Cloning the interferon regulatory factor 1 gene in lungfish (*Protopterus annectens*) and its molecular evolution among sarcopterygians

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Sarcopterygians is an important vertebrate clade that includes crossopterygians and tetrapods. Crossopterygians are lobe-finned fish that include lungfish and coelacanths. Tetrapods include amphibians, reptiles, avians and mammals. To compare the interferon regulatory factor 1 (*irf-1*) gene structure and to explore phylogenetic relationships among sarcopterygians, we cloned the cDNA sequence of *irf-1* from lungfish and compared it with *irf-1* orthologs in other sarcopterygian species. The lungfish is a primitive sarcopterygian that occupies a very important position in vertebrate phylogeny. Interferon regulatory factors (IRFs) are a family of proteins involved in innate immunity. To date, 11 IRF family members have been reported. All IRFs share homology in the first 115 amino acids, which encompasses a DNA binding domain containing a characteristic repeat of 5 tryptophan residues separated by 10–18 amino acids. IRF-1 and IRF-2 were the first members of this family to be reported and they have a very important role in innate immunity. However, studies of the *irf-1* and *irf-2* genes are mostly confined to mammals; very few non-mammalian *irf-1* genes have been reported. Consistent with the *irf-1* gene sequences already published, the first 345 nucleotides of lungfish *irf-1* are highly conserved. At the carboxyl terminal a C-terminal transactivating region motif and an interferon associated domain (IAD2) were identified. 417 million years separate the present from the closest common ancestor of lungfish and tetrapods; however, the *irf-1* genes among sarcopterygians are highly conserved and have very obvious phylogenetic relationships. Also the interrelationship tree of sarcopterygians, based on IRF-1 amino acid sequences, is identical with trees produced using other data, such as morphological characteristics or mitochondrial gene sequences.

IRF-1, innate immunity, Sarcopterygii, molecular evolution, phylogeny

Interferon (IFN) is a multifunctional cytokine that has a crucial role in innate immunity in animals. Interferon regulatory factor (IRF) has a very important role in interferon synthesis. IRF was first identified as a factor that regulates the transcription of the *IFN* gene and of IFN-induced genes [1]. IRF also has other metabolic functions, such as in antiviral defense, immune regulation and growth control [2]. To date, 11 members of the IRF family have been reported, mostly in mammals. IRF-1 regulates the virus-induced enhancer-like element in the human interferon-β (*IFN-β*) gene and is a transcriptional activator of the type I IFN gene and of the IFN-induced genes [3,4]. Another factor, structurally similar to IRF-1, was identified and termed IRF-2. IRF-2 is a repressor of transcription in IFN synthesis [5] and also has other important metabolic functions, for example, IRF-2 is a transcription activating in the transcription process of histone H4, VCAM-1, gp91phox and interleukin-7, also it is the transcript catalyst for class II transactivator [6–10]. IRF has a DNA binding domain (DBD) and an IRF association domain 1 (IAD1) or IAD2. Its first 115 amino acids are high homologous, and the tryptophan cluster consists of 5 tryptophans. On its carboxyl terminal region, there is lower

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1 Materials and methods

1.1 Sample collection and identification

Samples of African lungfish (*Protopterus annectens*) were obtained from an aquatic pet market in Guangzhou, China. Specimens were confirmed in the Institute of Hydrobiology, Chinese Academy of Sciences.

1.2 Extraction of RNA, synthesis of cDNA, gene amplification and sequencing

Total RNA was extracted using a Trizol reagent kit (TaKaRa, China) from a combination of heart, liver, kidney, spleen and intestine tissues. The first cDNA strand was synthesized using an M-MLV 1st strand kit (Invitrogen, USA) in accordance with the manufacturer’s instructions. Primers for the amplification of homologous fragments and RACE are listed in Table 1. Initially, we used degenerate primers (Lf-IRF-1F1 and Lf-IRF-1R1) to amplify a conserved fragment. Degenerate primers were designed according to published IRF-1 sequences. PCR was performed in a total volume of 50 μL that included 100 ng cDNA, 5 μL 10× buffer (TaKaRa), 1 μL each primer (10 μmol/L), 2 μL dNTPs (2.5 μmol/L each) (TaKaRa), 2.0 U Taq DNA polymerase (TaKaRa) and double distilled H₂O to the final volume to 50 μL. PCR cycling parameters were: 94°C for 3 min; followed by 34 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 1 min; and a final cycle of 72°C for 7 min. A control reaction without cDNA template was performed. To identify and clone a full-length cDNA, 3‘RACE and 5‘RACE were performed using gene specific primers and adaptor primers. RACE primers, 3‘Lf-IRF-1-RACEouter inner and 3‘Lf-IRF-1-RACEinner, were designed based on the amplified conserved fragment. PCR cycle parameters used were as above except that the annealing temperature was 54°C. 3 RACE was performed using the First Choice RLM-RACE kit (Ambion, USA). Primer sequences are listed in Table 1. PCR cycle parameters were: 94°C 3 min followed by 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min 40 s, and a final cycle at 72°C for 7 min. All PCR products were cloned into pMD18-T vector and sequenced. DNA sequencing was performed using the generic primer, M13.

1.3 Alignment and phylogenetic analysis of the irf-1 gene sequence

The IRF-1 gene sequences downloaded from GenBank are listed in Table 2. The multiple alignments of sequences were performed using Clustal X [13]. The editing of DNA sequences and protein translation were performed using SEAVIEW software [14]. Neighborhood joint (NJ), maximum likelihood (ML) and Bayesian analysis (BA) methods were used to construct the phylogenetic trees. In the ML and BA analyses, the models and parameters of nucleotide evolution were estimated using the Model-test software [15]. Each clade on the tree was evaluated with 1000 bootstrap samples. The software used in this study included PAUP 10.4b, MrBayes 3.12 and Mega 4.0 [16–18]. Grass carp (*Ctenopharynodon idellus*), Mandarin fish (*Siniperca chuatsi*) and snakehead (*Channa argus*) were assigned as out-groups.

2 Results

2.1 Characteristics of the lungfish irf-1 gene

The lungfish irf-1 gene has an open reading frame (ORF) of

| Name of primer | Sequence of primer | Application |
|----------------|--------------------|-------------|
| Lf-IRF-1F1     | 5’-CARATHCCCTGGGATGCAYGC-3’ | IRF-1 conserved fragment |
| Lf-IRF-1R1     | 5’-GCYTTCCAKGTYTTYNGLTRC-3’ | IRF-1 conserved fragment |
| Lf-IRF-1F2     | 5’-ATGCCNGTVGARMGRATGGMG-3’ | IRF-1 conserved fragment |
| Lf-IRF-1R2     | 5’-GCATCCAAAGTATAGGTTCTC-3’ | IRF-1 conserved fragment |
| 3‘Lf-IRF-1-RACEouter | 5’-GCTGGCATTTCTCAGAGTGGCG-3’ | 3’RACE |
| 3‘Lf-IRF-1-RACEinner | 5’-CTGGGCCTGTACAAACCTGGAGAG-3’ | 3’RACE |
942 bp, encoding 314 amino acids, and a 3-UTR of 399 bp. The stop codon is TAG. The putative IRF-1 peptide sequence contains a nuclear localization sequence (NLS), C-terminal transactivating region and an IAD-2. This sequence was submitted to GenBank with the accession number HQ873639.

2.2 Comparative analysis of IRF-1 sequences in sarcopterygians

Although there is variation in irf-1 gene size among the different sarcopterygian species, their ORFs are of similar length. The alignment of IRF-1 gene sequences of sarcopterygians (mammals, avian, reptiles, amphibians, sarcopterygian) and actinopterygii as an out-group show that the first 345 nucleotides, encoding 115 amino acids, are very conserved and that the other regions, such as the IAD-2, have higher levels of variation. The alignment of the putative IRF-1 amino acid sequences also show high levels of conservation, especially the DBD and the 5 tryptophan residues repeats in the first 115 amino acids.

2.3 Molecular evolution and phylogenetic analysis

The comparative analysis of IRF-1 amino acid sequences (Figure 1) indicated variation between tetrapods and sarcopterygians. Among tetrapods, avians and reptiles have a very high identity of the IRF-1 amino acid sequence, as do amphibians and lungfish (crossopterygian). In lungfish, the first 115 amino acid residues are conserved. Besides a conserved DBD in lungfish, on the carboxyl terminal region we also identified a conserved C-terminal transactivating region and IAD2.

The phylogenetic trees constructed using different methods showed a similar topological structure. The NJ tree, based on amino acid sequences with 1000 replicated bootstraps, is shown in Figure 2. When 3 actinopterygian species were assigned as an out-group, all the included sarcopterygian species form a monophyletic group, lungfish as the representative crossopterygian was located on the basal clade. The obtained clades were supported with high bootstrap values. Among tetrapods, the African claw frog is a primitive amphibian and is located on the base, followed by the avians (chick) and then the mammals. All clades obtained >90% bootstrap support. The same tree based on mitochondrial cytochrome b gene sequences is similar to that based on irf-1 sequences. Because the IRF-1 amino acid sequence from the Anolis genus of lizards obtained from GenBank is very short, we did not use it in phylogenetic analysis.

3 Discussion

Because of the high level of sequence diversity and a large intron, to design PCR primers and amplification of the IRF gene from genomic DNA of diverse species is very difficult. At present most of the IRF genes reported are mammalian, with few IRF genes reported in fish and primitive tetrapods. The sequence of the lungfish IRF-1 gene gives us a complete dataset of IRF-1 in sarcopterygians, so we have IRF-1 gene sequences from all the representative members of sarcopterygians. The fossil record indicates that the most recent common ancestor of lungfish and tetrapods is 417 million years old. However, over such a long time scale, the IRF-1 ORF has maintained a high level of conservation, indicating that IRF-1 has a very stable secondary structure of protein and a very important function. Also the involvement of IRF-1 and IRF-2 in innate immunity can be traced back to the time when osteichthyran species originated.

Sarcopterygians are very important vertebrates that include crossopterygians and tetrapods. In addition to the fossil record, the recent species of crossopterygians include two species of coelacanths and three species of lungfish. However, their molecular phylogeny based on innate immune genes is not known. Both the fossil record and morphological data indicated tetrapods originated from the early crossopterygians. The most primitive tetrapods may be the amphibian genus, ichthyostega. Reptiles are a huge group derived from amphibians that underwent rapid development in the Jurassic period. Avians constitute a branch of special reptiles, as are dinosaurs. Mammals are derived from another branch of reptiles. But the molecular phylogeny evidence has not provided conclusive evidence of interrelationships among avians, reptiles and mammals. Based on 18S rRNA gene sequences, Xia et al. [19], Hedges et al. [20] and Bininda-Emonds et al. [21] explored the phylogeny of tetrapods using different gene markers and character sets. Our results, based on irf-1 gene sequences, show similar phylogenetic trees to those in the above studies. In our tree, lungfish were located on the base and form a sister group with tetrapods with very high bootstrap support. Following this are amphibians, avian and mammals. Only a very short
IRF-1 gene sequence was available for the Anolis genus of lizards; therefore we did not include it in the phylogenetic analysis. Compared with full length IRF-1 gene cDNA, the phylogenetic tree based on only the first 345 nucleotides, which codes for the DNA binding domain, obtained higher bootstrap support for each clade on the tree. We also found that trees based on functional gene sequences yielded higher bootstrap support than those based on mitochondrial gene sequences. Our research also found that trees based on putative amino acid sequences can obtain higher bootstrap support than those based on DNA sequences. Finally, it is worth noting that because IRF-1 has such an important function and has remained stable over such a long time scale, it is a very good phylogenetic marker.
Figure 2  Phylogenetic tree based on the amino acid sequence of sarcopterygian IRF-1s. Genetic distance is estimated using the Kimura 2 parameter model. The tree is constructed using N-J methods, clades are estimated using bootstrap with 1000 resamples. Protopterus annectens, lungfish.

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