Developmental and Comparative Immunology 45 (2014) 21–34

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Analysis of 52 Rab GTPases from channel catfish and their involvement in immune responses after bacterial infections

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ARTICLE INFO

Article history:
Received 24 December 2013
Revised 31 January 2014
Accepted 31 January 2014
Available online 7 February 2014

Keywords:
Catfish
Fish
Rab
Innate immunity
Acute phase, infection
Disease

ABSTRACT

Rab genes, encoding a large family of monomeric G-proteins, contain over 60 members in the human genome. They have been recognized as crucial regulators for membrane trafficking including cargo sorting, vesicle formation, budding, motility, docking, fusion, secretory and endocytic pathway of host immune responses. However, little is known of the Rab gene family in teleost fish species. The development of full-length transcripts and whole genome sequences allow the identification and annotation of Rab GTPase gene family in catfish. In this study, a total of 52 Rab genes were identified from catfish cDNA and genome databases. Phylogenetic analysis assigned them into eleven subfamilies. Most Rab GTPases are conserved among vertebrates, though some of which are absent in fish genomes. Analysis of multiple RNA-seq datasets, along with real time PCR analysis revealed up-regulation of 10 Rab genes after bacterial infection. These included Rab3a, Rab4a, Rab4b, Rab5a, Rab5c, Rab7a, Rab9a, Rab11a, Rab11b, and Rab33a. Their up-regulation are temporally and spatially regulated in various tissues, but mostly induced at early stages after infection and in the gill and liver tissues, with the exception of Rab5c that is mostly up-regulated in the head kidney and trunk kidney. The complex pattern of their induced expression suggested both specific and cooperative actions of a these Rab genes in the acute immune responses to bacterial infection.

1. Introduction

Rab GTPases are members of the Ras superfamily (Ras, Rho/Rac/Cdc42, Ran, Sar/Arf and Rab) found to be present in all eukaryotes (Rink et al., 2005; Segev, 2001; Zerial and McBride, 2001). They are important components of the endomembrane systems including endoplasmic reticulum, Golgi, endosomes, lysosomes as well as nucleus, the plasma membrane, mitochondria and centrioles. Rab GTPases are essential for membrane trafficking and signaling, and are involved in cell proliferation and differentiation (Schwartz et al., 2007). In the process of exocytic and endocytic membrane trafficking, Rab proteins regulate plasma membrane delivery, organelle biogenesis and degradative pathways (Kroemer and Jäättelä, 2005) through Rab cycling (Pan et al., 2006), changing their conformations from inactive form (GDP-bound) to active form.

Rab proteins also have specialized functions including regulation of secretion in endocrine and exocrine cells, synaptic transmission in neuron cells, and phagocytosis in macrophages and dendritic cells. In the downstream signaling cascades, some Rab family members (Rab5, Rab8, Rab24) controlling the signaling to the nucleus work in concert with other Ras GTPases that control cytoplasmic shuttling, to bring rapid responses to signaling in cell growth or differentiation (Miaczynska et al., 2004; Wu et al., 2006). Cell growth and differentiation, in turn, may be modulated through the coordinated actions of Rab GTPases regulating cell–matrix and cell–cell adhesion (Rab4a, Rab8b, Rab13 and Rab21) and of those involved in growth regulatory signaling and mitosis or apoptosis (Rab6a, Rab11, Rab12, Rab23, Rab25, Rab35) (Bucci and Chiariello, 2006; Del Nery et al., 2006; Iida et al., 2005; Kouranti et al., 2006; Yang et al., 2006; Yu et al., 2007).

Rab proteins were first described in yeast (reviewed by Eoin et al., 2012). More than 60 unique Rab genes have been found to date from various species. The number of Rab genes varies depending on the species. In humans, 63 Rab genes were identified, while 11, 26 and 29 Rab GTPases were found in the genomes of *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *C. elegans*, respectively (Bock et al., 2001; Pereira-Leal and Seabra, 2001). The difference in the number of Rab genes are mainly caused by gene duplications, leading to the presence of paralogs (e.g. Rab5a, Rab5b and Rab5c). Orthologous Rab genes from various species, as well as

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http://dx.doi.org/10.1016/j.dci.2014.01.026
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their paralogs, are structurally highly conserved (Eoin et al., 2012; Touchot et al., 1987). For instance, human Rab1 gene shared 70% amino acid identity with the yeast Rab gene. Rab paralogs share significant degrees of sequence homology, but are typically divergent at C-termini (Stenmark and Olkkonen, 2001).

Systematic analysis of the Rab families have not been conducted in fish species, although isolated studies were conducted with *Danio rerio*, *Gadus morhua*, *Oryzias latipes*, *Gasterosteidae*, *Tetraodon nigroviridis* and *Oreochromis niloticus* (Team, 2002; Kasahara et al., 2007; Jones et al., 2012; Clark et al., 2011; Jaillon et al., 2004; Ber and Daniel, 1993). However, in a number of teleost fish studies, Rab genes were found to be involved in immune responses. For instance, Rab27 was inducible after the infection of *Hirame rhabdovirus* in Japanese flounder (Nam et al., 2000). In large yellow croaker, Rab8 was up-regulated after infection with *Vibrio parahaemolyticus* (Han et al., 2011). Of the large number of Rab genes, 11 Rab genes were known to be involved in immune response endocytic pathway, including Rab3a, Rab4a, Rab4b, Rab5a, Rab5b, Rab5c, Rab7a, Rab9a, Rab9b, Rab11a and Rab11b (Schwartz et al., 2007).

With the interest of understanding Rab genes in the catfish genome, including gene identification, genomic location and copy number variations, in this study, we conducted analysis for a complete set of 52 Rab GTPases in catfish. Their orthologies and paralogies were established through phylogenetic analysis, and their expression was analyzed using multiple RNA-seq datasets and real-time RT-PCR.

2. Materials and methods

2.1. Identification of Rab genes and homologs

In order to identify the Rab genes from catfish, the transcriptome database (Liu et al., 2012; Sun et al., 2012) and the draft whole genome sequence database of catfish (unpublished) were searched using all available zebrafish Rab proteins as queries. TBLASTN (available from NCBI) was used to obtain the initial pool of Rab gene sequences, with a cutoff *E*-value of 1e−5. Upon identification of the initial pool of catfish Rab-related sequences, sequences were aligned to delete the repeated entries and a unique set of sequences was subjected to further analysis. The cDNA sequences obtained from RNA-seq database was confirmed by comparison with the draft catfish whole genome sequence database. The open reading frame (ORF) was identified by using ORF finder (http://www.ncbi.nlm.nih.gov) and validated by BLASTP against NCBI non-redundant protein sequence database (nr). The full-length amino acid sequences were used in the phylogenetic analysis.

2.2. Phylogenetic and sequences analysis

The amino acid sequences of Rab genes from various organisms along the evolutionary spectrum were selected and retrieved from NCBI and Ensembl genome database (Release 68) to conduct phylogenetic analysis, including those from human, mouse, chicken, frog and various fish species including zebrafish, tilapia, stickleback, medaka, fugu, Atlantic cod, and *Tetraodon*. The amino acid sequences were aligned using CLUSTALW2 (Larkin et al., 2007). Phylogenetic analyses were performed with MEGA 5.1 using the neighbor-joining method (Stein et al., 2003). Bootstrapping with 10,000 replications was conducted to evaluate the phylogenetic tree (Tamura et al., 2011). The overall subfamily assignment of catfish Rab proteins was determined by phylogenetic analysis with Rab proteins from zebrafish and human. The subfamily assignments were guided by the human dendrogram clustering result in the previous study (Schwartz et al., 2007). Separate phylogenetic analyses were then reconstructed per subfamily using Rab GTPases sequences of catfish and other representative vertebrate species including zebrafish, tilapia, stickleback, medaka, fugu, Atlantic cod, and *Tetraodon* with the same methodology.

2.3. Bacterial challenge and sample collection

All the procedures involved in handling and treatment of fish during this study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) prior to the initiation of the study. ESC bacterial challenge was conducted as previously described (Liu et al., 2010). Briefly, *Edwardsiella ictaluri* was cultured from a single colony, re-isolated from a symptomatic fish and biochemically confirmed before being inoculated into brain heart infusion (BHI) medium and incubated in a shaker incubator at 28 °C overnight. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating 10 μl of 10-fold serial dilutions onto BHI agar plates. Channel catfish (mean weight 11.2 g and mean length 9.3 cm) were reared at the Fish Genetics Research Unit of Auburn University. Prior to experiments, fish were maintained in 30 L tanks and acclimatized for 5 days before immersion bath at a temperature of 27 °C. During the challenge, experimental fish were immersed at a concentration of 4×10^6 CFU/ml bacterial in 15 L aerated freshwater. Another set of unexposed fish were used as controls. Symptomatic fish and control fish were collected and confirmed to be infected with *E. ictaluri* and pathogen-free, respectively, at the Fish Disease Diagnostic Laboratory, Auburn University.

At each time point of 0 h, 4 h, 24 h, 48 h and 4 day post infection, 15 fish were collected from each of control and treatment group and euthanized with MS-222 (300 mg/L). Nine tissues including brain, head kidney, trunk kidney, liver, spleen, gill, skin, intestine and heart were collected from 45 fish (three pools of 15 fish per pool) of both the treatment group and the control group at each time point. Samples were flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.4. RNA extraction

Tissue samples were homogenized with a mortar and pestle in the presence of liquid nitrogen. Total RNA was then extracted using the RNeasy Plus Mini Kit (Qiagen, USA). RNA concentration and integrity were measured using a NanoDrop ND-1000 UV–VIS Spectrophotometer (NanoDrop Technologies, USA). First-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, USA) following the manufacturer's instructions. The cDNA samples were subsequently used for determination of Rab genes expression by quantitative real-time RT-PCR.

2.5. RNA-Seq analysis of Rab genes following bacterial challenge

The high quality reads from multiple RNA-seq datasets including *E. ictaluri* and *Flavobacterium columnare* infection RNA-seq studies (Li et al., 2012; Liu et al., 2012; Sun et al., 2012) were mapped onto the deduced channel catfish Rab genes using CLC Genomics Workbench software package (version 6.5.1). Reads per kilobase of exon model per million mapped reads (RPKM) (Brockman et al., 2008) was calculated as the original expression value. After scaling normalization of the RPKM values, the expression fold change was then calculated between the treatment and control groups. The Kal's test (Kal et al., 1999) was used to test the significance of the expression fold change. The expression analysis was performed using the RNA-seq module and the expression analysis module in CLC. The threshold of gene expression selection was set to: p-value <0.05, mapped reads >5, weighted proportions of fold change $\geqslant |2|$.

2.6. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed to determine Rab GTPase mRNA expression in different tissues before and after bacterial challenge. All the cDNA products were first diluted to 250 ng/μl and utilized for quantitative real-time PCR using the SsoFast™ EvaGreen® Supermix on a CFX96 real-time PCR Detection System (Bio-Rad, USA). The primers used in quantitative real-time RT-PCR are listed in Table 1. The thermal cycling profile consisted of an initial denaturation at 95 °C (for 30 s), followed by 40 cycles of denaturation at 94 °C (5 s), an appropriate annealing/extension temperature (58 °C, 5 s). An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 °C to 95 °C. The housekeeping gene 18S rRNA was set as the reference gene, relative fold changes were calculated in the Relative Expression Software Tool version 2009 based on the cycle threshold (Ct) values generated by q-RT-PCR (Pfaffl et al., 2002).

3. Results

3.1. Identification of Rab GTPases in catfish

A total of 52 Rab GTPase genes were identified from the doubled haploid channel catfish which harbors two identical sets of chromosomes (Liu et al., 2012) and confirmed with the draft genome databases. The deduced amino acid sequences were then further validated by BLASTP against NCBI non-redundant protein sequence database (nr). The names of various Rab genes were first assigned based on the BLASTP results on multiple fish species including zebrafish, tilapia, stickleback, medaka, fugu, Atlantic cod, and *Tetraodon*, and then validated and confirmed by phylogenetic analysis. The complete cDNA sequences of all these Rab genes have been submitted to the GenBank with accession numbers as listed in Table 2, which also summarized the size of transcripts, open reading frames, and 5′- and 3-UTR sequences.

3.2. Phylogenetic analysis and Rab subfamilies

Phylogenetic analyses were conducted to identify the Rab genes in catfish. Phylogenetic trees were generated per subfamily using Rab GTPases sequences of catfish, other representative vertebrate species including human, mouse, chicken, frog and various fish species including zebrafish, tilapia, stickleback, medaka, fugu, Atlantic cod, and *Tetraodon* (Figs. 1–7). Subfamilies were then categorized by the number of their family members including three single-member subfamilies, three two-member subfamilies and five multiple-member subfamilies. Phylogenetic trees of each subfamily were established.

Table 1
Primers used for quantitative RT-PCR in this study.

Primer name	Primer sequence 5′–3′ (bp)
18S-F	GAGAAACGGCTACCACATCC
18S-R	GATACGCTCATTCCGATTACAG
Rab3a-F	CCGTTATCCTGTGTATGGTA
Rab3a-R	GACATCATCTGCGAGAAGA
Rab4a-F	GGAACTGGGAAATCATGTCTAC
Rab4a-R	TGCTGCCCCTCTGTAATAG
Rab4b-F	TGTCCGAGACATACGATTTC
Rab4b-R	CCACCAACATTGACAACTC
Rab5a-F	GTTTGGCTCCGATGTATTAC
Rab5a-R	TGAAAGTCCAGTGCTCTCT
Rab5b-F	CCTCTTCTTATGTGCTCTCC
Rab5b-R	CTGTGTGACCTCTGTGTTC
Rab5c-F	TCTGCCTGGATGATACAAC
Rab5c-R	GTCTCTGTGTTAGTGATGTC
Rab7a-F	CCATCCATCTACATCACATC
Rab7a-R	GTTCTTCAGACTTCCTTCAG
Rab9a-F	AGGCTCCAATAGTCTGAAG
Rab9a-R	GGTCTGTGAAATCATCTGAG
Rab9b-F	CTCTCACTTCTCGTTTCTC
Rab9b-R	GGTCTTCCACTTCTACATAC
Rab11a-F	CATCCTGACTGAAATCTACC
Rab11a-R	CTAGATGTTCTGGCAACAC
Rab11b-F	GGACTCCACAAATGTTGAAG
Rab11b-R	TTTCAGCCCATCAGTGGTA
Rab33a-F	GGACATTGAGTTGGTTGGT
Rab33a-R	CATAGTTCTTGTGCCTTCTG

Table 2
Summary of 52 Rab GTPase genes identified in catfish genome.

Gene symbol	mRNA (bp)	ORF (bp)	5′-UTR (bp)	3′-UTR (bp)	Accession
Rab1a	2396	609	1321	466	JT348629
Rab1b	4184	606	340	3238	JT410249
Rab2a	1020	639	297	84	JT252821
Rab3a	1335	663	605	67	JT211172
Rab3c	1567	837	5	725	JT278705
Rab3d	1256	672	278	306	NM_001201098
Rab4a	2119	642	1388	89	JT407900
Rab4b	1021	642	139	240	NP_001188141.1
Rab5a	917	654	177	86	NP_001187496.1
Rab5b	2584	645	276	1663	JT412247
Rab5c	2126	669	255	1202	KF987076
Rab6a	2199	627	1350	222	JT347479
Rab6b	960	627	110	223	JT190246
Rab7a	1964	624	1013	327	KF987077
Rab8a	812	624	89	99	KF987078
Rab8b	3067	624	2223	220	JT480133
Rab9a	2515	609	1244	662	JT413092
Rab9b	3063	672	27	2364	JT414581
Rab10	1181	603	417	161	NM_001200713
Rab11a	1651	648	143	860	JT405486
Rab11b	1227	657	57	513	GU588777
Rab12	1882	735	966	181	JT408566
Rab13	2356	606	448	1302	JT417195
Rab14	1072	648	81	343	JT466719
Rab15	1744	639	960	145	JT180894
Rab18b	2786	618	79	2089	JT410154
Rab19	986	663	289	34	JT163758
Rab20	1379	735	336	308	JT418440
Rab21	1536	663	212	661	JT416918
Rab22a	1174	594	298	282	NM_001201157
Rab23	2566	711	1558	297	JT407815
Rab24	1098	612	429	57	JT348680
Rab25	2877	639	149	2089	JT405468
Rab26	3172	693	383	2096	JT417363
Rab27a	2851	669	1875	307	JT401066
Rab27b	1132	678	268	186	NM_001200832
Rab28	1074	666	161	247	NM_001201277
Rab30	5310	606	894	3810	JT415421
Rab31	4168	585	225	3358	JT405711
Rab32	1495	642	516	337	JT416014
Rab33a	2970	696	2254	20	JT406311
Rab33b	2189	702	1463	24	JT408097
Rab34	1291	780	198	313	JT209641
Rab35	1687	606	418	663	JT278592
Rab36	1283	789	394	100	JT414955
Rab37	3810	687	3060	63	JT244169
Rab38	1912	633	1213	66	JT406948
Rab39a	4048	642	3317	89	JT414876
Rab39b	2115	642	385	1088	JT278403
Rab40b	2008	837	758	413	JT418380
Rab40c	4245	837	1106	2302	JT415552
Rab43	1816	855	39	922	JT407682

3.2.1. Single-member subfamily

The catfish Rab23, Rab28 and Rab6 were divided into three independent single-member subfamilies in this study. Coding sequences (CDS) of all these Rab GTPases were obtained in full-length. The Rab6 has two duplicates: Rab6a and Rab6b. Both of them encoded a protein with the length of 208 amino acids, sharing 87% similarity between them. The Rab23 gene encoded a

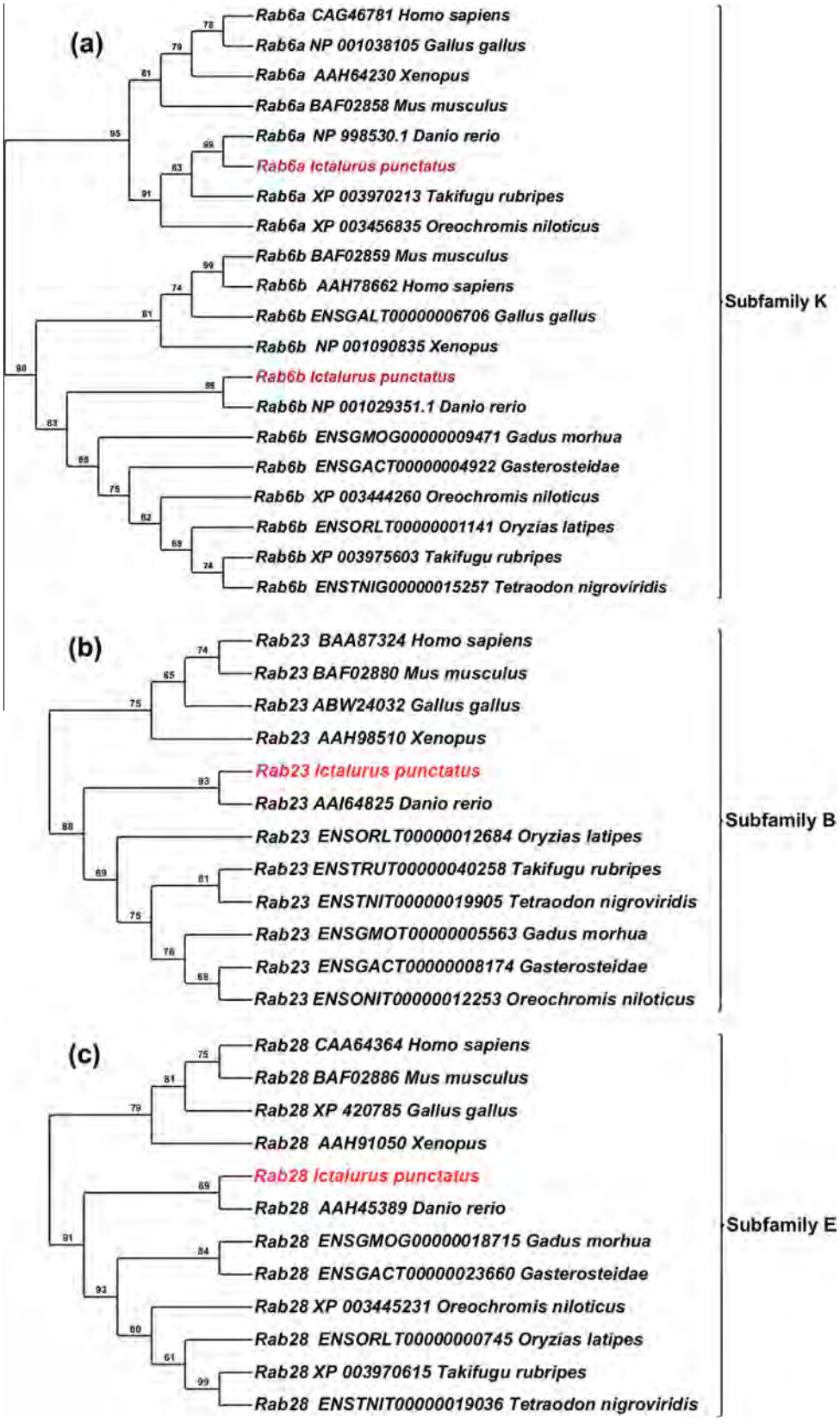

Fig. 1. Phylogenetic tree of catfish Rab GTPase subfamily K, B, and E. The phylogenetic tree was constructed based on the amino acid sequences of Rab6 (a) Rab23 (b) and Rab28 (c). Genes from selected species of fish, birds, and mammals were used in phylogenetic analysis. The accession numbers of the protein sequences followed the name of the species. Bootstrapping values are indicated by numbers at the nodes.

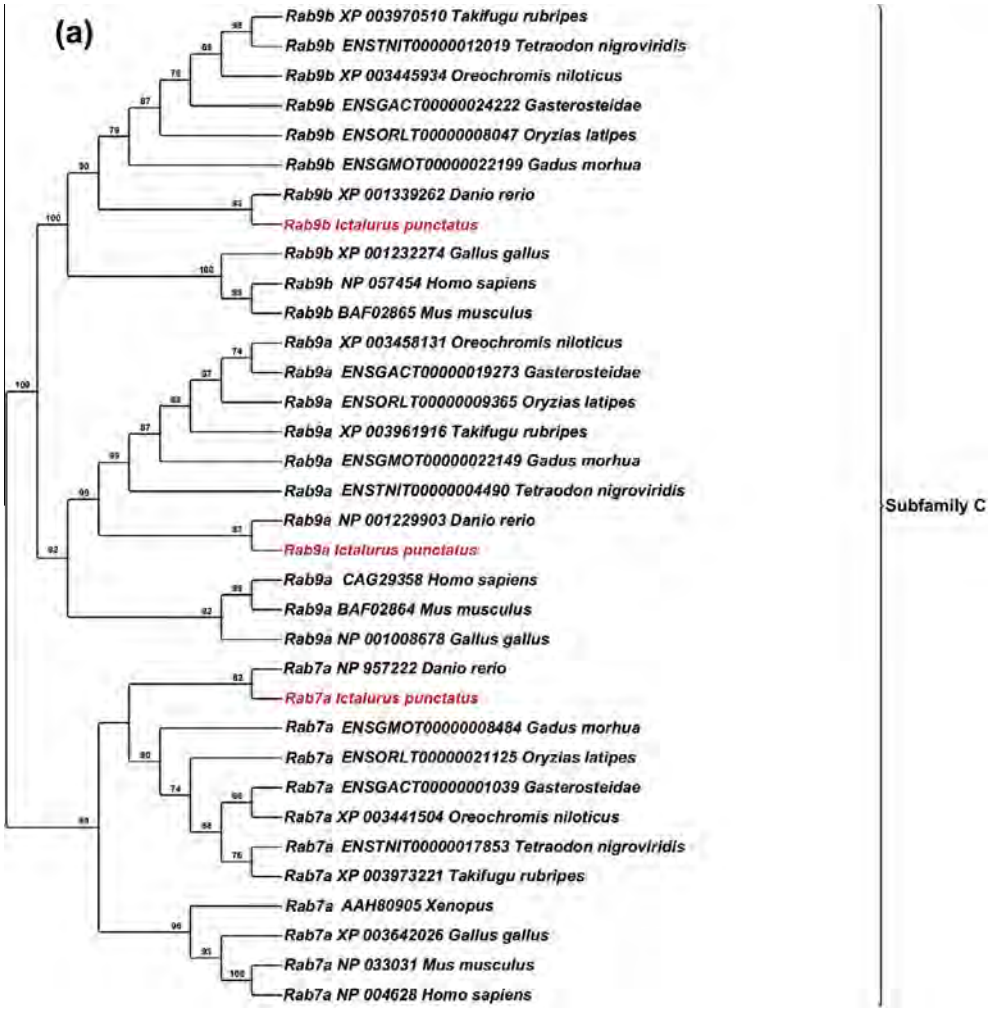

Fig. 2. Phylogenetic tree of catfish Rab GTPase subfamily C, J, and A. The phylogenetic tree was constructed based on the amino acid sequences of Rab9 and Rab7 (a), Rab32 and Rab38 (b) and Rab34 and Rab36 (c). The accession numbers of the protein sequences followed the name of the species. The bootstrapping values are indicated by numbers at the nodes.

protein of 236 amino acids, while Rab28 encoded a protein of 221 amino acids (Table 2).

The phylogenetic tree generated with these three Rab subfamilies well reflected the phylogenetic relationship of the involved organisms, suggesting a parallel evolution of the Rab proteins with organism level of evolution. Clearly, Rab6 (Fig. 1a), Rab23 (Fig. 1b) and Rab28 (Fig. 1c) formed separate clades as expected (Fig. S1). Two separate subclades formed among each Rab gene. The first subclade included all the teleost fish species involved in this study, the second subclade contained the higher level vertebrates including human, frog, mouse and chicken. In the subclade of fish species, catfish Rabs were first clustered with zebrafish and then clustered with other fishes (Fig. 1). Such relationships were consistent with the phylogenies of these fish species. For instance, channel catfish is phylogenetically closer to the zebrafish than to medaka, so is the Rab genes. For the duplicated Rab6, three duplications, Rab6a, Rab6b and Rab6c were found in human, but only Rab6a and Rab6b were found in all the teleost fish species except in Atlantic salmon (Leong et al., 2010) (Table 3).

3.2.2. Two-member subfamily

A total of three subfamilies contained two members including subfamilies C, J, and A. Subfamily C included Rab7 and Rab9 (Fig. 2a); subfamily J included Rab32 and Rab38 (Fig. 2b); and subfamily A included Rab34 and Rab36 (Fig. 2c). The catfish Rab9a encoded a protein with the length of 202 amino acids while its Rab9b encoded a protein of 223 amino acids, and they shared 70% similarity with amino acid sequences. The catfish Rab32 gene encoded a protein of 213 amino acids, while its Rab38 encoded a protein of 210 amino acids. The catfish Rab34 gene encoded a protein of 259 amino acids, while its Rab36 encoded a protein of 262 amino acids.

All the three two-member subfamilies were clearly clustered into separate clades. For each Rab gene, two similar subclades were observed as in single-member subfamilies, in which one contained all the higher level vertebrates and the other one contained all the teleost fish (Fig. 2).

3.2.3. Multiple-member subfamily

A total of five subfamilies contained more than two members including subfamily D (Rab5, Rab17, Rab20, Rab21, Rab22, Rab24 and Rab31) (Fig. 3), subfamily F (Rab1, Rab3, Rab8, Rab10, Rab12, Rab13, Rab15, Rab35 and Rab40) (Fig. 4), subfamily G (Rab26, Rab27 and Rab37) (Fig. 5), subfamily H (Rab19, Rab30, Rab33 and Rab43) (Fig. 6), and subfamily I (Rab2, Rab4, Rab11, Rab14, Rab25 and Rab39) (Fig. 7). Like in the situation of single-member subfamilies and two-member subfamilies, the catfish Rab genes in the multi-member families were placed into their respective clades closest to the zebrafish genes. The teleost fish genes

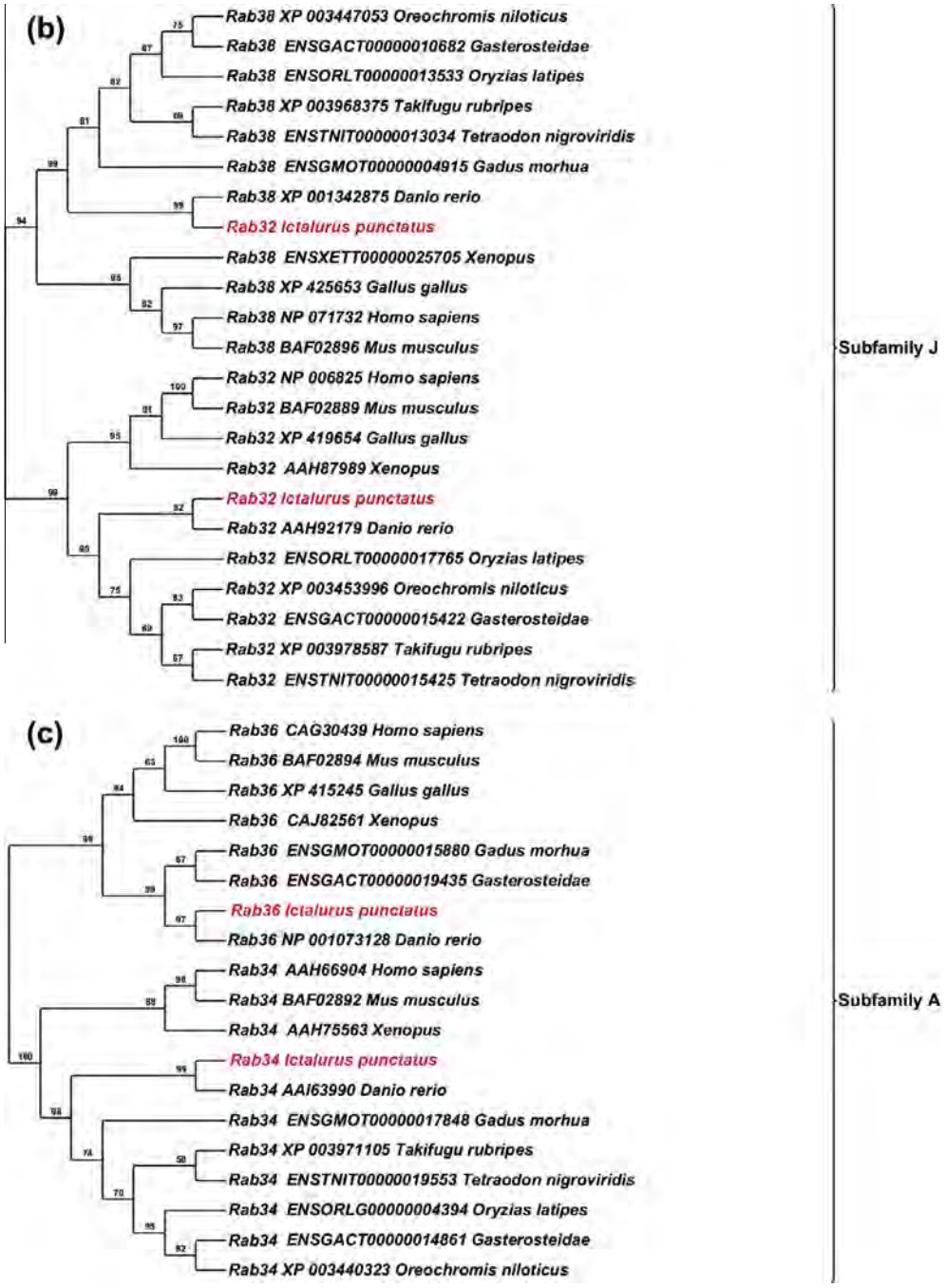

Fig. 2 (*continued*)

themselves formed distinct major clades while the genes from chicken, frog and mammals formed their separate clades.

3.3. Copy number variations across species

The copy numbers of Rab genes in human, mouse, and various teleost fish species are summarized in Table 3. In general, the copy numbers of the 43 Rab genes are well conserved, with several exceptions: (1) Catfish has only one copy of Rab2a, missing Rab2b; (2) catfish has three copies of Rab3, Rab3a, Rab3c, and Rab3d, but missing Rab3b; (3) in mammals, Rab7 has two duplicates: Rab7a and Rab7b, but in catfish and all other fish species studied so far, only Rab7a was found; (4) Rab16 was found only in the mouse, but not from human and all fish species; (5) Rab17 is missing in channel catfish and all other fish except fugu; (6) there is only a single gene of Rab22 in all fish species, but two copies in the mouse and three copies in the human; (7) Rab29 is missing in catfish and all other fish species except zebrafish; (8) Rab41 is missing from all fish species; and (9) Rab43 was found in catfish and zebrafish, but not from other fish species (Table 3). Rab9 has two duplicates: Rab9a and Rab9b, and the duplicates exist for all the species under analysis (Table 3).

3.4. Expression analysis of the Rab genes after bacterial infection using RNA-seq datasets

We previously conducted RNA-seq analysis using gill tissue after infection with *F. columnar* (Sun et al., 2012). Here we analyzed

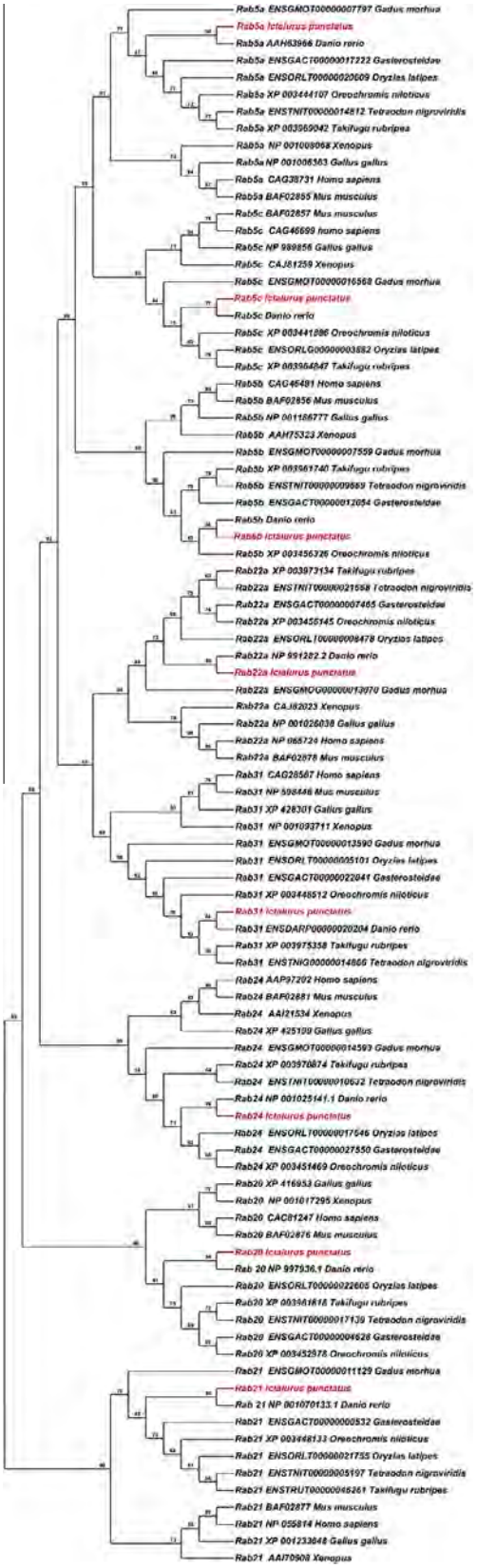

Fig. 3. Phylogenetic tree of catfish Rab GTPase subfamily D. The accession numbers of the protein sequences followed the name of the species. The bootstrapping values are indicated by numbers at the nodes.

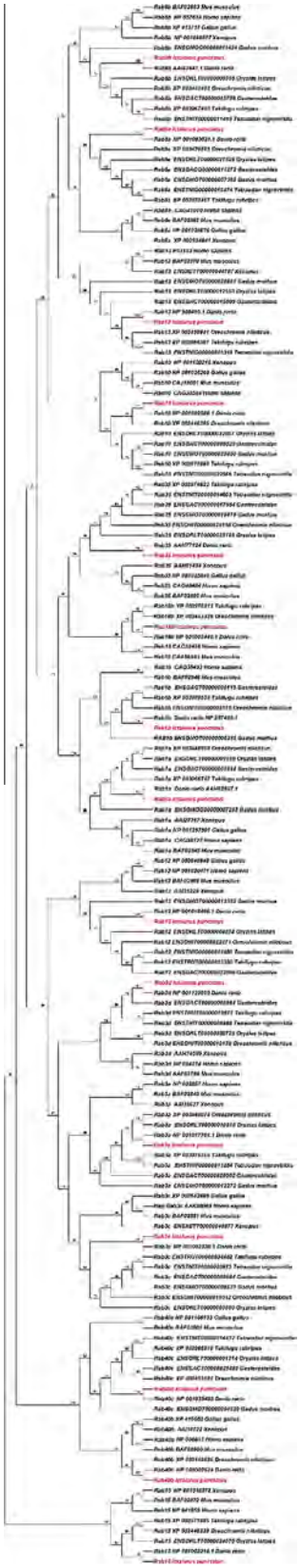

Fig. 4. Phylogenetic tree of catfish Rab GTPase subfamily F. The accession numbers of the protein sequences followed the name of the species. The bootstrapping values are indicated by numbers at the nodes.

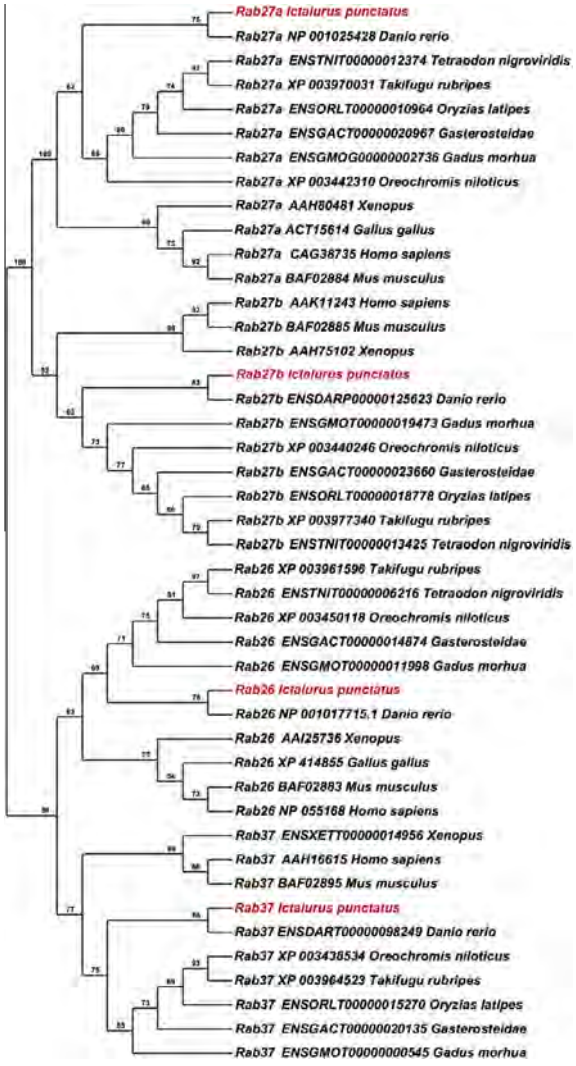

Fig. 5. Phylogenetic tree of catfish Rab GTPase subfamily G. The accession numbers of the protein sequences followed the name of the species. The bootstrapping values are indicated by numbers at the nodes.

expression of Rab genes using the RNA-seq dataset. Eight Rab genes were significantly induced after *F. columnar* bacterial infection in the gill including Rab3a, Rab4a, Rab4b, Rab5a, Rab7a, Rab9a, Rab11a and Rab33a. All these genes were up-regulated early after infection (4 h and 24 h). Most of these eight Rab genes were induced 2–5 folds, but Rab4b and Rab5a were more dramatically up-regulated with Rab4b being up-regulated 12-fold after 4 h, and Rab5a up-regulated 6-fold after 4 h following the bacterial infection.

With the RNA-seq dataset from the intestine after ESC infection (Li et al., 2012), six genes were significantly up-regulated after infection (3 h, 24 h, and 3 day). These included Rab3a, Rab4a, Rab4b, Rab5a, Rab7a and Rab11b. The extent of the infection-induced expression was very high. For instance, Rab4a was up-regulated 11-fold at 3 h and 17-fold at 24 h after the bacterial infection, Rab4b and Rab5a were up-regulated 7-fold and 9-fold at 3 h after the bacterial infection, respectively (Table 4).

3.5. Expression analysis of selected Rab genes after ESC infection using real time PCR

Although meta-analysis of RNA-seq datasets was very effective, there were only limited datasets for analysis. In order to gain into expression of a set of selected Rab genes after infection, real time PCR was performed to determine their expression in various tissues. Eleven Rab genes were analyzed including Rab3a, Rab4a, Rab4b, Rab5a, Rab5b, Rab5c, Rab7a, Rab9b, Rab11a, Rab11b, and Rab33a (Fig. 8). In general, all the Rab genes (except Rab5b, data not shown) were up-regulated in all tested tissues except in the skin and brain. However, the detailed patterns of expression after infection varied among the ten genes with elevated expression after infection. Nine of ten genes, Rab3a, Rab4a, Rab4b, Rab5a, Rab7a, Rab9a, Rab11a, Rab11b and Rab33a were most highly induced in the gill and liver, while Rab5c was the most highly induced in head kidney and trunk kidney. The extent of induction was quite large with these genes (Fig. 8). It is noteworthy that all the induced expression was at early stages after infection, mostly at 3 h and 24 h after infection.

4. Discussion

Rab GTPases play central roles in a number of intracellular processes including signal transduction, cell proliferation, cytoskeletal organization, and intracellular membrane trafficking. Up to date, more than 60 RabGTPases have been identified in mammalian species (Table 3, Pereira-Leal and Seabra, 2000a; Schwartz et al., 2007). Rab proteins regulate key steps in membrane traffic transport, including vesicle formation, vesicle motility, membrane remodeling, vesicle docking, and membrane exchanges. The C-termini of Rab proteins have one cysteine residues that may be isoprenylated, which enable Rab proteins to associate and target to cell membranes by post-translational modification (Wu and Zhang, 2007). In spite of their importance, including their involvement in disease and immune responses, systematic analysis of the Rab families has not been conducted in fish species. In the present study, we identified a complete set of 52 Rab GTPase genes in the catfish genome, conducted phylogenetic and expression analyses of the Rab genes in order to provide insight into their gene identities, orthologies, expression, and their involvement in the immune responses of catfish after bacterial infection.

Phylogenetic analysis allowed classification of the catfish Rab genes into eleven subfamilies including three single-member subfamilies, three two-member subfamilies, and five multiple-member subfamilies (Fig. S1). For the most part, their placements into subfamilies were consistent with those reported from human (Schwartz et al., 2007). The only exception was Rab18 which was clustered with Rab1, Rab3, Rab8, Rab10, Rab12, Rab13, Rab15, Rab35 and Rab40 in the present study, but was assigned into an independent subfamily in a previous study (Pereira-Leal and Seabra, 2000a). This is clearly due to the inclusion of the additional Rab genes from fish species that otherwise was not available for analysis when the human study was conducted. In addition, Rab41 and Rab29 were not identified from any teleost fish species (Table 3).

The functions of three single-member subfamilies including Rab6, Rab23 and Rab28 have been well-studied in mammals while barely known in fish. Rab6 genes in mammals are required for retrograde transport including transport from Golgi to endoplasmic reticulum (ER) (Rab6a and Rab6b) and multi-drug resistance regulation (Rab6c) (Schoebel et al., 2010; Schoebel et al., 2009; Suh et al., 2009). The expression of Rab23 in mammals is specific to the trafficking of sonic hedgehog signaling components, embryogenesis and ciliary trafficking (Machner and Isberg, 2006, 2007). A recent study of Rab28 in mammals suggested that it is an important regulator in ESCRT (endosomal sorting complex required for transport) pathway (Lumb et al., 2011). The function of these three Rab genes in fish is unknown at present. Establishing their orthologies from this work should allow inference of their similar roles in transport and trafficking.

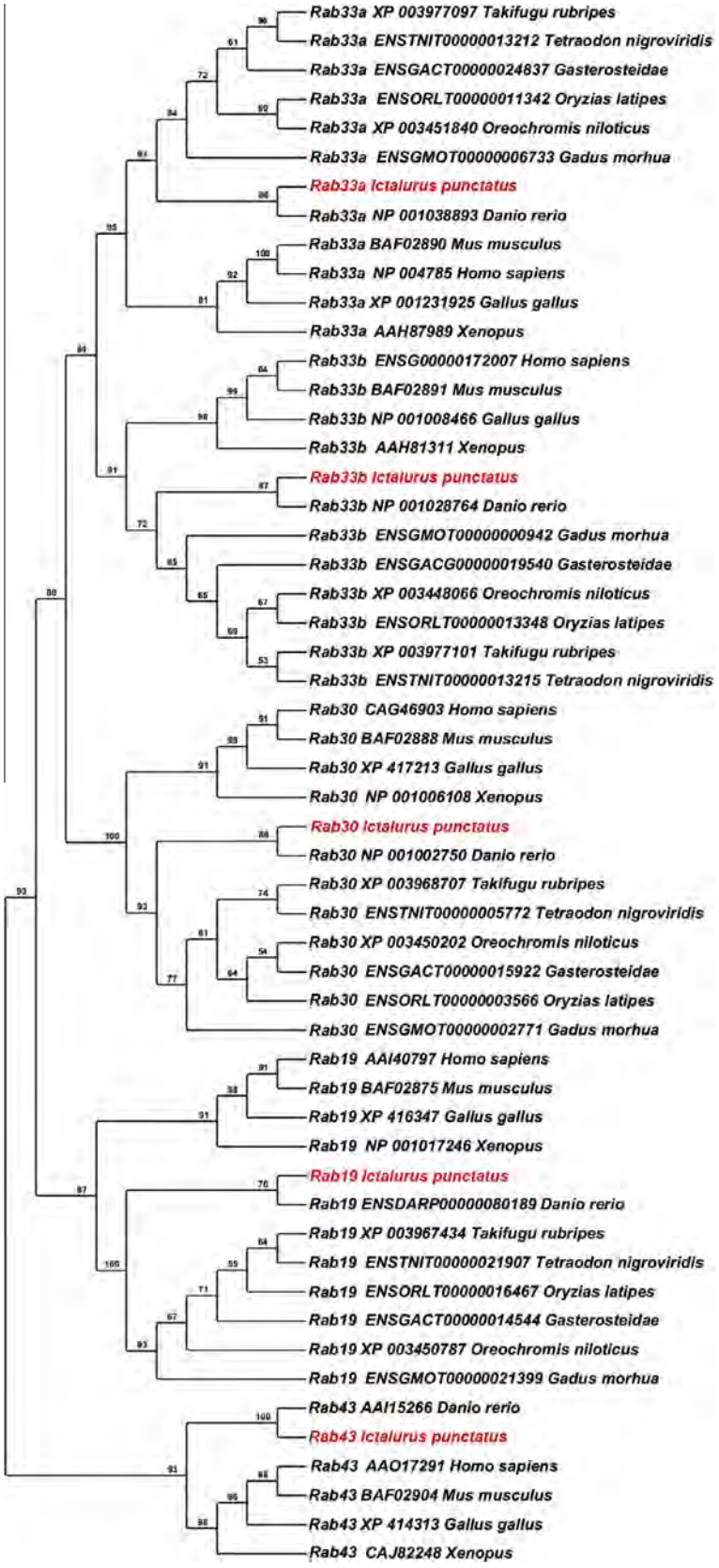

Fig. 6. Phylogenetic tree of catfish Rab GTPase subfamily H. The accession numbers of the protein sequences followed the name of the species. The bootstrapping values are indicated by numbers at the nodes.

The Rab genes included in the two-member subfamilies have been reported in mammals to be involved in immune response and enzymatic trafficking. Rab7 and Rab9 are two critical regulators in the endocytic pathway. Rab7a facilitates the transport of epidermal growth factor receptor (EGFR) complex from early endosomes to late endosomes (Feng et al., 2001; Vonderheit and

Table 3
Comparison of copy number of Rab GTPases in several vertebrate genomes. Data were collected from Ensembl database (Release 68), with a focus on catfish and other fish species whose genome was sequenced. Yellow shaded areas indicated major differences in copy numbers between catfish (or other fish species) and the mammalian species.

	Human	Mouse	Catfish	Zebrafish	Medaka	Fugu	Stickleback	Tetraodon	Tilapia	Cod
Rab1	2	2	2	2	1	2	2	0	2	2
Rab2	2	2	1	2	1	1	1	1	1	0
Rab3	4	4	3	4	4	3	4	4	4	2
Rab4	2	2	2	2	2	2	2	1	2	2
Rab5	3	3	3	3	2	3	2	2	3	3
Rab6	3	2	2	2	1	2	1	1	2	1
Rab7	2	2	1	1	1	1	1	1	1	1
Rab8	2	2	2	2	2	2	2	2	2	2
Rab9	2	2	2	2	2	2	2	2	2	2
Rab10	1	1	1	1	1	1	1	1	1	1
Rab11	2	2	2	2	0	2	1	0	2	0
Rab12	1	1	1	1	1	1	1	1	1	1
Rab13	1	1	1	1	1	1	1	1	1	1
Rab14	1	1	1	1	1	1	0	1	1	1
Rab15	1	1	1	1	1	1	0	0	1	0
Rab16	0	1	0	0	0	0	0	0	0	0
Rab17	1	1	0	0	0	1	0	0	0	0
Rab18	1	1	1	2	0	1	1	0	1	0
Rab19	1	1	1	1	1	1	1	1	1	1
Rab20	1	1	1	1	1	1	1	1	1	0
Rab21	1	1	1	1	1	1	1	1	1	1
Rab22	3	2	1	1	1	1	1	1	1	1
Rab23	1	1	1	1	1	1	1	1	1	1
Rab24	1	1	1	3	1	1	1	1	1	1
Rab25	1	1	1	1	1	1	1	1	1	1
Rab26	1	1	1	1	0	1	1	1	1	1
Rab27	2	2	2	0	2	2	2	2	2	2
Rab28	1	1	1	1	1	1	1	1	1	1
Rab29	1	1	0	1	0	0	0	0	0	0
Rab30	1	1	1	1	1	1	1	1	1	0
Rab31	1	1	1	2	1	1	1	1	1	1
Rab32	1	1	1	1	1	1	1	1	1	0
Rab33	2	2	2	2	2	2	2	2	2	2
Rab34	1	1	1	1	1	1	1	1	1	1
Rab35	1	1	1	1	1	1	1	1	1	1
Rab36	1	1	1	1	0	0	1	0	0	1
Rab37	1	1	1	1	1	1	1	0	1	1
Rab38	1	1	1	1	1	1	1	1	1	1
Rab39	2	2	2	2	1	2	1	1	2	1
Rab40	3	2	2	2	1	1	1	1	3	1
Rab41	1	1	0	0	0	0	0	0	0	0
Rab42	1	1	0	2	1	1	1	1	1	1
Rab43	1	1	1	1	0	0	0	0	0	0
Total	63	61	52	59	43	51	46	40	53	40

Helenius, 2005), while Rab9 mediates lysosomal enzyme trafficking and cycling of molecules from late endosomes to the trans-Golgi network (Ganley et al., 2004; Lombardi et al., 1993). Rab32 functions in trafficking of melanogenic enzymes, while Rab38 is believed to play an important role in post-Golgi biogenesis of melanosomes (Alto et al., 2002; Wasmeier et al., 2006). Rab34 is required for regulation of spatial distribution of lysosomes and formation of macropinosomes. The function of Rab36 is still unclear, but some studies reported that it has similar functions as Rab34 (Rosaria Colucci et al., 2005; Sun and Endo, 2005).

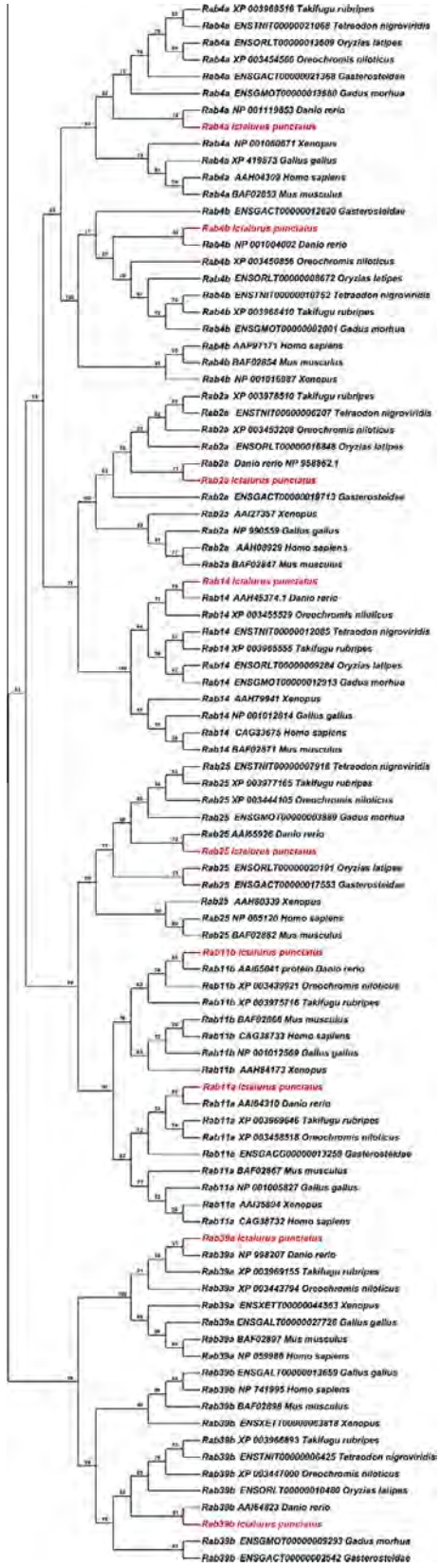

Fig. 7. Phylogenetic tree of catfish Rab GTPase subfamily I. The accession numbers of the protein sequences followed the name of the species. The bootstrapping values are indicated by numbers at the nodes.

The functions of multiple member subfamilies are quite complex. In subfamily D, Rab5 is involved in endocytosis pathway by the regulation of early endosome fusion and the caveolar vesicle targeting to early endosomes (Arnett et al., 2004; Barbieri et al., 2000; Pelkmans et al., 2004). Rab17 is required for transporting through apical recycling endosomes and polarized sorting (Zacchi et al., 1998); and Rab20 is specific to V-ATPase trafficking (Curtis and Gluck, 2005). Rab21 in mammals is essential for cell-extracellular matrix (Pellinen et al., 2006). Rab22a and Rab31 have very similar functions including two-directional transport from endosome to Golgi (Kauppi et al., 2002; Roberts et al., 2006); and Rab24 is involved in tyrosine phosphorylation (Munafó and Colombo, 2002). In subfamily I, Rab4 and Rab11 are important transport regulators in endocytic pathway (Bottger et al., 1996; Nagelkerken et al., 2000; Ullrich et al., 1996; van der Sluijs et al., 1992; Wilcke et al., 2000); Rab1 can control the transport from endoplasmic reticulum to Golgi (Tisdale et al., 1992); Rab14 and Rab25 are also transport regulators. Rab14 works between early endosomes and Golgi, while Rab25 facilitates the transport of apical recycling endosomes (Junutula et al., 2004; Pereira-Leal and Seabra, 2000b). In subfamily F, Rab1 has the similar function to Rab2 for ER to Golgi transport (Tisdale et al., 1992); Rab3 can control the Ca^{2+}-dependent secretion and dense-core vesicle docking to the plasma membrane (Rupnik et al., 2007; Tsuboi and Fukuda, 2006); Rab8 is broadly involved in the trafficking between Golgi, endosomes and plasma membrane, basolateral transport in epithelia and dendritic transport in neurons (Rab8a) and adherens junction assembly (Rab8b) (Chen and Wandinger-Ness, 2001; Nachury et al., 2007; Peränen et al., 1996); Rab12 controls the transport from cell periphery to perinuclear centrosomes (Iida et al., 2005); Rab13 is required for the formation of tight junctions (Marzesco et al., 2002); the expression of Rab15 is specific for trafficking through recycling endosomes (Elferink and Strick, 2005). In subfamily G, Rab26 can regulate secretion of granules (Yoshie et al., 2000); Rab27 is the interaction protein of Rab3 (Tsuboi and Fukuda, 2006); and Rab37 is required for mast cell activation and degranulation (Masuda et al., 2000). The function of subfamily H is not well studied. Only Rab33 is known as regulator of retrograde Golgi transport to ER (Valsdottir et al., 2001). However this gene surprisingly displayed potential function related to acute phase immune response and even endocytic pathway in this study.

Table 4
Differentially expressed Rab genes following bacterial infections as determined by analysis of RNA-seq datasets. Initial RNA-seq reads files were collected from previous studies (Li et al., 2012; Liu et al., 2012; Sun et al., 2012), the significantly expressed genes were identified based on the following criteria: threshold of p-value <0.05, mapped reads >5, weighted proportions of fold change $\geqslant|2|$.

Rab genes	Columnaris gill			ESC intestine		
	4 h	24 h	48 h	3 h	24 h	3d
Rab3a	3.38	2.05	–	2.85	–	–
Rab4a	3.31	5.08	–	11.21	17.44	2.69
Rab4b	12.08	4.05	–	7.34	–	2.24
Rab5a	6.22	4.15	–	9.03	3.01	–
Rab7a	3.13	2.01	–	2.02	2.19	3.14
Rab9a	2.41	2.11	–	–	–	–
Rab11a	3.03	2.13	–	–	–	–
Rab11b	–	–	–	–	–	2.02
Rab33a	–	2.09	–	–	–	–

The objective of this study was not to determine the functions of the Rab genes, but rather to make the initial assessment of their involvement in immune responses after bacterial infection. To achieve this goal, meta-analysis was first performed using RNA-seq datasets after infection with *E. ictaluri* and *F. columnare* (Li et al., 2012; Liu et al., 2012; Sun et al., 2012). Of the 52 catfish Rab genes, nine Rab genes were found to be up-regulated from the RNA-seq analysis (Table 4) including Rab3, Rab4a, Rab4b, Rab5, Rab7, Rab9, Rab11a, Rab11b, and Rab33. Of these nine genes, eight were up-regulated with columnaris infection, and six were

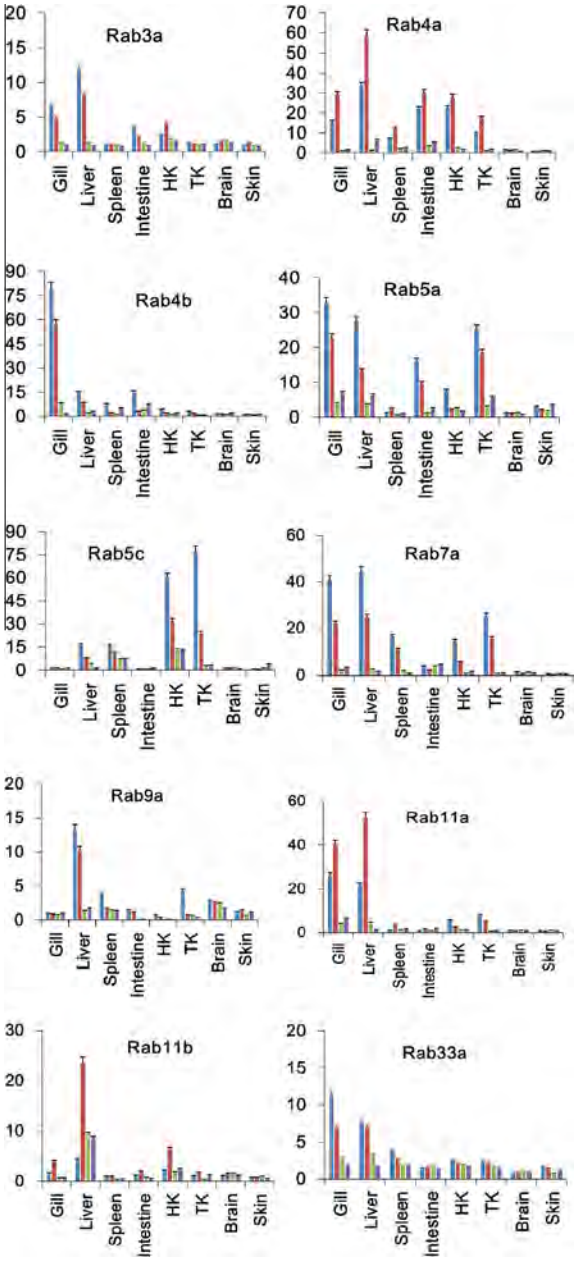

Fig. 8. Quantitative real-time RT-PCR analysis for the expression of ten endocytic Rab genes in catfish after challenge with *Edwardsiella ictaluri*. Tissues are indicated on the *X*-axis including gill, liver, spleen, intestine, head kidney (HK), trunk kidney (TK), brain and skin. Fold of induction as compared with controls is indicated on the *Y*-axis. Gene names are indicated on each panel of the figure. Blue, 4 h; red, 24 h, green, 48 h, and purple, 96 h after infection. Please note the difference of scales on the *Y*-axis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

up-regulated with ESC infection. These genes were expressed at the early phases after infection (3 h to 3 d), consistent with the understanding of endocytic process in host immune response of bacterial infection in fish species (Uribe et al., 2011). Of the 9 Rab genes involved in the response after infection, Rab33a was not previously reported to be involved in immune responses. Its up-regulation was also validated by real-time quantitative PCR after ESC infection. This gene had been less studied previously and its only known function was related to Intra-Golgi transport (Pereira-Leal and Seabra, 2001). The observed differential expression of Rab33a in our study suggested that this gene was also involved in immune responses against Gram negative bacteria.

In order to provide additional information of Rab expression after infection in various tissues as well as to validate the expression analysis using RNA-seq datasets, real time quantitative PCR was conducted. In general, the real time PCR data were consistent with RNA-seq data. All six genes that were found up-regulated using RNA-seq datasets were found up-regulated using real time PCR in the intestine (Fig. 8). However, it appeared that the relative extent of up-regulation differed somewhat between the results using the two methods. In general, the expression fold changes observed from RNA-seq were smaller than those observed from real-time PCR. This raises the question of the accuracy of quantitative analysis. Although sequencing biases can cause a certain level of variations, quantitation from direct counting of short reads as adjusted to RPKM (Li et al., 2012; Liu et al., 2012; Sun et al., 2012) could be more accurate because there are no additional complications. It is possible that the differences between samples could be signified with real time PCR. The more abundant templates could have supported more efficient PCR amplifications during real time PCR.

Based on the expression patterns of Rab genes, the general Rab-regulated endocytic pathway in catfish can be proposed: the molecules internalized by endocytosis are initially transported to early endosomal compartments which are characterized by the presence of Rab3a, Rab5a, Rab5c and rab4b. Transport of EGFR complex from early endosomes to late endosomes is facilitated by Rab7a, and cycling of molecules from late endosomes to the trans-Golgi network is mediated by Rab9. Transport to perinuclear recycling endosomes is subsequently followed by Rab11-mediated recycling to the plasma membrane. The transport of molecules to lysosomes is mediated by rab7 and my also mediated by Rab33a, leading to the secretion of lysosomes, which are delivered back to the plasma membrane in a calcium-regulated process. The proposed process of this pathway was consistent with its counterpart found in Rab GTPase studies in human except the involvement of Rab33a in our study (Stein et al., 2003).

5. Conclusion

The identification and phylogenetic analysis of Rab GTPases in catfish genome has revealed interesting features of this important group of membrane proteins in fish species. The analysis of the complete set of Rab proteins revealed parallels between catfish and zebrafish in that both lack homologues to certain human Rab proteins, e.g., Rab16, Rab17 and Rab41. The high conservation of Rab proteins involved in fundamental physiological processes suggests an evolutionary ancestral origin of these proteins. Clear orthologous relationships were established for the majority of Rab genes, enabling the potential for functional inference of the orthologous genes. All the catfish Rab genes were strongly supported by the phylogenetic trees, indicating the feasibility to annotate most catfish gene families. The Rab genes were significantly induced at early stages after bacterial infection, but exhibited differences in the extent of induction, and temporal and spatial expression, suggesting their distinct roles and cooperative actions in the immune responses.

Acknowledgements

This project was supported by Agriculture and Food Research Initiative Competitive Grant Nos. 2009-35205-05101, 2010-65205-20356 and 2012-67015-19410 from the USDA National Institute of Food and Agriculture (NIFA). The authors wish to thank Geoffrey C. Waldbieser, Huseyin Kucuktas, Ludmilla Kaltenboeck, Xin Geng, Jiaren Zhang, Jun Yao and Lin Song for their technical

assistance in various experiments. Ruijia Wang was supported by a scholarship from the China Scholarship Council.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2014.01.026.

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