injected intraperitoneally with 2 mg Poly I: C (Sigma) dissolved in phosphate-buffered saline (PBS), and another group of three fish used as controls with similar size were injected with PBS. Forty eight hours later, the head kidney, kidney, spleen, liver, heart, intestine, and gill were dissected out for isolating total RNA using Trizol reagent (Life Technologies, USA). Before the stimulation and tissue sampling, fish were anesthetized with MS-222 (Sigma). RNA samples were treated with RQ1 RNase-free DNase (Promega) and then desalted before cDNA synthesis using the Absolute RNA

RT-PCR Miniprep Kit. Total RNA was quantified by determination at OD₂₆₀. The purified total RNA (2 µg) was then reverse transcribed into cDNA using Powerscript II RT (Clontech). The final volume of the cDNA synthesis reaction was 20 µl.

External controls consisted of plasmid cDNA standards for IRF-2a, IRF-2b, and β-actin. IRFs and β-actin cDNA fragments were generated by RT-PCR, and each amplicon was purified using the DNA Gel Exaction Kit (Qiagen, Germany) and cloned using pMD18-T vector (Takara) according to standard protocols. Plasmid DNA was prepared with the Plasmid Mini Kit (Qiagen) and the identities of purified cDNA constructs were verified by sequencing. The cDNA plasmid concentrations were measured at OD₂₆₀ and the corresponding copy numbers were calculated based on the formula that 1 µg of 1,000-bp DNA is equivalent to 9.1×10^9 molecules.

Quantitative real-time RT-PCR was conducted by amplifying 0.5 µl cDNA with the SYBR Green qPCR Kit (Finnzymes, Finland) on a Chromo4 Real-Time Detection

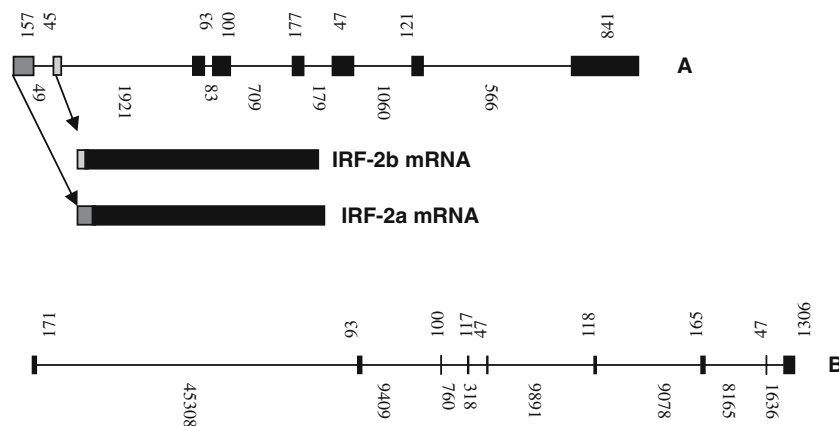


Fig. 1 Schematic representation of the genomic organization of IRF-2. **a** Mandarin fish IRF-2 (GenBank accession number AY395717); **b** human IRF-2 (NC_000004). The nt numbers of each exon and intron are also indicated

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CTGGACACAGAAACAGAAATCCACGACGGCCACACTTTCCTTCTTCTTTTTTTTTTATTA 60
AAACCACAAATAAATTAACCTTGCCAACCTTCAGCTGCGCTTGTATTCTGACAGCAGGAC 120
TGTGAAGCTAACGTTACAGGCCGTCCAAACTGAGCAGGACACAATG  CCAGTAGAGAGAAT 180
                                     M P V E R M 6
GAGGATGCGGCCGTGGCTGGAGGAACAGATCGACTCCTGTGATACACAGGGCTCAAATG 240
  R M R P W L E E Q I D S C Q I P G L K W 26
GGTTAACAAAGAAAAGAGAATCTTCCAGATCCCATGGATGCATGCTGCGCGTCATGGCTG 300
  V N K E K R I F Q I P W M H A A R H G W 46
GGACCTGGAGAAAAGATGCTCCGCTCTTCATGAGATGGGCCATACATACTGGTAAATACCA 360
  D L E K D A P L F M R W A I H T G K Y Q 66
GCCAGGCATAGACCGTCCAGATCCCAAGACGTGGAAGGCTAATTTCCGCTGTGCCATGAA 420
  P G I D R P D P K T W K A N F R C A M N 86
CAGCCTGCCAGACATCGAGGAGGTGAAGGATAAAAGCATCAAAAAGGGAACCTAATGCCTT 480
  S L P D I E E V K D K S I K K G T N A F 106
CAGGGTCTATAAGATGCTCTCCTCCTCAGAGAGAAGCATGAAGAAAGGAAAGAAGAC 540
  R V Y K M L S S S E R S M K K G K K K T 126
AGACAAAGAGGGGAGGCCAAGGAAAACAAAGAGGTAGCTTCTCCATCTCCAGACAGGAC 600
  D K E G R P K G N K E V A S P S P D R T 146
TCCATCTGATGCTCCTGTTGGACCCATGACTTCACCAAACAGGAAGTGATCAAGCAGGA 660
  P S D A P V G P I D F T K Q E V I K Q E 166
AACAGTAGAGCTTACAGTGATGGACAACGCCTCAGCCATTACAGTTCGGTAGAAGACCA 720
  T V E L T V M D N A S A I H S S V E D H 186
TGTGATCACCAGCAGCAGCTTCCATTCTGTCAGACGATTGAAGTGACCACAGAGAA 780
  V I T S E Q L P F V C Q T I E V T T E N 206
CGAAGAGCAGACTGTTAGCTCCTCCACTCGTACCCGCTCCAAATCTCTCCCGTGTCTTC 840
  E E Q T V S S S H S Y P L Q I S P V S S 226
ATGCTGCGGTGAGACAA  CACACACACA  TGCT  CACACACACACACACACACACACAC  900
  C C G E T T H T H A H T H T H T H T H T 246
  ACACACACA  CGTACGTGCACGCTGCACACATCACCCCTTACCAGATTATGTTATGAGGAG 960
  H T H V R A R C T H H P L P D Y V M R R 266
GAGTATAAATAGGTTAGTTACATGGGACCGGGCTCGTAGAAGCAGTAGGTGTTGTAGTTT 1020
  S I N R L V T W D R A R R S S R C C S F 286
TTCAGAGCCACAGTTT  TGA  CCACAGTTACAATCAAGCAAGCTTTTCTGGAACATAGTGGT 1080
  S E P Q F Stop 291
TACCACACATACAAAGGAACAACCTTACGAGGTTAATGGTACGTGTGTGTTAAAAACACA 1140
CAGCTAAATATGGCTGTATGCTCAAATGAGGTATTGTTTTTTTTTTTTTGGTTCGGATT 1200
AATTGGTGTGGAATAGCCAGCAAACAACAAACTGTGGTTCTTATGGTGATCCCATGAC 1260
CTGTTAATATCATGTCAATGTCAATACCATCCCCTATTACATCATCCACCTAAAGTGT 1320
CTGCATGTTCTGTTTTACAGTCCCCGGGTCTTATCTTAAGTGGGAACAGCATGGGCAA 1380
CCAGCTGAATGTTGAGAATAACCAACCATAGATGTGATCAGACTGCTAACAGTAATTGA 1440
ATTCGACTTGCTCAGGCTGTTTTTTCAACTTGTAGTTGGAACAGCAGAATGGCTTTTGTCT 1500
AGAATTAATTCAAAACAATTTAAAAAGTGCACCTTGAAAAAAAAAAAAA 1598
    
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Fig. 2 Compiled nt and aa sequence for mandarin IRF-2a cDNA sequence (accession number: AY395717). Tryptophan repeats are shown in gray; the CA repeats and start and stop codons are boxed. Polyadenylation, RNA instability motifs are underlined

System (MJ Research, USA). Amplification conditions were 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 58°C for 25 s, and 72°C for 30 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Quantities of specific mRNAs in samples were measured according to the corresponding gene specific standard curves. Each sample was run in three tubes, and PCR reactions without the addition of the template were used as blanks. After completion of the

PCR amplification, data were analyzed with the Opticon-Monitor software 2.03 version (MJ Research). The exact copy number of IRF mRNA in each tissue before Poly I: C stimulation was derived from each threshold value in correspondence to the standard curve, and the relative change after stimulation was calculated based on the method using actin as the internal control (Livak and Schmittgen 2001). *T* test was used to compare the mean±SD value between two groups of samples with or without Poly I: C stimulation, and the difference ($P<0.05$) between

two groups would indicate that there was a significant change.

Confirmation of microsatellites polymorphism in mandarin fish IRF-2 and detection microsatellites in some perciform fish IRF-2 genes

To confirm the influence of the microsatellites on the open reading frame (ORF) of mandarin fish IRF-2 and microsatellites polymorphism, fragments were amplified from DNA samples of 16 mandarin fish, and nine clones from each fish were sequenced from both directions.

To examine whether the microsatellites exist in other perciform fish IRF-2, degenerate primers were designed by comparing all IRF-2 sequences available to amplify the corresponding region from isolated DNA. Fish used in this experiment were Korean aucha perch *Coreoperca herzi* (Herzenstein), Japanese aucha perch *Coreoperca kawamebari* (Temminck et Schlegel), *Coreoperca roulei* (Wu), *Coreoperca whiteheadi* (Boulenger), spotted mandarin fish *Siniperca scherzeri* (Steindachner), wavyline mandarin fish *Siniperca undulates* (Fang et Chong), *Siniperca kneri* (Garman), and *Siniperca fortis* (Lin). Due to the limitation of sampling, only one individual each of *C. herzi*, *C. kawamebari*, and *C. roulei* were analyzed in this study, while three individuals used in the other species. Nine clones of the amplified fragment from each individual were sequenced from both directions.

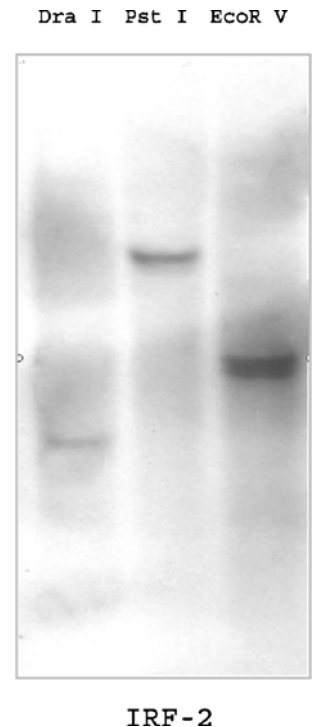
Results

Two transcripts of the mandarin fish IRF-2 and its gene structure

Two IRF-2 mRNAs, named as IRF-2a and IRF-2b, differed in the 5' UTR. The two IRF-2 mRNAs contain an ORF of 873 nucleotides (nt) translating into the same 291 aa. But, they have totally different sequences in the 5' UTR: the 5' UTR of IRF-2a contains 163 nt, while the 5' UTR of IRF-2b contains 51 nt. The same 3' UTR in the two mRNA contains 518 nt with an atypical polyadenylation signal (AATTAAA) starting 34 nt upstream from the PolyA tail and contains only one mRNA instability motif (Fig. 1).

Based on the results of Southern blotting (Fig. 2) and the corresponding genomic sequence, the two different mRNAs were different transcripts of the same gene. From the first transcription initiation site, the mandarin fish IRF-2 gene extends 6,418 bp to the end of the 3' UTR, and it contains eight exons and seven introns (Fig. 3). All the 5' and 3' ends of the introns, except the first one, show canonical splicing motifs (GT/intron/AG). The IRF-2a starts its transcription initiation from the exon 1 bypassing exon 2,

Fig. 3 Southern blotting analysis of mandarin fish IRF-2



while the IRF-2b just starts from exon 2. The difference of the two molecules was due to their transcription initiation site and alternative splicing. The mandarin fish IRF-2 DNA is a much compact one compared with human IRF-2, which extends 86,789 bp (Fig. 1), and the third, fourth, fifth, and sixth exons of mandarin fish IRF-2 contain the same number of nt with the second, third, fourth, and fifth exons of human IRF-2, respectively.

The amino-terminal DNA-binding domain (first 115 aa) of the mandarin fish IRF-2 contains the repeated tryptophan motif characteristic of all members of the IRF family (Fig. 4). The unrooted phylogenetic tree was constructed (Fig. 5), and the mandarin fish IRF-2 has the most similarity with rainbow trout IRF-2 (Table 2). When compared to other IRF-1 and IRF-2, the full-length aa identities were 53.3% with rainbow trout IRF-2, 48.5% with human and chicken IRF-2, 37.8% with chicken IRF-1, and 34.0–34.7% with IRF-1 from fish and mammals. When comparing the DNA binding domain, the identity was 88.7% with rainbow trout IRF-2, 87.0% with chicken and mouse IRF-2, 75.7% with chicken IRF-1, 72.2% with human and mouse IRF-1, 71.3% with rainbow trout IRF-1, 67.8% with Japanese flounder IRF-1, and 65.2% with the pufferfish IRF-1.

The isolated 5' flanking region of the mandarin fish IRF-2 gene contains 1,103 nt (GenBank accession number DQ472018). When compared with the promoters of human IRF-2 and rainbow trout IRF-2 (AF455112), two same binding motifs were found. One NFkappaB binding site



Fig. 4 The alignment of the aa sequence of the mandarin fish IRF-2 and in comparison with IRF-2 sequences of vertebrates by using CLUSTAL W program. The abbreviation names and GenBank accession numbers of the genes are shown in Table 2. The conserved

residues are indicated with *asterisks* (*), and the similar residues with *periods* (.). The conserved tryptophans are shown in *gray*, and the microsatellites region is *boxed*

could be found in the mandarin fish (−56 to −47) (position+1 of the nt sequence is defined as the first transcription initiation site), whereas the NFkappaB binding site could be found in human (−101 to −92) and rainbow trout (−33 to −24). It was interesting to find that the sequence GAG GAAGTGAAAATGAAAT could be found both in manda-

rin fish (−689 to −671) and rainbow trout IRF-2 (−158 to −140) 5' flanking regions, which has only two nt different from the GGGGAAGCGAAAATGAAAT in the promoter of human IRF-2 (−334 to −316). Several GAAA motifs were also found in mandarin fish IRF-2 5' flanking region. Two putative IRF-8 binding sites were predicted in the first

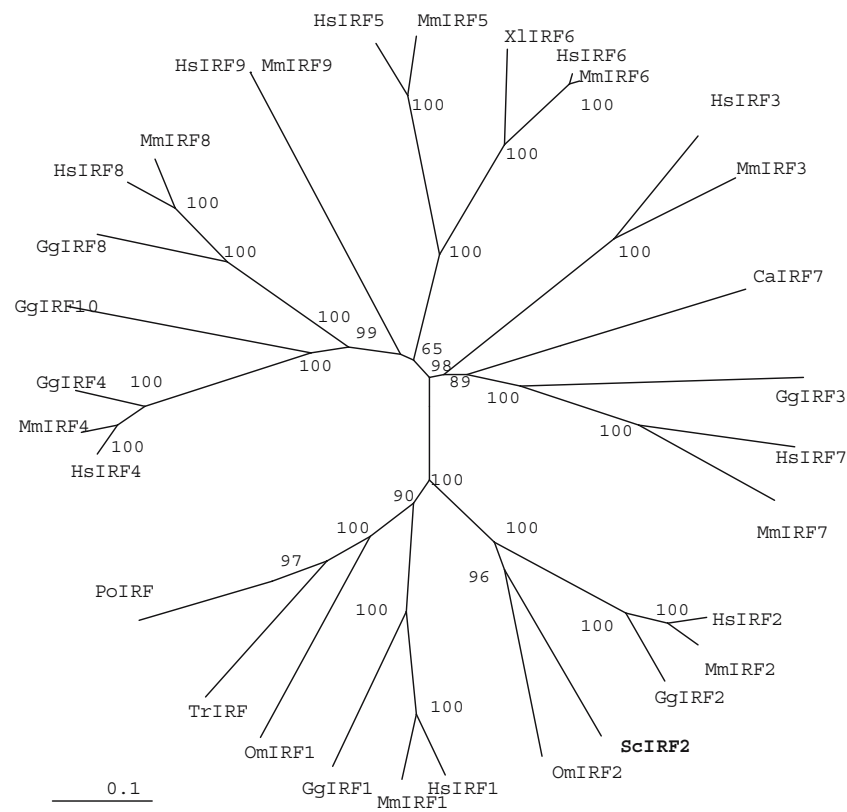


Fig. 5 Unrooted phylogenetic tree of IRF sequences. Complete aa sequences were aligned by using ClustalW, and the tree was constructed with unweighted pair group method with arithmetic mean

in MEGA 2 and a bootstrap analysis was performed using 1,000 replicates to test the relative support for particular clades. GenBank accession numbers of these genes are shown in Table 2

exon (+10 to +19 and +33 to +42); however, no significant similarity could be found in the first intron of mandarin fish IRF-2 when compared with human and rainbow trout flanking regions.

Transcription of IRF-2a and IRF-2b in different tissues

The typical standard curve for IRF-2a, IRF-2b, and actin are constructed using a series of 10-fold dilutions of each plasmid DNA from 10^4 – 10^9 copies. For each assay, the linear correlation ($r > 0.99$) between the crossing point and log concentration of IRFs or actin mRNA copy number was observed. The amplification efficiencies during the exponential phase were highly reproducible.

Both the IRF-2a and IRF-2b were constitutively expressed genes, and the IRF-2a was the predominant IRF-2 molecule in the tissues examined (Fig. 6). The copy number of IRF-2a mRNA (copies/ μ g total RNA) before stimulation was about 5.9×10^7 in the liver, 6.4×10^7 in the intestine, 9.6×10^7 in the kidney, 1.4×10^8 in the heart, 1.4×10^8 in the pronephros, 2.4×10^8 in the gill, and 3.5×10^8 in the spleen, after stimulation with Poly I: C, and significant increases in transcripts observed in kidney and

pronephros ($P < 0.05$). The copy number of IRF-2b mRNA before stimulation was about 8.0×10^6 in the liver, 1.1×10^7 in the heart, 1.6×10^7 in the intestine, 2.3×10^7 in the kidney, 2.8×10^7 in the head kidney, 2.9×10^7 in the spleen, and 2.8×10^7 in the gill, with a significant increase in transcripts in the kidney after stimulation ($P < 0.05$).

The microsatellites in the coding region of other perform fish IRF-2

All the 16 mandarin fish analyzed were heterozygous with two different alleles, and eight different alleles were identified (Fig. 7). The insertions of TGCT or CGCT found in seven alleles separate the CA repeat into two parts, and the number of the second CA repeat varies from 19 to 27. All the alleles identified in this investigation form the truncated molecules, and the stop codon in each allele was also shown in Fig. 7.

The IRF-2 molecules in other perciform fish investigated in this study contain the microsatellites, although the length of the CA repeats varies (Fig. 8). All the fish were heterozygous, and both alleles form the truncated form IRF-2. One allele from each fish was shown in Fig. 8.

Table 2 IRF sequences used for phylogenetic tree construction and multiple sequence alignment

Species	Protein	Accession no.
<i>Homo sapiens</i>	HsIRF1	NM_002198
	HsIRF2	NM_002199
	HsIRF3	Q14653
	HsIRF4	AAH15752
	HsIRF5	Q13568
	HsIRF6	AF027292
	HsIRF7	NM_004029
	HsIRF8	A45064
	HsIRF9	Q00978
<i>Mus musculus</i>	MmIRF1	CAB9163
	MmIRF2	NM_008391
	MmIRF3	P70671
	MmIRF4	NM_013674
	MmIRF5	P56477
	MmIRF6	AAB36714
	MmIRF7	NM_016850
	MmIRF8	NM_008320
	MmIRF9	NM_008394
<i>Gallus gallus</i>	GgIRF1	L39766
	GgIRF2	Q98925
	GgIRF3	NP_990703
	GgIRF4	AF320331
	GgIRF8	Q90781
	GgIRF10	AF380350
<i>Xenopus laevis</i>	XlIRF6	D86492
<i>Siniperca chuatsi</i>	ScIRF2	AY395717
<i>Paralichthys olivaceus</i>	PoIRF	AB005883
<i>Takifugu rubripes</i>	TrIRF	AAK28340
<i>Carassius auratus</i>	CaIRF7	AY177629
<i>Danio rerio</i>	DrIRF7	BQ074286
<i>Oncorhynchus mykiss</i>	OmIRF1	AF332147
	OmIRF2	AY034055

Discussion

The mandarin fish IRF-2 gene found in the present study has some unique features in respects of gene organization, alternative transcripts, and microsatellites. The eight exon and seven intron organization differs from the clarified human IRF-2 gene structure, which extends 86,789 bp and contains nine exons and eight introns (Cha and Deisseroth 1994). The observation that two mRNAs are transcribed from the same mandarin fish IRF-2 gene with the difference in their transcriptional initiation sites has not been reported for other IRF genes for vertebrates from teleosts to mammals, although alternative splicing could be found in mammal IRF-7 (Zhang and Pagano 1997). In addition, alternative splicing has been shown to be involved in the generation of various immuno-modulatory proteins, such as EGF, transforming growth factor-β1, IL-2, IL-4, and others (Atamas 1997).

It was not surprising to find similar transcriptional factor binding motifs in the flanking regions of teleosts and mammals. Previous research indicates that genes involved in the same biological process are often regulated by similar transcriptional mechanisms, and their proximal promoters may contain similar transcriptional factor binding sites (Pilpel et al. 2001; Elkon et al. 2003). The similar sequence and motif that are found in IRF-2 flanking regions of fish and mammal might indicate their functional importance. The sequence GAGGAAGTGAAAATGAAAT found in mandarin fish and rainbow trout was interesting because the sequence (GAAAN₁₋₂GAAA) being the recognition sequence of IRF-1 (Escalante et al. 1998) suggests that the expression of IRF1 could induce the IRF-2 transcription in teleosts just as in mammals (Harada et al. 1994). The binding site of NFκB, which is required for IRF-family-mediated IFN-β induction (Kirchhoff et al. 1999), has been confirmed to be involved in the IRF-2 induction (Harada et al. 1994). The promoters of human IRF-2 could be constitutively expressed and activated by IFN α and IFN γ in the transient expression assays (Cha and Deisseroth 1994), and the promoter of rainbow trout IRF-2 could be activated by poly I: C (Collet et al. 2003b). Whether or not the transcription of mandarin fish IRF-2 is regulated by different promoters still remains to be clarified in future work.

The constitutive transcriptions of both mandarin fish IRF-2 mRNAs were similar to those in previous reports. The IRF-2 is a constitutively expressed molecule in mammals (Cha and Deisseroth 1994), and the constitutive transcription of IRF-2 was also reported in cultured rainbow trout cells and tissues (Collet et al. 2003b). The elevation of mandarin fish IRF-2a in pronephros and kidney may be induced by IFN and IFN-induced genes because Poly I: C could effectively induce IFN and IFN-induced

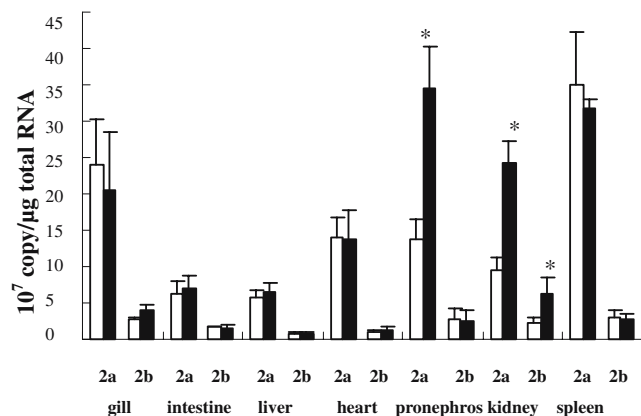


Fig. 6 Transcripts of mandarin fish IRF-2a (2a) and IRF-2b (2b) in different tissues. The white column represents tissue without Poly I: C stimulation, and the black column represents tissue with Poly I: C stimulation. Each column and bar represents the mean±SD of from three individuals, and an asterisk (*) indicates a significant change (P<0.05)


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CAA (CA) 5 TGCT (CA) 19 CGTA.....TTATGAGGA.....AAATAGGTT.....TCGTAGAAG.....TTTTGACCA.....
CAA (CA) 5 TGCT (CA) 20 CGTA.....TTATGAGGA.....AAATAGGTT.....TCGTAGAAG.....TTTTGACCA.....
CAA (CA) 5 TGCT (CA) 21 CGTA.....TTATGAGGA.....AAATAGGTT.....TCGTAGAAG.....TTTTGACCA.....
CAA (CA) 5 TGCT (CA) 22 CGTA.....TTATGAGGA.....AAATAGGTT.....TCGTAGAAG.....TTTTGACCA.....
CAA (CA) 5 CGCT (CA) 24 CGTA.....TTATGAGGA.....AAATAGGTT.....TCGTAGAAG.....TTTTGACCA.....
CAA (CA) 5 CGCT (CA) 25 CGTA.....TTATGAGGA.....AAATAGGTT.....TCGTAGAAG.....TTTTGACCA.....
CAA (CA) 5 TGCT (CA) 27 CGTA.....TTATGAGGA.....AAATAGGTT.....TCGTAGAAG.....TTTTGACCA.....
CAA (CA) 26 CGTA.....TTATGAGGA.....AAATAGGTT.....TCGTAGAAG.....TTTTGACCA.....

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Fig. 7 Confirmation of microsatellites' polymorphism in mandarin fish IRF-2. Eight alleles were found in the 16 fish investigated. The CA repeat number is indicated with (CA)*n*, and stop codon of each allele is boxed in sequence

Fig. 8 Comparison of some perciform fish IRF-2 microsatellite regions with rainbow trout sequence. Fish used including *Coreoperca kawamebari* (Ck), *Coreoperca herzi* (Ch), *Coreoperca whiteheadi* (Cw), *Siniperca scherzeri* (Ss), *Siniperca fortis* (Sf), *Coreosiniperca roulei* (Cr), *Siniperca kneri* (Sk), *Siniperca undulates* (Su), *Onorhynchus mykiss* (Om). The stop codon is boxed

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Ck CACTCGTACCCGCTCCAATCTCTCCTGTGTCTTCGTGCTGCG---GTGAGACGACACACACACACACACACACACACACACACACACAC
Ch CACTCGTACCCGCTCCAATCTCTCCTGTGTCTTCGTGCTGCG---GTGAGAC-----CACACACACACACACACACACACACAC
Cw CACTCGTACCCGCTCCAGATCTCTCCCGTGTCTTCATGCTGCG---GTGAGACG-----AGACGCAC
Sf CACTCGTACCCGCTCCAATCTCTCCCGTGTCTTCATGCTGCG---GTGAGACAACACACAC-----ATGCTCAT
Su CACTCGTACCCGCTCCAATCTCTCCCGTGTCTTCATGCTGCG---GTGAGACAACACACACA-----CATGCTCAC
Ss CACTCGTACCCGCTCCAATCTCTCCCGTGTCTTCATGCTGCG---GTGAGACAACACACAC-----ATGCTCAC
Sk CACTCGTACCCGCTCCAATCTCTCCCGTGTCTTCATGCTGCG---GTGAGACAACACACACA-----CATGCTCAC
Cr CACTCGTACCCGCTCCAATCTCTCCCGTGTCTTCATGCTGCG---GTGAGACAACACACACATGCTCACACGCACACGCACACGCACAC
Om CACCCGTACCCACTCCAGATCTCCCTGTGTCTTCGTATGAGAGAGTGACCCAGACAGTGTGACAGTGAAGAGGACTCCAAGGAGGAT
*** ***** ***** ***** ** ***** * * * * ***** *

Ck ACACACACACACACACACA-----CACACAGAGCGGCTGTACCAATTACGTTTTGAGAAGG-AGCATAAATAGGTTAGTT
Ch ACACACACACACACACACA-----CACACGGAGCAGCTGTACCAATTACGTTTTGAGAAGG-AGCATAAATAGGTTAGTT
Cw GCACGCACGCACACACACA-----CAGGCACGCACGCTGTACCAAGTTACGTTTTGGGAAGG-GGCATAAAATAGGTTAGTT
Sf ACACACACACACACACGC-----GCGCTCCACACATCACCCTTTACCAGATTATGTTATGAGGAGG-AGTATAAAATAGGTTAGTT
Su ACACACACACACACACACACACACATACGCGCTCCACACATCACCCTTTACCAGATTATGTTATGAGGAGG-AGTATAAAATAGGTTAGTT
Ss ACACGC-----GCGCTCCACACATCACCCTTTACCAGATTATGTTATGAGGAGG-AGTATAAAATAGGTTAGTT
Sk ACACACACACACACACGTACG--TGCACGCTGCACACATCACCCTTTACCAGATTATGTTATGAGGAGG-AGTATAAAATAGGTTAGTT
Cr ACACACACACACACTCACACGTACGTCACGCTGCACACATCACCCTTTACCAGATTATGTTATGAGGAGG-AGTATAAAATAGGTTAGTT
Om CTTCGTGGTGGGCTGTGGGAATC---AAGTTTCACTTCTCTGTGCTGAGAGTGCCCTCGTGTCTATTACCAGCATGGCCACCTTTGCTC
** * * * *

Ck A-CAGTACGTGGG--ACCGGCTTGTGGAAG---CAGTAGCTGTGCG-AAATTTTCAG-AGCCACAGTTTTGACCACA
Ch A-CAGTACGTGGG--ACTGGCTTGTGGAAG---CAGTAGCTGTGCG-AAGTTTTTCAG-AGCCACAGTTTTGACCACA
Cw AACAGTGCCTGGG--ACCGGCTTGTGGAAG---CAGTAGCTGTGCGAAGTTTTTCAG-AGCCACAGTTTTGACCACA
Sf A-----CATGGG--ACCGGCTCGTAGAAG---CAGTAGGTGTTG-TAGTTTTTCAG-AGCCACAGTTTTGACCACA
Su A-----CATGGG--ACCGGCTTGTGGAAG---CAGTAGGTGTTG-TAGTTTTTCAG-AGCCACAGTTTTGACCACA
Ss A-----CATGGG--ACCGGCTCGTAGAAG---CAGTAGGTGTTG-TAGTTTTTCAG-AGCCACAGTTTTGACCACA
Sk A-----CATGGG--ACCTGGCTCGTAGAAG---CAGTAGGTGTTG-TAGTTTTTCAG-AGCCACAGTTTGACCACA
Cr A-----CATGGG--ACCGGCTCGTAGAAG---CAGTAGGTGTTG-TAGTTTTTCAG-AGCCACAGTTTTGACCACA
Om ACTGGTGGAAAGGTAACCAACTTCAAAGTGACCAGCACCATGGACCCCATGCCCTTATTAGCTACAGCAACAGCCTCTG
* ** * * * * * *

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genes, and the elevated expression of IFN and IFN-induced genes could promote strong expression of IRF-2 (Cha and Deisseroth 1994). The pronephros and kidney are important lymphoid and hematopoietic organs of fish, which are rich in leukocytes; elevated expression of IRF-2 would keep the antiviral state in an elaborate way to prevent the host defense system from overreacting (Barnes et al. 2002). The high transcription of IRF-2a may be explained by the nearby 5' flanking region being a strong promoter because it contains motifs similar to human and rainbow trout IRF-2. The different transcription of mandarin fish IRF-2a and IRF-2b may be due to different promoters or regulated by cooperation of different transcriptional factors and coactivators.

Another feature of mandarin fish IRF-2 was the microsatellites (CA repeats) in the C terminus of the ORF. Microsatellites are short tandemly repeated sequence motifs consisting of 1–6 bp. Over the past decade, microsatellites have attracted considerable attention due to their involvement in some neurodegenerative diseases and their high polymorphism (Schlötterer 2000). Microsatellites in the ORF region are not rare cases, and in fugu genome, 11.55% of the microsatellites are detected in ORF regions (Edwards et al. 1998). Most of the microsatellites in coding regions have repeat unit sizes that are multiples of three, and the dominance of triplets and hexanucleotide repeats may be due to the limitation by nonperturbation of the reading frame because frameshift mutation would produce a wrong corresponding protein, lead to a gain or loss of gene function, and eventually cause phenotypic changes (Young et al. 2000). However, the CA repeats in the mandarin fish IRF-2 lead to frameshift and resulted in the truncated form IRF-2.

The high polymorphism of CA repeats in mandarin fish IRF-2 is surprising, and the elongation and shorting of the CA repeat resulted in the polymorphism in this investigation. All the IRF-2 alleles investigated encode the truncated form molecule with a threonine and histidine tract. Microsatellites within genes should be subjected to stronger selective pressure than other regions because of their functional importance (Young et al. 2000; Li et al. 2004); thus, the C terminus containing the basic aa seems not to be functionally indispensable for the mandarin fish. In addition, a truncated form of IRF-2 has been observed in previous research. IRF-2 can be processed by proteolysis after viral infection, resulting in an approximately 160-aa N-terminal peptide, which can also bind to target sequences, and some studies have shown that the truncated versions of IRF-2 repress reporter genes more efficiently than full-length IRF-2 does (Whiteside et al. 1994). The existence and polymorphism of CA repeats in mandarin IRF-2 may indicate different roles from its homologues of mammals.

The fish in genera *Coreoperca* and *Siniperca* used in this experiment are freshwater perciform fishes endemic to East Asia including China, Vietnam, Japan, and Korea. Because Perciformes is the largest and most diversified vertebrate group and contains 37.7% of the total fish species (Nelson 1994), whether or not the truncated form IRF-2 only exist within perciform fishes awaits further research.

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