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Evolution of *akirin* family in gene and genome levels and coexpressed patterns among family members and *rel* gene in croaker

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ABSTRACT

Akirins, which are highly conserved nuclear proteins, are present throughout the metazoan and regulate innate immunity, embryogenesis, myogenesis, and carcinogenesis. This study reports all *akirin* genes from miiuy croaker and analyzes comprehensively the *akirin* gene family combined with *akirin* genes from other species. A second nuclear localization signal (NLS) is observed in *akirin2* homologues, which is not in *akirin1* homologues in all teleosts and most other vertebrates. Thus, we deduced that the loss of second NLS in *akirin1* homologues in teleosts likely occurred in an ancestor to all Osteichthyes after splitting with cartilaginous fish. Significantly, the *akirin2(2)* gene included six exons interrupted by five introns in the miiuy croaker, which may be caused by the intron insertion event as a novel evidence for the variation of *akirin* gene structure in some species. In addition, comparison of the genomic neighborhood genes of *akirin1*, *akirin2(1)*, and *akirin2(2)* demonstrates a strong level of conserved synteny across the teleost classes, which further proved the deduction of Macqueen and Johnston 2009 that the produce of *akirin* paralogues can be attributed to whole-genome duplications and the loss of some *akirin* paralogues after genome duplications. Furthermore, *akirin* gene family members and *relish* gene are ubiquitously expressed across all tissues, and their expression levels are increased in three immune tissues after infection with *Vibrio anguillarum*. Combined with the expression patterns of LEAP-1 and LEAP-2 from miiuy croaker, an intricate network of co-regulation among family members is established. Thus, it is further proved that *akirins* acted in concert with the *relish* protein to induce the expression of a subset of downstream pathway elements in the NF-kB dependent signaling pathway.

1. Introduction

Akirins, conserved nuclear resident NF-kB signaling pathway molecules, are present throughout the metazoan. To date, *akirin* genes are identified from eukaryotes, including coelenterates, arthropods, fish, amphibians, birds, reptiles, and mammals (Chen et al., 2012; Dai et al., 2011; Goto et al., 2008; Macqueen et al., 2010a, 2010b; Man et al., 2011; Yang et al., 2011). Furthermore, the *akirin* gene family consists of two members in amphibians and mammals (*akirin1* and *akirin2*), a single member in birds and reptiles (*akirin2*), and two to three members in teleosts (*akirin1(1)* and *akirin2(1)* and/or *akirin2(2)*) (Macqueen et al., 2010a, 2010b). However, teleost species of the Salmonidae family include eight *akirin* family members (*akirin1(1a), 1(1b), 1(2a), 1(2b), 2(1a), 2(1b), 2(2a),* and *2(2b)*) (Macqueen et al., 2010a). Meanwhile, Macqueen and Johnston (2009) and Macqueen et al. (2010a) proposed that the number of *akirin* gene family members is closely related with whole genome duplications (WGDs). *Akirin1* and *akirin2* are derived from basal vertebrate WGD (2R) (Putnam et al., 2008). *Akirin1(1)* and *1(2)* along with *akirin2(1)* and *2(2)* are retained from the teleost WGD (3R) (Jaillon et al., 2004). However, *akirin1(2)* is lost in most species. Eight members of the *akirin* gene family in the Salmonidae family are paralogues retained from the salmonid WGD (4R) (Macqueen and Johnston, 2014).

The innate immune system, which serves as the first line of defense, shields all metazoans against invasion of pathogens and deals with any foreign material until the adaptive immune system can sufficiently take over (Medzhitov and Janeway, 1997; Sinyakov et al., 2002). As an indispensable component of the innate immune responses, *akirins* can coordinate with "14-3-3" proteins to promote or to inhibit mRNA transcription (Gonzalez and Baylies, 2005; Komiya et al., 2008) and interact genetically and physically with the basic helix–loop–helix transcription factor (Twist) to facilitate the expression of a number of Twist-regulated genes during embryonic myogenesis (Nowak et al., 2012). *Akirins* are essential in animal development, owing to the lethal embryonic phenotype of mice

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knockouts as well as the lethal or reduced growth phenotypes demonstrated by targeted knockdown in *Drosophila*, ticks, and nematodes (de la Fuente et al., 2006; Goto et al., 2008; Maeda et al., 2001). Meanwhile, *akirins* participate in regulating gene expressions in numerous physiological processes, including the innate immune responses of mammalians and insects (Goto et al., 2008; Tartey et al., 2014), metazoan myogenesis (Marshall et al., 2008; Salerno et al., 2009), mammalian carcinogenesis (Komiya et al., 2008), insect reproduction, and arthropod growth (Almazán et al., 2005; de la Fuente et al., 2006, 2008). Furthermore, the study demonstrated that *akirins* are required to activate a subset of Relish-dependent genes (Bonnay et al., 2014). *Akirins* acted in concert with *relish* (*rel*) protein in the NF-kB dependent signaling pathway, which induces the expression of a subset of downstream pathway elements (e.g., LEAP-1 and LEAP-2) (Goto et al., 2008; Hou et al., 2013; Xue et al., 2014).

In this study, we extended the comparative genomics and phylogenetics work by Macqueen and Johnston (2009) and analyzed the *akirin* gene structure and the genomic neighborhood surrounding of *akirin* genes to better understand *akirin* genes in miiuy croaker (*Miichthys miiuy*) and to establish the chromosomal locations of *akirin* genes in teleosts. Furthermore, we conducted real-time quantitative PCR (qRT-PCR) assays by using *akirin* genes and *relish* (*rel*) gene of the miiuy croaker to investigate the regulation relationship among *akirin* gene family members, along with *rel* gene.

2. Materials and methods

2.1. Sequence analysis

To identify the *akirin1*, *akirin2(1)*, and *akirin2(2)* genes from the miiuy croaker, we used the available *akirin* homologues reported in other organisms as queries to seek for the transcriptome (Che et al., 2014) and whole-genome sequences of the miiuy croaker (unpublished) by using local BLASTN program. Three corresponding scaffolds were identified. The cDNA sequences were aligned with the corresponding scaffolds by using MAFFT (http://mafft.cbrc.jp/alignment/software/) to determine the exon–intron structure of *akirin* genes from the miiuy croaker. Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to predict the open reading frame (ORF) and to translate nucleotide into protein. Nuclear localization signal (NLS) was forecasted using PSORT II server (http://psort.hgc.jp/form2.html), and SignalP 4.1 server was used to predict the presence and location of signal peptide cleavage sites (Nielsen et al., 1997).

2.2. Sequence alignment and phylogenetic analyses

The *akirin* genes used in this study were derived from GenBank (http://www.ncbi.nlm.nih.gov) and Ensemble database (http://www.ensembl.org) (Supporting information, Table S1). Multiple alignments of the *akirin* sequences from different species were performed under codon model by using MUSCLE software (Edgar, 2004). Before constructing the phylogenetic tree, jModeltest software was used to select an optimal substitution model (Posada, 2008), through which GTR + I + G was considered the best-fit model based on Bayesian information criterion (Posada and Buckley, 2004). Phylogenetic analysis was performed using Bayesian approach in MrBayes v3.2 (Ronquist and Huelsenbeck, 2003), which was ran for 5,000,000 generation with the first 25% of trees burned.

2.3. Comparative analysis of gene synteny

To obtain insight on whether the genomic neighborhoods surrounding *akirin* genes are evolutionary conserved, synteny maps for the genomic neighborhoods surrounding *akirin1*, *akirin2(1)*, and *akirin2(2)* were constructed. Data were manually obtained from release Ensembl genome assemblies for teleost and lamprey (*Petromyzon marinus*). The miiuy croaker corresponding data were obtained from the whole-genome sequences of miiuy croaker (unpublished).

2.4. Fish sampling and challenge experiment

Healthy miiuy croakers (mean weight: 800g) were collected from Zhoushan Fisheries Research Institute (Zhejiang, China). Fish were acclimatized in a recirculating seawater system at an ambient temperature (25 °C) for one week. Challenge experiments, as previously described by Zhu et al. (2013), were performed on the miiuy croaker. Fish were randomly divided into two groups, namely, injection and control groups. In the injection group, fish were challenged with 1 mL of *Vibrio anguillarum* suspension (approximately 3.0×10^7 CFU/mL) by intraperitoneal injection as the control fish were injected with 1 mL of phosphate-buffered saline. The infected and control fish samples were killed at 6, 12, 24, 36, 48, and 72 h after the challenge. Three immune tissues (head kidney, liver, and spleen) from three individuals were sampled. Furthermore, 10 tissues (head kidney, liver, spleen, intestines, heart, muscle, gill, brain, eye, and skin) from healthy miiuy croaker were removed. All tissues were immediately frozen in liquid nitrogen after dissection and then separately stored at –80 °C until RNA extraction.

2.5. RNA isolation and cDNA synthesis

Total RNA was extracted from diverse tissues from individuals following the manufacturer's instructions by using RNAiso Reagent (Takara). The cDNA templates were synthesized through reverse transcriptase M-MLV (Takara) according to the manufacturer's protocol. Extracted RNA samples were stored at –80 °C until its use, whereas cDNA samples were diluted 10-fold and stored at –20 °C until further analysis.

2.6. Expression analysis of akirin genes and rel gene

To examine the tissue distribution of *akirin* mRNAs in different tissues of healthy miiuy croaker and to investigate the regulation relationship among *akirin* and *rel* genes after immune challenges, real-time qPCR was conducted on a 7300 Real-time PCR system (Applied Biosystems, USA) by using a RealMaster Mix kit (TIANGEN) following the manufacturer's instructions. Reaction was carried out without the template used as blank control. Five pairs of qPCR primers were used to amplify *akirin*, *rel*, and β-actin genes as normalizer gene fragments (Supporting information, Table S2). Real-time qPCR reactions were carried out as described by Liu et al. (2014). The reaction for each sample was conducted in triplicate, and the cycling conditions were as follows: 15 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 60 s at 60 °C, and 31 s at 72 °C. Dissociation curve analysis was performed after each assay to determine target specificity. The PCR efficiency was determined using LinRegPCR (Karlen et al., 2007; Ruijter et al., 2014). Pfaffl (2001) method was selected as the relative quantification calculation method. Statistical analysis was performed using one-way analysis of variance statistical test followed by Duncan's multiple comparison tests. *P*-values of less than 0.05 were considered statistically significant. All data were expressed as mean ± standard error.

3. Results

3.1. Characterization and structure of akirin genes from miiuy croaker

The molecular characteristics of *akirin1*, *akirin2(1)*, and *akirin2(2)* from the miiuy croaker are compiled in Table 1. The complete cDNA