Fish and Shellfish Immunology 84 (2019) 726–732

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular characteristics and functional study of tumor necrosis factor receptor-associated factor 2 from the orange-spotted grouper (*Epinephelus coioides*)

Man Yang[a], Rui Han[a], Lu-Yun Ni[a], Xiao-Chun Luo[b], An-Xing Li[c], Xue-Ming Dan[a,*], Yan-Wei Li[a,**]

[a] Joint Laboratory of Guangdong Province and Hong Kong Regions on Marine Bioresource Conservation and Exploitation, College of Marine Sciences, South China, Agricultural University, Guangzhou, 510642, China
[b] School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, 510006, China
[c] State Key Laboratory of Biocontrol/Guangdong Provincial Key Lab for Aquatic Economic Animals, School of Life Sciences, Sun Yat-sen University, Guangzhou, 510275, Guangdong Province, PR China

ARTICLE INFO

Keywords:
TRAF2
Epinephelus coioides
NF-κB
Cryptocaryon irritans

ABSTRACT

In mammals, tumor necrosis factor receptor-associated factor 2 (TRAF2) is a crucial intracellular adaptor protein, which performs a vital role in numerous signaling pathways that activate NF-κB, MAPKs, and IRFs. In the present study, three TRAF2 sequences were identified from the orange-spotted grouper (*Epinephelus coioides*), and named EcTRAF2-1, EcTRAF2-2, and EcTRAF2-3. These sequences contained conserved structure features that were similar to those of mammals. EcTRAF2-1 shared relatively low sequence identity with the other two EcTRAF2s. In healthy *E. coioides*, EcTRAF2s were widely expressed in all tissues tested, but with distinct expression profiles. After infection with *Cryptocaryon irritans*, EcTRAF2s was markedly upregulated in the gill and head kidney at most time points, implying that EcTRAF2s may be involved in host defense against *C. irritans* infection. In HEK293T cells, EcTRAF2s were scattered in the cytoplasm. EcTRAF2-1 and EcTRAF2-2 increased the activity of NF-κB, while EcTRAF2-3 reduced NF-κB activation mediated by EcTRAF2-1 implying that EcTRAF2-3 might be a negative regulator of EcTRAF2-1.

1. Introduction

Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) are involved in multiple signaling pathways that are initiated by Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors, and cytokine receptors such as TNFR and IL-1 receptor (IL-1R). TRAF mediates the activation of nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs), and interferon-regulatory factors (IRFs), and controls various cellular responses [1,2].

Seven TRAFs (TRAF1–7) have been identified in mammals; they are characterized by a C-terminal TRAF domain that is highly conserved in all TRAFs (with the exception of TRAF7 that contains a seven WD40 repeat at the C-terminus) [3]. The TRAF domain is subdivided into an N-terminal coiled-coil region and a C-terminal domain (TRAF-C) (also named MATH domain), which is mainly responsible for protein-protein interactions, such as TRAF oligomerization, and interaction with other adaptor proteins and/or receptors [4]. TRAFs (except TRAF1) contain an additional N-terminal domain composed of a RING finger domain, and a zinc finger domain that includes a variable number of zinc finger motifs. The RING finger domains of TRAF2, TRAF3, TRAF5, and TRAF6 possess E3 ubiquitin ligase activity, which mediates protein ubiquitylation through lysine-63 linkage and is required for the activation of downstream signaling pathways [5].

TRAF2 was originally identified as an adaptor protein interacting with the cytoplasmic domain of human TNFR2 [6]. Additional studies have demonstrated that TRAF2 is required for activation of NF-κB, MAPKs, and IRFs; activation is mediated by a variety of receptors including TNFR family members, NLRs, and RIG-I-like receptors [1,7]. Although in mammals significant progress has been made to elucidate the role of TRAF2 in signaling, functional studies of TRAF2 is scarce in fish. The first fish TRAF2 was reported in grass carp; it was then reported that rock bream TRAF2 activates NF-κB [8,9]. Recently, Chen et al. reported that black carp TRAF2 upregulates NF-κB activity but not IFN activity. TRAF2 increased mitochondrial antiviral signaling (MAVS)-mediated interferon production and increased antiviral activity mediated by MAVS [10]. To our best knowledge, there are no

* Corresponding author.
** Corresponding author.
E-mail addresses: dxm72@scau.edu.cn (X.-M. Dan), yanweili@scau.edu.cn (Y.-W. Li).

https://doi.org/10.1016/j.fsi.2018.10.070
Received 29 June 2018; Received in revised form 9 October 2018; Accepted 26 October 2018
Available online 28 October 2018

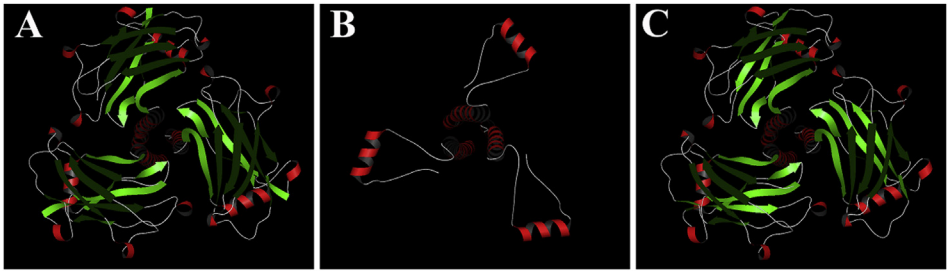

Fig. 1. Three dimensional model of the MATH domain of EcTRAF2-1 (A), EcTRAF2-2 (B), and EcTRAF2-3 (C). The model was created using human TRAF2 MATH domain (1ca9.2.A) as a template.

Table 1
Protein sequence identity of TRAF2 between grouper and other species.

Species	Identity (%)		
	EcTRAF2-1	EcTRAF2-2	EcTRAF2-3
EcTRAF2-1 (AME21332.1)	100	51	58
EcTRAF2-2 (AME21331.1)	51	100	77
EcTRAF2-3 (AME21330.1)	58	77	100
Lates calcarifer (XP_018518421.1)	94	51	58
Lates calcarifer (XP_018549809.1)	58	76	98
Lates calcarifer (XP_018524804.1)	60	95	82
Larimichthys crocea (XP_010736869.2)	91	50	59
Larimichthys crocea (KKF33157.1)	58	76	97
Larimichthys crocea (KKF24896.1)	61	95	81
Takifugu rubripes (XP_003965428.1)	86	47	56
Takifugu rubripes (XP_011617744.1)	59	76	97
Takifugu rubripes (XP_011617713.1)	61	87	78
Stegastes partitus (XP_008303697.1)	94	52	59
Stegastes partitus (XP_008278833.1)	60	95	82
Notothenia coriiceps (XP_010778810.1)	88	51	59
Oncorhynchus mykiss (NP_001117865.1)	82	50	58
Ctenopharyngodon idella (ABE99696.1)	78	49	58
Mylopharyngodon piceus (ASO96850.1)	78	49	58
Xenopus tropicalis (XP_017945881.1)	57	50	61
Alligator mississippiensis (XP_019331323.1)	61	59	67
Gallus gallus (CDZ92726.1)	60	57	66
Mus musculus (NP_001277342.1)	58	57	64
Homo sapiens (ADQ89802.1)	58	57	64

additional reports on the function of TRAF2 in fish.

Orange-spotted grouper (*Epinephelus coioides*) is one of the most important marine fish species cultured in south China; it is often infected by *Cryptocaryon irritans*. *C. irritans* is an obligate ecto-parasite, which mainly infects the skin, gills, and fins of marine fish causing heavy economic losses [11].

We have used *E. coioides* and *C. irritans* as a model to study immune mechanisms against parasites. In order to explore the role of *E. coioides* TRAF2 (EcTRAF2), we analyzed the transcriptome database of *E. coioides*, TRAF2 sequences were used as query in a sequence homology search. Three TRAF2 sequences with complete open reading frames (ORFs) were identified. The expression of TRAF2s was determined by real-time PCR before and after infection with *C. irritans*. To analyze the ability to activate NF-κB, TRAF2s were overexpressed in HEK293T cells and NF-κB luciferase activity was detected.

2. Materials and methods

2.1. Fish, parasites, and cells

Orange-spotted groupers (60.3 ± 7.3 g) were purchased from the Marine Fisheries Development Center of Guangdong Province, Guangdong, China. Fish were then reared in a recirculating seawater system and fed daily with commercial feed at 25 °C for 2 weeks.

C. irritans was maintained by serial passage on *Trachinotus ovatus* following a method described previously [12].

HEK293T cells were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (Gibco) under 5% CO_2, at 37 °C, and subcultured at 2-day intervals.

2.2. RNA extraction and cDNA synthesis

Samples of gill, muscle, thymus, head kidney, liver, spleen, heart, stomach, foregut, midgut, hindgut, trunk kidney, and brain were isolated from three *E. coioides*, and used to analyze the constitutive expression of EcTRAF2s. Total RNA was isolated from these tissues using the HiPure Universal RNA Kit (Magen, China) according the manufacturer instructions. RNA quality and concentration were determined by agarose gel electrophoresis and OD260/280 analysis. After removal of contaminating DNA with RNase-free DNase I (Thermo Fisher Scientific, USA), 1 μg of total RNA from each sample was used to synthesize first strand cDNA with the RevertAid Frist Strand cDNA Synthesis Kit (Thermo Fisher Scientific), following the manufacturer protocol; cDNA was then stored at −20 °C.

2.3. Sequence analysis of EcTRAF2s

Three EST sequences sharing high identity with TRAF2 of other species were identified in the *E. coioides* transcriptome data (unpublished data). Sequence analysis with ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) indicated that these TRAF2s contained a complete ORF. Primers for TRAF2-1 F/R, TRAF2-2 F/R, and TRAF2-3 F/R (STable 1) were used to amplify the ORF of each TRAF2, and then the PCR product was sequenced.

Isoelectric point (pI) and molecular weight (Mw) were calculated using Expasy program (http://web.expasy.org/compute_pi/). Protein structure was predicted using SMART program (http://smart.embl-heidelberg.de/). Multiple sequence alignment was performed with ClustalW2 (http://www.ebi.ac.uk/Tools/clusta-lw2/index.html). A three dimensional model was constructed using SWISS-MODLE software (http://swissmodel.expasy.org/interactive). A phylogenetic tree was constructed with MEGA 5.0 program using the neighbor-joining method and 1000 bootstrap replications.

2.4. Expression of EcTRAF2s in different tissues

Real-time PCR was performed to analyze the expression of EcTRAF2s. TRAF2-1qF/R, TRAF2-2qF/R, and TRAF2-3qF/R (STable 1) were the primers for EcTRAF2-1, EcTRAF2-2, and EcTRAF2-3, respectively. Beta-actin primers of β-actinF/R were used as internal control. The volume of the PCR reaction was 10 μL; it contained 0.5 μL of cDNA, 0.3 μL of each primer, 5 μL of iTaq Universal SYBR Green Supermix (Bio-Rad, USA), and 3.9 μL of water. The PCR reaction protocol was 95 °C for 30 s, and then 95 °C for 15 s, and 60 °C for 60 s for 40 cycles. The specificity of PCR products was determined by melting curve analysis and sequencing. All samples were analyzed in triplicate. The expression of the target gene was normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method [13]. The expression level in each tissue was represented as the ratio to that in the muscle for each gene.

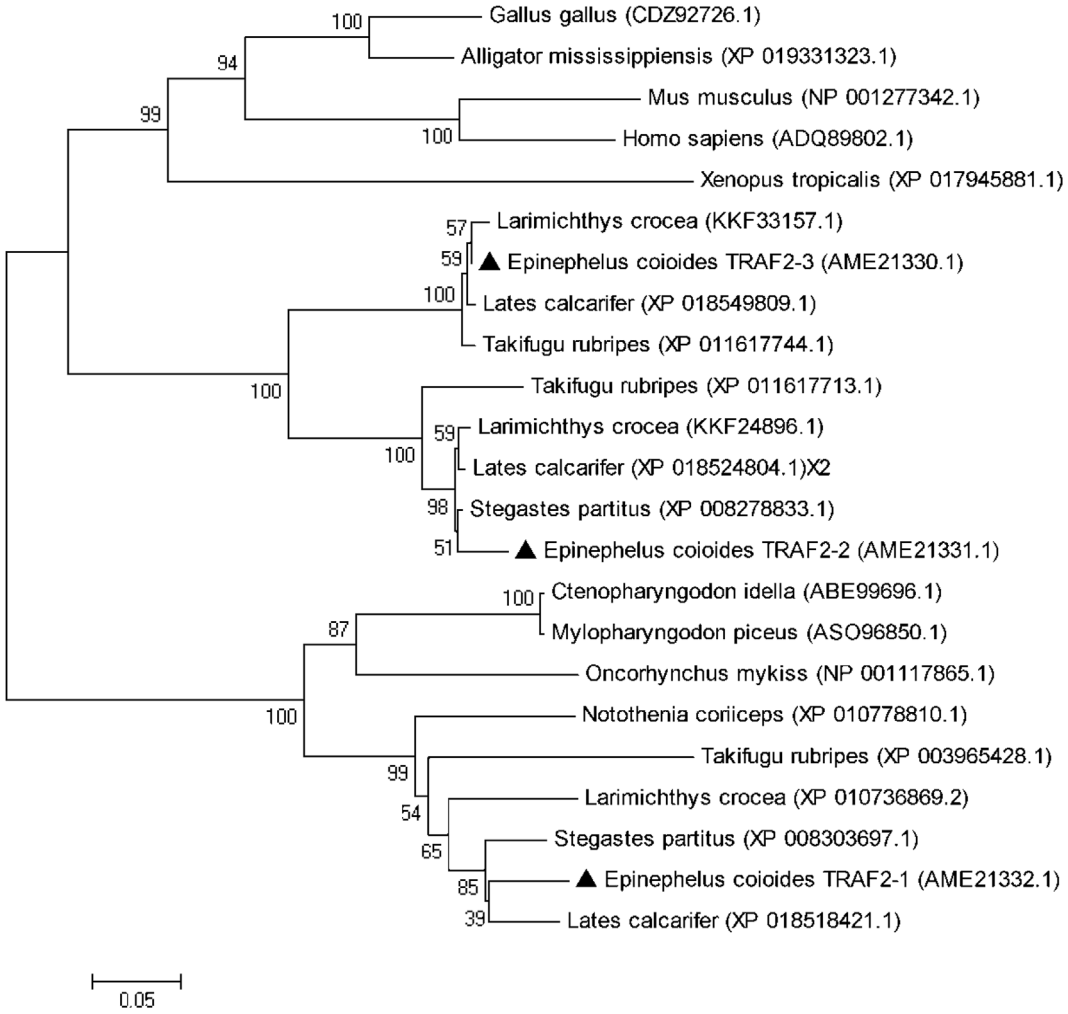

Fig. 2. Phylogenetic analysis of the EcTRAF2 proteins. The predicted amino acid sequences of TRAF2 were aligned and a neighbor-joining tree was constructed. Numbers on the lines indicate the percentage bootstrap values after 1000 replicates.

2.5. Expression of EcTRAF2s after C. irritans infection

To assess the expression of EcTRAF2s after infection, *E. coioides* was infected with *C. irritans*, and the expression of these genes was measured in gills and head kidney by real-time PCR. 80 E. *coioides* were randomly divided into two groups, namely the infection group, and uninfected group, with one tank in each group. In infection group, fish were exposed to the *C. irritans* with a dose of 25,000 theronts per fish for 2 h. Fish in the uninfected group were treated in the same way without *C. irritans*. At 6 h, 12 h, 1 d, 2 d, 3 d, and 5 d after infection, gills and head kidney from both groups were isolated, and gene expression analyzed using real-time PCR as described in part 2.4.

2.6. Plasmid construction

Full-length EcTRAF2s were inserted into pFlag-CMV2 plasmid to detect their role in signaling. In brief, EcTRAF2s were amplified using Prime STAR Max Premix (Takara, Japan) with primers containing KpnI(forward primer) and SmaI(reverse primer) restriction sites at the 5′-terminus. All the primers used to amplify the target genes are listed in STable 1. After double enzyme digestion, each sequence was ligated into a pFlag-CMV2 vector, transferred into *Escherichia coli* TG1, sequenced, and stored at −80 °C.

2.7. Transfection

Before transfection, the endo-free plasmids were extracted from *E. coli* using the E.Z.N.A Endo-free Plasmid Mini Kit I (Omega, USA) according to the manufacturer instructions. For transfection, the HEK293T cells were seeded either into 24-well plates for analysis of intracellular localization, or into 96-well plates for reporter gene assays of EcTRAF2s. Cells were cultured for 24 h, and then transfected with each plasmid using Lipofectamine™ 2000 Reagent (Invitrogen, USA) following the manufacturer protocol.

2.8. Intracellular localization of EcTRAF2s

L-lysine-treated coverslips were placed in 24-well plates. Cells were transfected with 1 μg of target plasmid, and cultured for 24 h. The cells were fixed with immunostaining fixative (Beyotime, China) for 10 min, then 100 μL of 0.5% Triton X-100 was added for 15 min for membrane permeabilization. Cells were blocked with goat serum for 30 min, and then incubated with mouse anti flag-tag mAb (Abmart, China) for 1 h. Alexa Fluor 488-conjugated rabbit anti-mouse IgG antibody (Cell Signaling Technology, USA) was then added into each well for 1 h. Finally, 4′,6-diamidino-2-phenylindole (DAPI), at a final concentration of 1 μg/mL was added for 5 min for nuclear staining. A drop of anti-fade mounting medium was added to microscope slides (Beyotime) and coverslips were placed on top. Localization of EcTRAF2s was analyzed using a Zeiss LSM DUO confocal laser scanning microscope (Carl Zeiss,

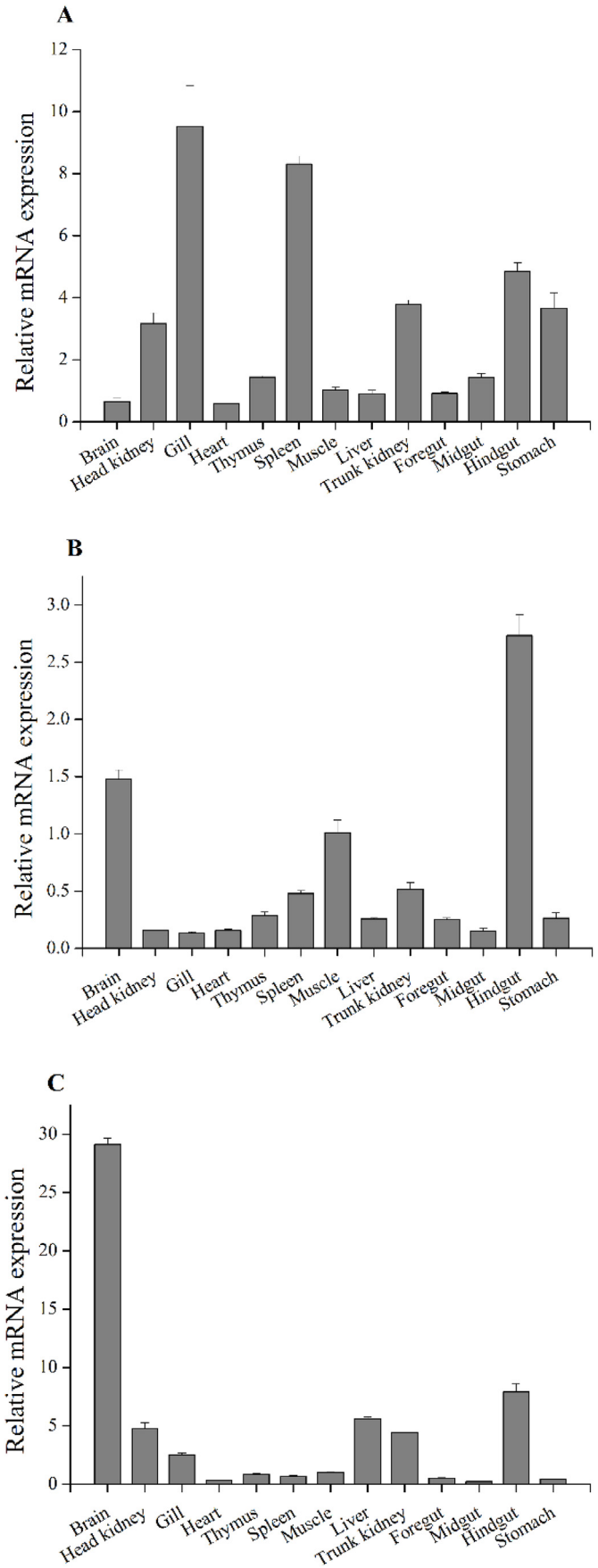

Fig. 3. Tissue distribution of EcTRAF2-1 (A), EcTRAF2-2 (B), and EcTRAF2-3 (C) in healthy *E. coioides*. Expression levels were normalized to β-actin. Data are expressed as mean ± standard error (N = 3).

Germany).

2.9. Dual luciferase reporter assay

Cells were transfected with 200 ng of plasmid containing 150 ng of target plasmid, 40 ng of NF-κB reporter plasmid, and 10 ng of the pRL-TK internal reference plasmid. After 24 h, the cells were washed three times with phosphate-buffered saline, and lysed with passive lysis buffer (Promega, USA). Firefly and Renilla luciferase activities were determined with a Dual Luciferase Reporter Assay System (Promega, USA) following the manufacturer protocol. Each experiment was performed in triplicate. The relative luciferase activity of each well was calculated as the firefly luciferase activity relative to the Renilla luciferase activity.

2.10. Statistical analysis

All data were analyzed with the SPSS version 16.0 software, using Duncan's test. $P < 0.05$ was considered significant. Data are expressed as mean ± standard error.

3. Results

3.1. Sequence of EcTRAF2s and phylogenetic analysis

Three TRAF2 sequences were identified from the transcriptome of *E. coioides*; their ORFs were amplified, sequenced, and named EcTRAF2-1 (KR005608), EcTRAF2-2 (KR005607), and EcTRAF2-3 (KR005606), respectively. The ORFs of EcTRAF2-1, EcTRAF2-2, and EcTRAF2-3 were 1563 bp (encoding 520 aa), 1356 bp (encoding 451 aa), and 1797 bp (encoding 598 aa), respectively (SFig. 1). The theoretical Mw of EcTARF2-1, EcTRAF2-2, and EcTRAF2-3 were 59 kDa, 51 kDa, and 67 kDa, respectively. The theoretical pI of cTARF2-1, EcTRAF2-2, and EcTRAF2-3 were 8.16, 7.91 and 8.48, respectively.

SMART structure analysis indicated that EcTRAF2s contained an N-terminal RING finger domain, a zinc finger domain, a coiled-coil region, and a C-terminal MATH domain (SFig. 1). Through homology modeling with human TRAF domain of TRAF2 as a model (1ca9.2.A), we found that trimeric self-association was possible through the C-terminal TRAF domain of EcTRAF2s (Fig. 1). Each TRAF domain of EcTRAF2-1 and EcTRAF2-3 in the trimer formed a mushroom shape, with an α-helix forming the stalk and an eight antiparallel β-sandwich constituting the cap. However, the TRAF domain of EcTRAF2-2 just contained one α-helix but no β-sandwich.

Sequence alignment indicated that EcTRAF2-1 had 51% and 58% sequence identity with EcTRAF2-2 and EcTRAF2-3, respectively; EcTRAF2-2 had high sequence identity with EcTRAF2-3 (77%) (Table 1). EcTRAF2-1, EcTRAF2-2, and EcTRAF2-3 respectively displayed 57%–94%, 47%–95% and 58%–98% sequence identity with that of other fish species; additionally, EcTRAF2-1, EcTRAF2-2, and EcTRAF2-3 respectively shared 57%–61%, 50%–59% and 61%–67% sequence identity with that of higher vertebrates (Table 1). Full-length of EcTRAF2-1, EcTRAF2-2 and EcTRAF2-3 respectively had the highest sequence identity with that of *Lates calcarifer* (XP_018518421.1) (94%) and *Stegastes partitus* (XP_008303697.1) (94%), *L. calcarifer* (XP_018524804.1) (95%), *S. partitus* (XP_008278833.1) (95%), and *Larimichthys crocea* (KKF24896.1) (95%), as well as *L. calcarifer* (XP_018549809.1) (98%) (Table 1).

To analyze the evolutionary relationship of EcTRAF2s with other species, two phylogenic trees were constructed. In SFig. 2, all TRAF2s, including three grouper TRAF2s clustered into one group apart from the other TRAFs, implying all three TRAFs identified in this study were TRAF2. In Fig. 2, the tree was divided into two groups, one group contained fish TRAF2s (including EcTRAF2-1); the other group contained TRAF2s of higher vertebrates and other fish. In the latter group, TRAF2s of higher vertebrates were clustered into one subgroup, and

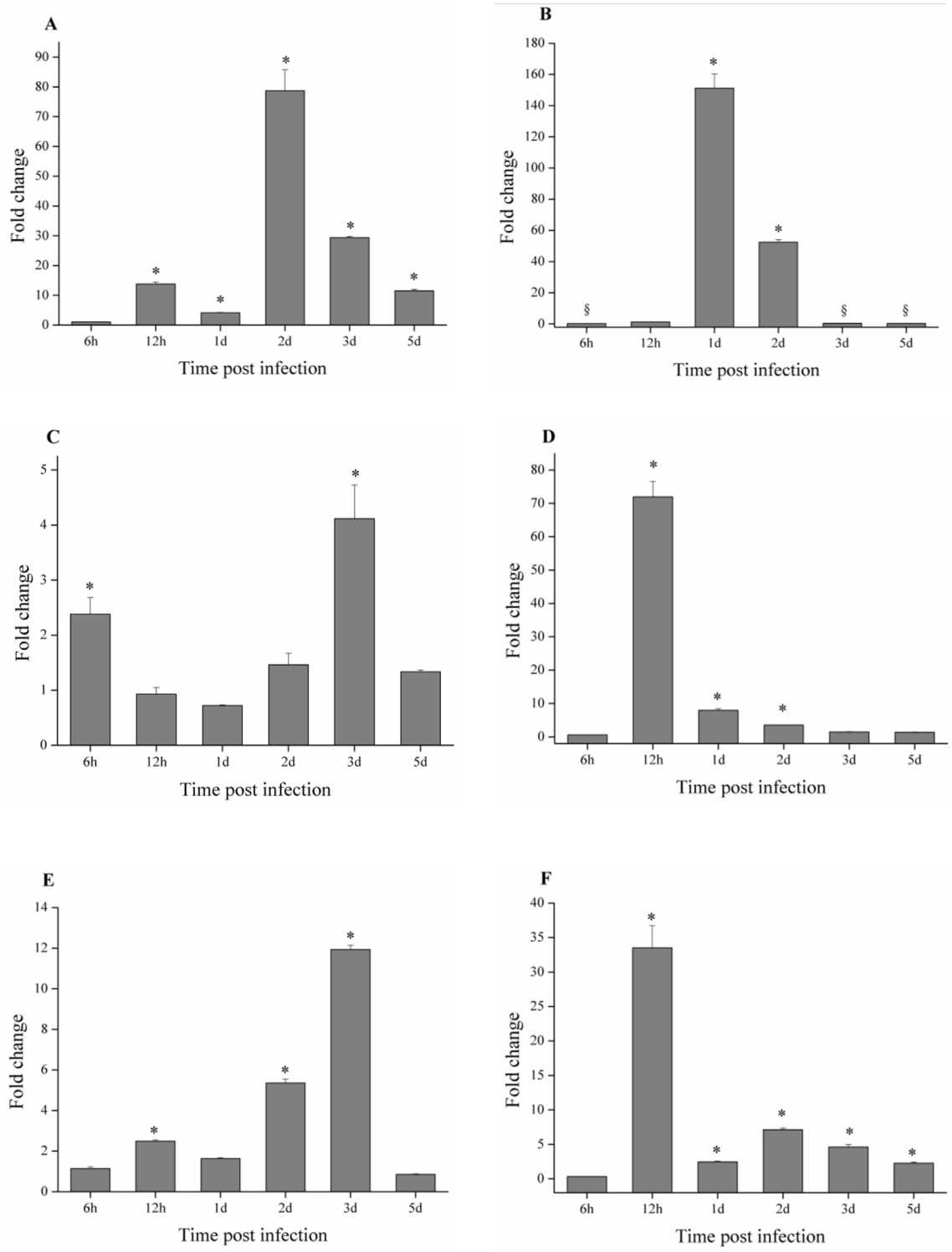

Fig. 4. Expression of EcTRAF2-1 (A, gills; B, head kidney), EcTRAF2-2 (C, gills; D, head kidney), and EcTRAF2-3 (E, gills; F, head kidney) after infection with *C. irritans*. Expression levels were firstly normalized to β-actin, and fold change calculated relative to uninfected control at the same time point. Data are expressed as mean ± standard error (N = 3). Significant differences in gene expression between *C. irritans*-infected and control groups are indicated by symbols * (increase) or § (decrease) ($P < 0.05$). The Ct value was in the STable 2.

fish TRAF2 were clustered into another subgroup. Within the fish TRAF2 subgroup, EcTRAF2-2 and EcTRAF2-3 were clustered into a different branch.

3.2. Tissue expression of EcTRAF2s

Constitutive expression of EcTRAF2s was analyzed in 14 tissues from healthy *E. coioides* using real-time PCR. As shown in Fig. 3, EcTRAF2s were expressed in all tissues analyzed. EcTRAF2-1 was highly expressed in the gills and spleen, but expressed at low levels in the brain

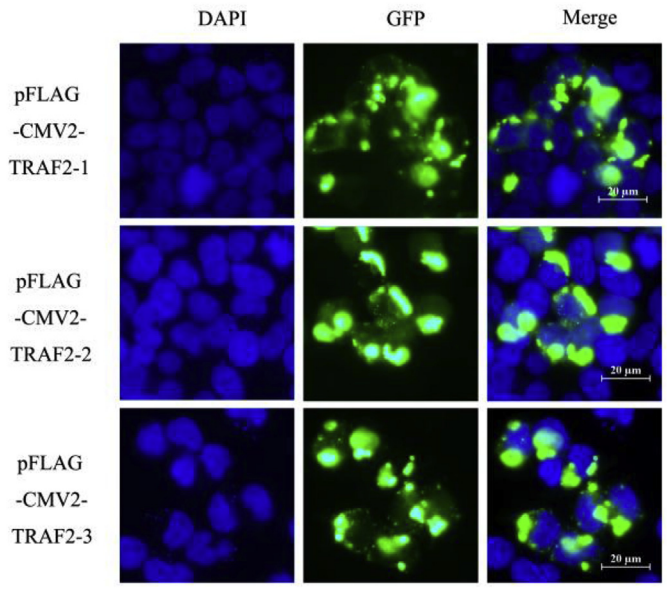

Fig. 5. Subcellular localization of EcTRAF2s in HEK293T cells. Cells were transfected with target gene vectors, and then stained with mouse anti-flag-tag antibody followed by Alexa Fluor 488-conjugated rabbit anti-mouse IgG antibody. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

and heart (Fig. 3A). High expression of EcTRAF2-2 mRNA was observed in the hindgut, brain, and muscle (Fig. 3B). EcTRAF2-3 was highly expressed in the brain, hindgut, and liver (Fig. 3C).

3.3. Expression of EcTRAF2s after C. irritans infection

The expression of EcTRAF2s in the gills and head kidney was determined by real-time PCR after fish were infected with *C. irritans* (Fig. 4). After *C. irritans* infection, expression of EcTRAF2-1 in the gills was significantly upregulated from 12 h to day 5; expression peaked at day 2 (Fig. 4A). In the head kidney, increased EcTRAF2-1 expression was observed at day 1 and day 2 post infection (Fig. 4B), after which the expression of EcTRAF2-1 decreased (Fig. 4B).

Increased expression of EcTRAF2-2 in the gill was detected at 6 h and 3 d after *C. irritans* infection (Fig. 4C). In the head kidney, EcTRAF2-2 expression increased only from 12 h to day 2 (Fig. 4D).

In the gills, EcTRAF2-3 expression increased 3 days after *C. irritans* infection, peaking at this time point (Fig. 4E). In the head kidney, EcTRAF2-3 expression increased from 12 h to 5 d, peaking at 12 h (Fig. 4F).

3.4. Reporter gene assay

To determine the intracellular localization of EcTRAF2s, their sequences were inserted into the pFlag-CMV2 vector and transfected into HEK293T cells. As shown in Fig. 5, EcTRAF2 proteins were localized in the cytoplasm with punctate manner.

To analyze the role of EcTRAF2s in signaling, EcTRAF2s were transfected into HEK293T cells together with a NF-κB luciferase vector. As indicated in Fig. 6, only EcTRAF2-1 and EcTRAF2-2 activated NF-κB (Fig. 6A). EcTRAF2-3 significantly reduced activation of NF-κB induced specifically by EcTRAF2-1 (Fig. 6B).

4. Discussion

In mouse, two TRAF2 sequences have been identified; they are alternatively splicing forms of the same gene [14]. In fish, only one TRAF2 sequence was found in grass carp, rock breeam and black carp [8–10]. In the present study, we identified three TRAF2 sequences in

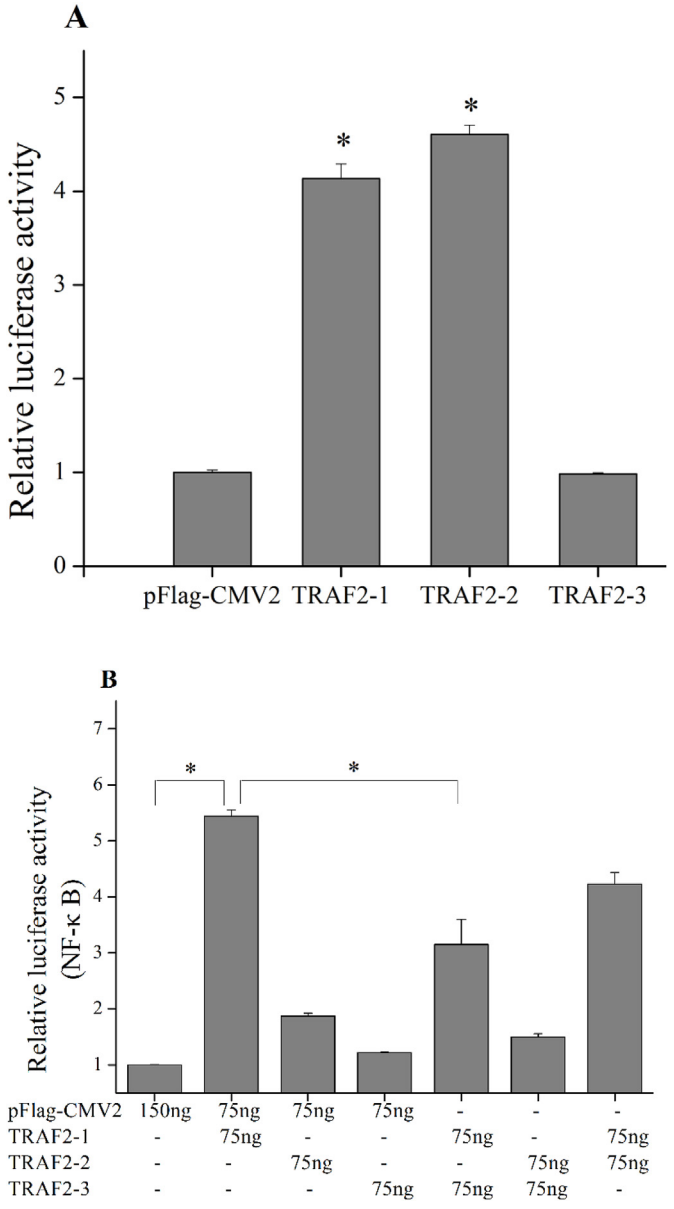

Fig. 6. NF-κB activity induced by EcTRAF2s. A, NF-κB activity induced by each EcTRAF2. B, NF-κB activity induced by co-transfection of EcTRAF2s. Firefly and Renilla luciferase activities were detected in cell lysates 24 h after transfection. Data represent fold changes relative to cells transfected with pFlag-CMV2. Results are expressed as mean ± standard error (N = 3). Significant differences are indicated by asterisks (*P < 0.05).

the transcriptome *E. coioides*. Sequence alignment demonstrated that EcTRAF2s shared 51%–77% sequence identity with each other, suggesting that they are encoded by different genes (SFig. 3). In NCBI GenBank database, we found numerous fish species that also had more than one TRAF2 sequence. For example, two TRAF2 sequences were found in *S. partitus*, and three TRAF2 sequences were found in *L. calcarifer*, *L. crocea*, and *T. rubripes*. These sequences were not alternative splicing forms of the same gene, and had relatively low sequence identity. These results indicated that the fish normally had more than one TRAF2 gene, which shared different evolutionary relationship with each other.

An N-terminal RING finger domain, a zinc finger domain, a coiled-coil region, and a C-terminal MATH domain were identified in EcTRAF2, and in TRAF2 of other fish species, indicating that the structure of TRAF2 was conserved in vertebrates. However, there are

five zinc finger motifs in mammalian TRAF2, but only four in fish TRAF2 [6]. The lack of the additional zinc finger motif may not affect the ability of fish TRAF2 to activate NF-κB (Fig. 6 and [9,10]).

Previous reports have demonstrated that the RING finger domain and zinc finger domain of TRAF2 has a typical motif, namely C-X_2-C-$X_{(9-39)}$-C-$X_{(l-3)}$-H-$X_{(2-3)}$-C-X_2-C-$X_{(4-48)}$-C-X_2-C motif and H-$X_{(3,4)}$-C-$X_{(6)}$-C-$X_{(2,6)}$-C-$X_{(11,12)}$ (X represents any amino acid), respectively [15,16]. By sequence alignment, we found that the structure of the RING finger domain and zinc finger domain of TRAF2 were conserved from fish to mammals (SFig. 3). In addition, the C-terminal TRAF domain of mammalian TRAF2 adopts a mushroom shape with the coiled-coil region forming a single α-helix as stalk and the TRAF-C domain forming a novel eight antiparallel β-sandwich as the cap, which was similar to the structure of other TRAFs [17–19]. The C-terminal TRAF domain of EcTRAF2-1 and EcTRAF2-3 had a similar three-dimensional mushroom-like structure, as does the mammalian TRAF2. EcTRAF2-2 only had the typical stalk but not the cap. Whether this change of structure effects the interaction of EcTRAF2-2 with other protein needs additional studies.

We have found that, similar to mice [6,20], to grass carp, and black carp [8,10], EcTRAF2s are constitutively expressed in healthy *E. coioides*. The highest expression of EcTRAF2-1, EcTRAF2-2, and EcTRAF2-3 was observed in the gills, hindgut, and brain, respectively. However, the highest expression of TRAF2 was observed in the heart (grass carp) and gills (black carp).

Chen et al. recently demonstrated that the expression of TRAF2 increased in *Mylopharyngodon piceus* fin (MPF) cells after spring viremia of carp virus (SVCV) or grass carp reovirus (GCRV) infection [10]. However, the role of TRAF2 during fish parasite infection is largely unknown. We found that EcTRAF2s expression increased in the gills and head kidney after *C. irritans* infection; however, the expression of each EcTRAF2 was different in different tissues. These results implied that EcTRAF2s may be involved in host defense against *C. irritans* infection.

TRAF family members were distributed in the cytoplasm in mammal, with the exception of TRAF4 that localized in the nucleus [16]. In resting cells, TRAF2 was uniformly distributed throughout the cytoplasm [21,22]. Unlike mammalian TRAF2, EcTRAF2s proteins were localized in the cytoplasm in a scattered manner; this localization pattern was similar to that of black carp TRAF2 [10].

We found that, similar to rock bream and black carp TRAF2 [9,10], expression of EcTRAF2-1 or EcTRAF2-2 induced the activation of NF-κB. EcTRAF2-3 reduced the activation of NF-κB mediated by EcTRAF2-1, which suggested that EcTRAF2-3 may be a negative regulator of EcTRAF2-1. Similarly, in mammals, overexpression of TRAF2 induced the activation of NF-κB, and TRAF2A reduced the activation of NF-κB mediated by TNFR2 [20,23].

In conclusion, herein, three EcTRAF2 sequences were identified from *E. coioides*, and which had distinct tissue distribution, and responded differently to *C. irritans* infection. Both EcTRAF2-1 and EcTRAF2-2 activated NF-κB, while EcTRAF2-3 reduced the activation of NF-κB mediated by EcTRAF2-1.

Acknowledgements

This work was funded by the Finance Supporting Specific Projects for Fish Diseases Control from the Government of Guangdong Province (grant No. YCN[2016]11H) to Dr. Yan-Wei Li, and China Modern Agricultural Industry Technology System (The Control of Parasites Infection on Marine Fish, CARS-47-18) to Prof. An-Xing Li.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.070.

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