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L-Rhamnose-binding lectins (RBLs) in channel catfish, *Ictalurus punctatus*: Characterization and expression profiling in mucosal tissues

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ABSTRACT

Rhamnose-binding lectins (RBLs) have recently emerged as important molecules in the context of innate immunity in teleost fishes. Previously, using RNA-seq technology, we observed marked up-regulation of a RBL in channel catfish (*Ictalurus punctatus*) gill following a challenge with the bacterial pathogen *Flavobacterium columnare*. Furthermore, the magnitude of RBL up-regulation positively correlated with disease susceptibility. Moving forward from these findings, we wished to more broadly understand RBL function, diversity, and expression kinetics in channel catfish. Therefore, in the present study we characterized the RBL gene family present in select channel catfish tissues and profiled family member expression after challenge with two different Gram-negative bacterial pathogens. Here, six RBLs were identified from channel catfish and were designated IpRBL1a, IpRBL1b, IpRBL1c, IpRBL3a, IpRBL3b, and IpRBL5a. These RBLs contained carbohydrate recognition domains (CRD) ranging from one to three domains and each CRD contained the conserved motifs of –YGR- and –DPC-. Despite a level of structural conservation, the catfish RBLs showed low full-length identity with RBLs from outside the order Siluriformes. IpRBL expression after bacterial infection varied depending on both pathogen and tissue type, suggesting that IpRBLs may exert disparate functions or exhibit distinct tissue-selective roles in the host immune response to bacterial pathogens.

1. Introduction

Lectins are a group of carbohydrate-binding proteins that recognize mono- or oligosaccharide moieties of their ligands. They are widely distributed in viruses, bacteria, plants, and animals, and function in a variety of biological processes (Ballarin et al., 2013; Gabius, 1997; Ogawa et al., 2011; Vasta et al., 2011). Types of lectins found in teleosts include C-type, F-type, lily type, galectin, intelectin, tectonin, and rhamnose-binding lectins (RBLs; Ogawa et al., 2011). RBLs are a new family of animal lectins that were first isolated in the sea urchin (Ozeki et al., 1991). RBLs specifically recognize L-rhamnose and/or D-galactose (Ogawa et al., 2011), function as Ca^{2+}-independent lectins (Hosono et al., 1999, 1993a,b), and are comprised of one or multiple homologous carbohydrate recognition domains (CRDs) with a unique α/β fold (Ballarin et al., 2013; Ogawa et al., 2011). The structure of the CRD is a key factor in the classification of lectins (Hosono et al., 2013). Each RBL CRD is composed of amino acids of about 100 residues in length that encode eight conserved cysteine residues, contributing to four disulfide bridges in their characteristic topology (Ballarin et al., 2013; Jimbo et al., 2007; Tateno et al., 2001; Terada et al., 2007) as well as containing the conserved motifs of -(AN)YGR- and -DPC-(KYL)- (Hosono et al., 1999, 2013; Ogawa et al., 2011; Terada et al., 2007). RBLs have been largely isolated from teleosts, tunicates, and other aquatic invertebrate species, such as bivalves and sea urchin (de Lorgeril et al., 2011; Gasparini et al., 2008; Naganuma et al., 2006; Ogawa et al., 2011; Ozeki et al., 1991, 1995). To date, members of the RBL family have been identified in more than 25 species of fish, with expression predominant in ovaries, eggs, and skin mucus (Ogawa et al., 2011; Watanabe et al., 2009).

RBLs have been reported to play roles as antibacterial and non-self-recognition molecules in the innate immune response (Booy et al., 2005; Shiina et al., 2002; Tateno et al., 2002b,c; Watanabe et al., 2008, 2009). RBLs from rainbow trout (*Oncorhynchus mykiss*) agglutinated both Gram-positive and Gram-negative bacteria and inhibited bacterial growth by binding to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) of bacterial surfaces (Tateno et al., 2002b). RBLs from chum salmon (*O. keta*) used globotriaosylceramide (Gb3) as a putative natural ligand of RBL to increase their expression in response to inflammatory stimuli, enhance

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phagocytosis, act as opsonins, and induce the synthesis and release of pro-inflammatory cytokines, including IL-1β1, IL-1β2, TNF-α1, TNF-α2, and IL-8 (Lam and Ng, 2002; Ogawa et al., 2011; Terada et al., 2007; Watanabe et al., 2009). The structure of the RBL gene promoter from snakehead (*Channa argus*) also indicated that RBL expression was potentially stimulated by inflammatory substances, such as LPS, IL-6, and IFN-γ (Jia et al., 2010).

The channel catfish (*Ictalurus punctatus*) is an economically important species of America's aquaculture industry (Shoemaker et al., 2008; USDA, 2013; Wagner et al., 2002). The value of America's channel catfish in farm-gate sales decreased by 20 percent between 2011 and 2012 (USDA, 2013). Bacterial diseases of *Edwardsiella ictaluri,* also known as the causative agent of enteric septicemia of catfish (ESC), and *Flavobacterium columnare,* the causative agent of columnaris disease, are two major Gram-negative bacterial pathogens that contribute heavily to production losses of channel catfish (Shoemaker et al., 2008; Wagner et al., 2002). Recent RNA-seq studies by our group revealed the striking early up-regulation of a RBL in the gill of channel catfish infected by *F. columnare* (Sun et al., 2012). Subsequent follow-up studies demonstrated higher basal and induced RBL levels in the gill of susceptible fish when compared to resistant fish (Beck et al., 2012; Peatman et al., 2013). Additionally, pre-exposure of channel catfish to the putative RBL ligands, L-rhamnose and D-galactose, decreased RBL expression and lowered columnaris mortality in a dosage-dependent manner (Beck et al., 2012). Finally, short-term fasting of channel catfish dramatically up-regulated RBL expression levels, revealing a novel mechanism potentially linking nutrition and immunity in the mucosa (Beck et al., 2012; Liu et al., 2013). Whereas we have previously characterized several other key families of innate signaling and effector molecules in catfish (Niu et al., 2011; Takano et al., 2008; Wang et al., 2006a,b; Zhang et al., 2012), nothing is known about the breadth and function of the RBL family in channel catfish. Therefore, here we characterized the RBL family of channel catfish using both RNA-seq and genomic databases and determined the expression of the RBL members in mucosal tissues following exposure to the Gram-negative bacterial pathogens, *E. ictaluri* and *F. columnare*. The present study is the most comprehensive examination of RBL structure and expression in a single species to-date and should provide a foundation for further functional characterization of RBLs in species of commercial and ecological importance.

2. Methods

2.1. Sequence identification and analysis

The RBL genes of the channel catfish were identified from the channel catfish databases including RNA-seq (Beck et al., 2012; Li et al., 2012; Liu et al., 2011, 2012; Sun et al., 2012) and the catfish genome scaffolds (unpublished data) using zebrafish (*Danio rerio*) RBL sequences (Ogawa et al., 2011) as queries. TBLASTN was used to obtain the initial pool of RBL sequences with a cutoff *E*-value of $1e^{-5}$. The RBL sequences were then aligned to delete the repeated entries and a unique set of sequences were subjected to further analysis. BLASTN was then used to verify the cDNA sequences through comparing the transcriptome sequences with the whole genome sequence. The obtained sequences were translated using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted amino acid sequences from ORFs were further verified by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against NCBI nr protein database. The simple modular architecture research tool (SMART) was used to identify the conserved domains. NCBI's Spidey (http://www.ncbi.nlm.nih.gov/spidey/) was used to align and compare transcript and genomic scaffold sequences.

2.2. Phylogenetic analysis

According to Ogawa et al. (2011), the RBL CRD amino acid sequences were used to conduct phylogenetic analysis to determine types of CRD in each RBL. RBL CRDs from chum salmon (*O. keta*), rainbow trout (*O. mykiss*), white-spotted char (*Salvelinus leucomaenis*), blue catfish (*Ictalurus furcatus*), amur catfish (*Silurus asotus*), far-east dace (*Tribolodon brandtii*), winged pearl oyster (*Pteria penguin*) and mouse latrophilin-1 Gpcr were chosen and retrieved from NCBI databases. Multiple RBL CRD protein sequences were aligned using the ClustalW program. Phylogenetic analyses were performed using MEGA 5.2 using the neighbor-joining method (Tamura et al., 2011) with 10,000 replications of bootstrapping to evaluate the phylogenetic tree topology. Furthermore, the full length amino acid sequences of RBLs of other species including amur catfish (*S. asotus*), Atlantic salmon (*Salmo salar*), blue catfish (*I. furcatus*), chum salmon (*O. keta*), far-east dace (*T. brandtii*), northern pike (*Esox lucius*), Pacific oyster (*Crassostrea gigas*), ponyfish (*Leiognathus nuchalis*), purple sea urchin (*Strongylocentrotus purpuratus*), rainbow trout (*O. mykiss*), red snapper (*Lutjanus sanguineus*), sea urchin (*Anthocidaris crassispina*), seahorse (*Hippocampus kuda*), smelt (*Spirinchus lanceolatus*), snakehead (*C. argus*), vase tunicate (*Ciona intestinalis*), winged pearl oyster (*P. penguin*), white-spotted charr (*S. leucomaenis*), and zebrafish (*Danio rerio*) were used to construct the full-length phylogenetic tree.

2.3. Bacterial challenge and sample collection

In order to evaluate the roles of RBL genes in the host immune response to bacterial infection, *E. ictaluri* and *F. columnare* challenges were conducted following established detailed protocols (Beck et al., 2012; Li et al., 2012; Sun et al., 2012). All 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 initiation. Pond-run (unselected) Marion channel catfish (average size 35 ± 1.3 g) were reared at the Auburn University Fish Genetics Research Unit. Prior to experiments, fish were maintained in 30 L tanks and acclimatized for 2 weeks at a temperature of 28 °C. Before challenge, the bacteria was cultured from a single colony, re-isolated from a symptomatic fish and biochemically confirmed before being inoculated in the shaker incubator overnight. The concentration of bacteria was determined using colony forming unit (CFU) per mL by plating 10 μl of 10-fold serial dilutions onto plates. During challenge, symptomatic fish and control fish were collected and confirmed to be infected with *E. ictaluri*, *F. columnare* and pathogen-free, respectively, at the Fish Disease Diagnostic Laboratory, Auburn University.

Briefly, *E. ictaluri* (MS-S97-773) was inoculated in brain heart infusion (BHI) medium in a shaker incubator at 28 °C overnight. Fish were challenged in 30 L aquaria with 4 control and 4 treatment groups. Aquaria were randomly divided into sampling time-points-4 h treatment, 24 h treatment, 3 d treatment and 7 d treatment, 4 h control, 24 h control, 3 d and 7 d control with forty fish in each aquarium. During challenge, the bacterial culture with a concentration of 4×10^8 CFU/ml was added into the treatment aquaria. Water was turned off in the aquaria for 2 h of immersion exposure, and then continuous water flow-through resumed for the duration of the challenge experiment. Control fish were exposed to the same routine as treatment fish, but were immersed in sterilized media alone. At 4 h, 24 h, 3 d and 7 d after challenge, 30 fish were collected from each of the appropriate control and treatment aquaria at each time point and euthanized with MS-222 (300 mg/L). Gill, skin and intestine from 10 fish (3 replicates of 10 fish each) were pooled together for each tissue, flash frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

F. columnare (BGFS-27; genomovar II; Olivares-Fuster et al., 2011) was inoculated in modified Shieh broth and grown for 24 h in a shaker incubator (100 rpm) at 28 °C. Fish were randomly divided into 8 rectangular 30 L aquaria of which 4 aquaria were designated control (4 h, 24 h, 2 d and 2.5 d) and the other 4 were designated challenge groups (4 h, 24 h, 2 d and 2.5 d). Challenge experiments were then conducted by immersion exposure for 2 h at a final concentration 3×10^6 CFU/ml. Control fish were exposed to the same routine as treatment fish, but were immersed in sterilized media alone. After the challenge, fish were incubated in aquaria with flow through water. Gill and skin were collected at 4 h, 24 h, 2 d and 2.5 d time points post challenge. At each time point, 18 fish from both control and treatment were randomly selected and divided into 3 replicate pools (6 fish each) respectively. The fish were euthanized with tricaine methanesulfonate (MS 222) at 300 mg/L (buffered with sodium bicarbonate) before tissues were collected. Gill and skin tissues in the 3 replicates were flash frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.4. RNA extraction and real-time PCR analysis

Prior to RNA extraction, samples were removed from the −80 °C freezer and ground with sterilized mortar and pestle in the presence of liquid nitrogen to a fine powder. Total RNA was extracted from tissue powder using the RNeasy Plus Kit (Qiagen) following manufacturer's instructions. RNA concentration and integrity was measured using a NanoDrop ND-1000 UV–VIS Spectrophotometer version 3.2.1. First strand cDNA was synthesized by iScript™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions. The iScript chemistry uses a blend of oligo-dT and random hexamer primers. All the cDNA products were diluted to 250 ng/μl and utilized for the real-time PCR reaction using the SsoFast™ EvaGreen® Supermix on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The thermal cycling profile consisted of an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 94 °C for 5 s, an appropriate annealing/extension temperature at 58 °C for 5 s. An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 °C to 95 °C for 5 s. All primers of channel catfish RBLs were listed in Table 1. 18S rRNA gene (forward primer-GAGAAACGGCTACCACATCC and reverse primer-GATACGCTCATTCCGATTACAG) was used as a reference gene (Small et al., 2008). Results were expressed relative to the expression levels of 18S rRNA in each sample using the Relative Expression Software Tool (REST) version 2009 (Pfaffl et al., 2002). The biological replicate fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (Ct) values, were compared and converted to fold differences by the relative quantification method. Expression differences between groups were assessed for statistical significance using a randomization test in the REST software. The mRNA expression levels of all samples were normalized to the levels of 18S rRNA gene in the same samples. Test amplifications were conducted to ensure that 18S and target genes were within an acceptable range. A no-template control was run on all plates. QPCR analysis was repeated in triplicate runs (technical replicates) to confirm expression patterns. For the expression analysis in healthy tissues, the tissue with the lowest Ct values for each gene were set as the control group (1) in REST, and the relative expression of each IpRBL gene in healthy tissues were obtained by comparing expression in other tissues with that tissue.

Table 1
Primer sequences of IpRBL genes.

	Name	Sequences (5′–3′)	Primer length	Product size
1	IpRBL1aF	GTGATGTCCAAAGACTCACGTG	22	210
2	IpRBL1aR	GGTCGGGGTTGCCAAGTAAATC	22	
3	IpRBL1bF	GTCATGTCCAAAGACTCACTTG	22	210
4	IpRBL1bR	GGTCAGGGTTGCCAAGTAATTC	22	
5	IpRBL1cF	TATTGCAGCTCAGGGCTTGT	20	167
6	IpRBL1cR	TGACAACCTCAGATGGCGAC	20	
7	IpRBL3aF	AGACGGATTTACTTGGCAACCC	22	171
8	IpRBL3aR	CAGCACGTCCGTAGTTCGCA	20	
9	IpRBL3bF	TGCTACGATGCCGAAACAAC	20	150
10	IpRBL3bR	CTTGGTCAAACCACTGGGGA	20	
11	IpRBL5aF	AATTTGCCCTGCTCTGGTGA	20	140
12	IpRBL5aR	GCACACGTTCGCGAATCAAT	20	

3. Results

3.1. Identification and classification of channel catfish RBL genes

Six different cDNA and putative amino acid sequences were identified from the channel catfish transcriptome using RBL

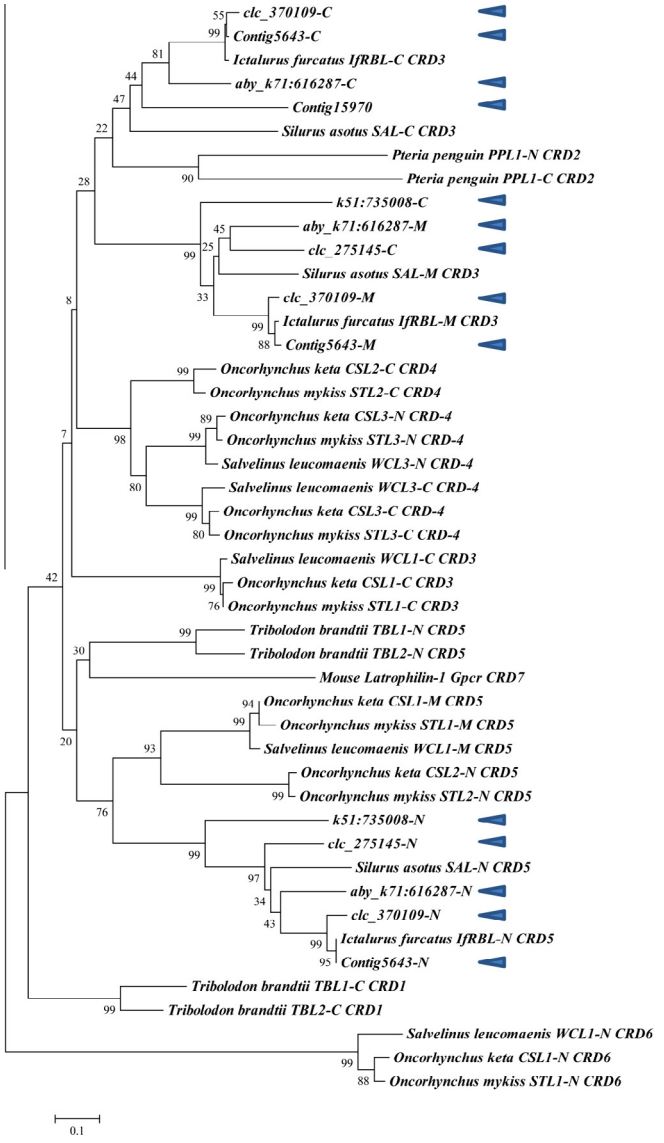

Fig. 1. CRD identification of channel catfish RBL through phylogenetic classification using the neighbor-joining method. Seven groups of well characterized RBL CRDs (Ogawa et al., 2011) were included and used to name CRDs of the channel catfish RBL. Contigs including Contig5643, clc_370109, clc_275145, aby_k71_616287, k51:735008, and Contig15970 are initial RBL CRDs from the channel catfish transcriptome. N, M, and C represent N-terminus, Middle, and C-terminus in each CRD, respectively.

sequences of zebrafish (*D. rerio*) as queries. In order to name these RBLs, the sequences were examined based on their CRD compositions. RBLs contain variable numbers of CRDs and, therefore, can vary significantly in length. According to Ogawa et al. (2011), animal RBL CRDs were clustered into seven groups. The composite CRD structure of RBLs allowed Ogawa et al. (2011) to identify 13 types of RBL genes. The seven CRDs were used to classify each channel catfish RBL constituent CRD. Phylogenetic tree analyses demonstrated that channel catfish RBL CRDs belong to the clades of CRD3 and CRD5 (Fig. 1). Moreover, to organize the channel catfish RBL into composite types, the organization of the CRD was constructed. As shown in Table 2, three RBL genes containing three domains (in a N–C orientation) CRD5- CRD3- CRD3 were classified as type Ia (Ogawa et al., 2011), and they were named as IpRBL1a, IpRBL1b, IpRBL1c. Two RBL genes composed of two domains CRD5- CRD3 were a new type termed here as IIIg and were named as IpRBL3a and IpRBL3b. The final RBL containing only one CRD3 domain was termed type Va and was named as IpRBL5a. Of note here, the RBL previously described by our group (Sun et al. 2012), is identified here as IpRBL1a.

Given the importance of the CRDs for RBL characterization, we carried out additional comparisons of the constitutive catfish CRDs with those of other well-studied RBLs. The amino acid length of each channel catfish RBL CRD was 95–98 residues (Fig. 2). Each deduced amino acid sequence of IpRBL CRDs possessed eight cysteine residues which are involved in the formation of four disulfide bonds. Each contained the conserved motifs of –YGR- and –DPC- with the exception of the C-terminus CRD domain of IpRBL3b (-FGR). In type I RBLs which contain three CRDs, the deduced amino acid sequences of IpRBL CRDs in the same gene shared low identities of 28–51% with one another (Table 3). Similarly, in type III RBLs which contain two

Table 2
RBL type and CRD composition for the six IpRBLs and key published RBLs from other fishes.

RBL*	Type/CRD composition**	Order	Protein ID	References
SAL	Ia 5 3 3	Siluriformes	BAA87860.1	Hosono et al. (1999)
IfRBL			ADO28323.1	Chen et al. (2010)
IpRBL1a				This paper
IpRBL1b				This paper
IpRBL1c				This paper
CSL1	II 6 5 3	Salmoniformes	P86177.1	Shiina et al. (2002)
STL1			NP_001117667.1	Tateno et al. (2001)
WCL1			BAB83629.1	Tateno et al. (2002a)
CSL3	IIIa 4 4	Salmoniformes	P86179.1	Shiina et al. (2002)
STL3			NP_001117669.1	Tateno et al. (2001)
WCL3			BAB83628.1	Tateno et al. (2002a)
CSL2	IIIb 5 4	Salmoniformes	P86178.1	Shiina et al. (2002)
STL2			NP_001117668.1	Tateno et al. (2001)
IpRBL3a	IIIg 5 3	Siluriformes		This paper
IpRBL3b				This paper
IpRBL5a	Va 3	Siluriformes		This paper

* SAL is RBL from amur catfish (*S. asotus*); IfRBL is RBL from blue catfish (*I. furcatus*); IpRBL is RBL from channel catfish (*I. punctatus*); CSL is RBL from chum salmon (*O. keta*); STL is RBL from stealhead trout or rainbow trout (*O. mykiss*) and WCL is RBL from white-spotted char (*S. leucomaenis*).
** RBL grouping was followed to the data reported by Ogawa et al. (2011). CRD group was phylogenetically classified and the number of CRD group was denoted in the boxes.

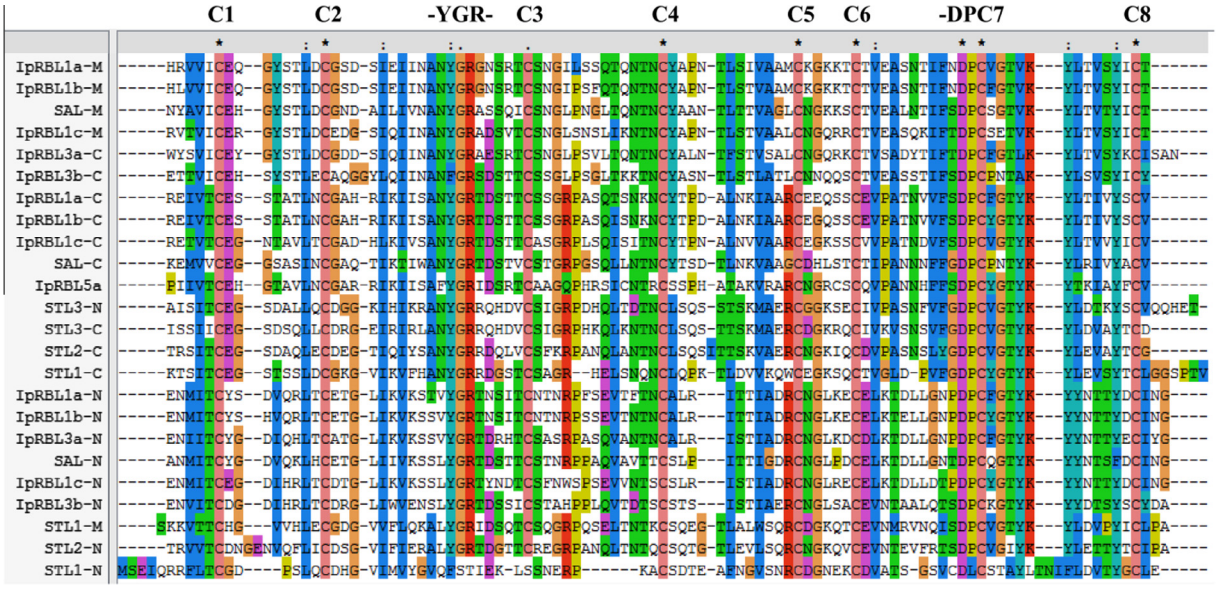

Fig. 2. Alignment of amino acid sequences of RBL CRD from channel catfish (IpRBL), amur catfish (SAL), and rainbow trout (STL). N, M, and C represent N-terminus, Middle, and C-terminus in each CRD, respectively. Multiple alignments were completed using the CLUSTAL X program. Eight cysteine residues (C1–C8) engaged in disulfide-bond pairs and the conserved motifs of YGR and DPC are indicated on the top. Note that STL1-N lacks characteristic RBL motifs.

CRDs, the deduced amino acid sequences of IpRBL CRDs in the same gene shared 30–31% identities with one another. Interestingly, the highest amino acid identities were found among the N-terminus domains of IpRBL1a, 1b, and 1c, middle domains of IpRBL1a, 1b, and 1c, and C-terminus domains of IpRBL1a, 1b, and 1c which shared 75–94%, 71–95%, and 74–97% identities, respectively. Comparison of each IpRBL CRD with RBL CRDs from other fish species revealed amino acid identities of less than 75% (Table 3).

Following CRD and type classification and naming of the catfish RBLs, the full length of amino acid sequences were used to construct a phylogenetic tree with RBLs from other fish, mollusks, primitive vertebrates, and invertebrates including amur catfish (*S. asotus*), Atlantic salmon (*S. salar*), blue catfish (*I. furcatus*), chum salmon (*O. keta*), far-east dace (*T. brandtii*), northern pike (*E. lucius*), Pacific oyster (*C. gigas*), ponyfish (*L. nuchalis*), purple sea urchin (*S. purpuratus*), rainbow trout (*O. mykiss*), red snapper (*L. sanguineus*), sea urchin (*A. crassispina*), seahorse (*H. kuda*), smelt (*S. lanceolatus*), snakehead (*C. argus*), vase tunicate (*C. intestinalis*), winged pearl oyster (*P. penguin*), white-spotted charr (*S. leucomaenis*), and zebrafish (*D. rerio*) (Fig. 3). Phylogenetic analysis placed all RBLs from channel catfish into the same clade (Fig. 3). The only other members of the clade came from other catfish species, the well-studied amur catfish SAL RBL (Hosono et al., 1993a, 1999) and a RBL from the closely related blue catfish, IfRBL. Other species from which multiple RBLs have been identified (e.g. *D. rerio*, *O. mykiss*) were also placed in either species-specific clades or in clades containing only closely related species (e.g. salmonids).

All six cDNA and putative amino acid sequences were submitted to NCBI GenBank with accession numbers **KF725628–KF725633** (Table 4). The features of each IpRBL including the nucleotide length of cDNA, 5′ untranslated region (UTR), and 3′UTR, the length of amino acid residues contained in the open reading frame (ORF), the position of the signal peptide, and the number of CRDs contained in each IpRBL are summarized in Table 4. The ORF length between the IpRBLs, as with other species, varies greatly from 129 amino acids to 308 amino acids depending on the number of CRDs within each RBL.

3.2. Channel catfish RBL genomic structure

We compared the catfish RBL cDNA transcripts with the draft catfish genome assembly scaffolds to gain a better understanding of their spatial arrangement in the genome and their intron/exon structure where possible. BLAST-based comparison revealed that five out of six of the RBLs were located on a single scaffold (jcf7180003676198) within 0.5 Mb of one another (Fig. 4). We were unsuccessful in locating the genomic position of IpRBL1b. However, due to significant gaps within some of the scaffold joins, the gene may be located in the proximity of the closely related IpRBL1a. The cluster of catfish RBL genes on one chromosomal region seems to mirror a similar cluster of RBLs on zebrafish (*D. rerio*) chromosome 22. However, zebrafish (*D. rerio*) appears to encode many more RBL than any other examined fish species (Ogawa et al., 2011), a phenomenon consistent across other families of innate immune genes (Peatman and Liu, 2007), and also encodes RBL genes on several other chromosomes. We also predicted intron and exon structure using NCBI's Spidey when scaffold sequences allowed for the analysis (Fig. 4). While further comparative analysis will be possible with the finishing of the catfish genome sequence, it appears clear that the RBL repertoire differs significantly in arrangement, diversity, and depth from one fish taxa to another.

3.3. Basal tissue expression of IpRBL genes

Basal-level tissue expression of RBLs in healthy catfish was determined using real-time qRT-PCR in ten tissues: brain, gill, head kidney, intestine, liver, muscle, ovary, skin, spleen, and trunk kidney. Each of the six IpRBLs exhibited distinct tissue expression profiles. As shown in Fig. 5, IpRBL1a was expressed at the highest level in skin, followed by trunk kidney. IpRBL1b expression showed the highest levels in trunk kidney followed by muscle and skin, while IpRBL1c had the highest level in liver followed by intestine. IpRBL3a was expressed most strongly in intestine, trunk kidney, and head kidney. IpRBL3b was expressed very highly in ovary followed by gill and skin. IpRBL5a was expressed at the highest level in intestine, followed by gill and ovary.

Table 3
Percentage of amino acid identity of each RBL CRD from channel catfish (*I. punctatus*) compared to CRDs from their internal domains, other IpRBLs, and other fish species. IpRBL is RBL from channel catfish. SAL is a RBL from amur catfish (*S. asotus*) and STL1 is a RBL from stealhead trout or rainbow trout (*O. mykiss*). N, M, and C represent N-terminus, Middle, and C-terminus in each CRD, respectively.

% aa	IpRBL													
Identity	1a-N	1a-M	1a-C	1b-N	1b-M	1b-C	1c-N	1c-M	1c-C	3a-N	3a-C	3b-N	3b-C	5a
Ip1a-N	100													
Ip1a-M	28.4	100												
Ip1a-C	35.8	50.5	100											
Ip1b-N	93.7	30.5	36.2	100										
Ip1b-M	30.5	94.8	51.5	31.6	100									
Ip1b-C	35.8	50.5	96.8	37.9	50.5	100								
Ip1c-N	74.7	27.1	33.0	75.8	27.1	34.7	100							
Ip1c-M	29.5	70.8	48.4	30.5	70.8	50.5	31.6	100						
Ip1c-C	33.7	54.8	74.2	33.7	52.6	76.8	31.6	50.5	100					
Ip3a-N	77.9	29.5	39.4	76.8	31.6	39.4	72.6	30.5	36.6	100				
p3a-C	30.5	68.8	47.9	30.5	71.9	46.8	29.6	71.1	46.2	31.6	100			
p3b-N	53.7	29.5	36.2	53.7	31.6	37.2	62.1	30.5	35.5	55.8	30.5	100		
Ip3b-C	24.2	54.2	44.7	25.3	56.3	43.6	22.9	62.5	45.2	25.3	57.3	30.5	100	
Ip5a	30.5	43.2	59.6	32.6	44.2	62.8	32.6	42.1	58.1	33.7	43.2	31.6	33.7	100
SAL-N	73.7	26.3	34.0	72.6	28.4	35.1	68.4	30.5	33.3	70.5	30.5	56.8	24.2	31.6
SAL-M	30.5	70.5	47.9	31.6	72.6	47.9	27.4	69.5	52.7	30.5	69.5	30.5	60.0	43.2
SAL-C	30.5	47.4	62.8	31.6	47.4	62.8	29.5	44.2	57.0	31.6	42.1	31.6	41.1	48.4
STL1-N	11.7	18.1	16.0	12.8	16.0	18.1	16.0	17.0	20.4	13.8	21.3	13.8	11.7	14.9
STL1-M	32.6	40.6	40.4	33.7	39.6	41.5	29.9	38.1	44.1	30.5	38.1	37.9	34.4	41.1
STL1-C	28.4	38.5	41.5	29.5	38.5	43.6	31.6	37.1	44.1	33.7	36.7	31.6	29.2	34.7
STL2-N	35.8	36.5	39.4	37.9	35.4	40.4	33.7	34.0	43.0	35.8	33.7	41.1	31.3	36.8
STL2-C	32.6	41.7	44.7	33.7	40.6	45.7	36.5	39.6	46.2	41.1	38.5	37.9	35.4	42.1
STL3-N	34.7	41.7	50.0	34.7	41.7	51.1	35.7	37.1	50.5	36.8	36.7	42.1	34.4	43.2
STL3-C	31.6	46.3	43.6	32.6	46.3	44.7	34.7	44.2	45.2	34.7	43.2	35.8	37.9	40.0

3.4. Expression profiles of RBL genes after challenge with E. ictaluri

We examined the expression of the six IpRBLs after challenge with *E. ictaluri* at several timepoints in critical tissues for pathogen adhesion: gill, intestine, and skin (Fig. 6; Supplementary Table 1S). In the gill, a general, significant trend of RBL up-regulation was observed following infection. As early as 4 h, IpRBL1b and IpRBL3b were induced greater than 5-fold. By 24 h post-infection, IpRBL1a,

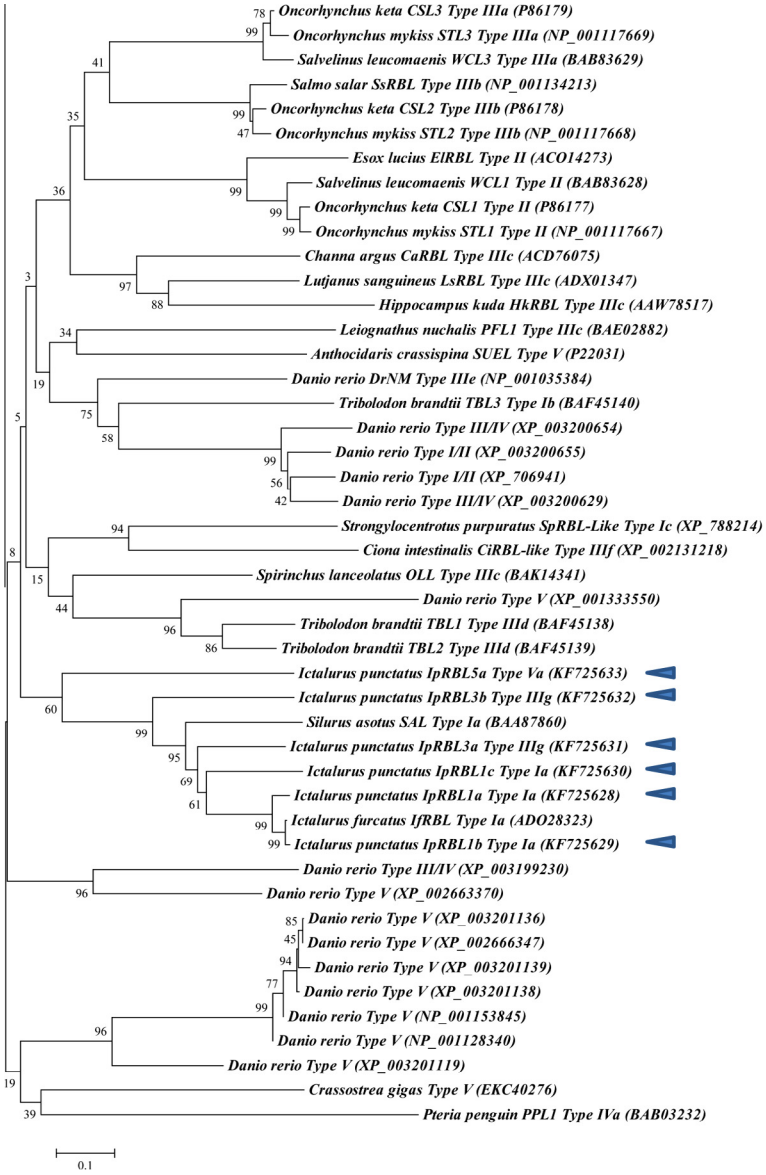

Fig. 3. Phylogenetic tree for RBL genes. The phylogenetic tree was constructed based on 46 full-length amino acid sequences of RBL from channel catfish, other fish, and sea urchin using the neighbor-joining method in MEGA 5.2. The accession number and type of RBL (Ogawa et al., 2011) is indicated following each RBL.

Table 4
The sequence features of six IpRBLs. The IpRBL features include their accession numbers, the length of nucleotides in each cDNA, 5′UTR, and 3′UTR, the length of amino acid residues contained in the ORF, the position of signal peptide cleavage, and the number of CRDs of each IpRBL.

Name	GenBank accession No.	# nt of cDNA	# nt of 5′UTR	# nt of 3′UTR	# aa ORF	Signal peptide	# of CRDs
IpRBL1a	KF725628	2495	101	>1467	308	23/24: VSG-EN	3
IpRBL1b	KF725629	1179	25	>227	308	22/23: IVS-RE	3
IpRBL1c	KF725630	2079	307	>851	306	16/17: AQG-LF	3
IpRBL3a	KF725631	772	38	>74	219	22/23: VSG-EN	2
IpRBL3b	KF725632	883	33	>199	216	23/24: VSG-EN	2
IpRBL5a	KF725633	959	138	>431	129	22/23: VSG-EN	1

IpRBL1b, IpRBL3a, and IpRBL3b were all significantly up-regulated, with the highest induction observed in IpRBL1a and IpRBL1b. This trend continued to 3 d and 7 d post-infection with up-regulation relative to control reaching greater than 650-fold in IpRBL1b. IpRBL5a, on the other hand, showed no significant changes following infection, and IpRBL1c showed consistent, modest down-regulation at all timepoints (Fig. 6A).

In the intestine of catfish challenged with *E. ictaluri* the expression patterns of IpRBLs were quite different. There, most genes showed reduced expression following infection with significant down-regulation of IpRBL1c at all timepoints (reaching greater than 400-fold at 3 d). IpRBL1a, IpRBL3a, and IpRBL5a also were down-regulated at one or more timepoints following infection. IpRBL1a and IpRBL1b were significantly, if modestly, up-regulated at 4 h and 3 d, respectively (Fig. 6B).

Patterns of IpRBL gene expression in catfish skin following ESC challenge were similar to those observed in intestine. Largest down-regulation of expression was again seen in IpRBL1c. Significant down-regulation was also observed in IpRBL1a, IpRBL3a, and IpRBL3b. IpRBL1b was the only RBL induced in the skin, up-regulated greater than 5-fold at 7 d post-infection (Fig. 6C).

3.5. Expression profiles of RBL genes after challenge with F. columnare

We also examined expression changes of IpRBLs in relevant mucosal tissues (gill and skin) following *F. columnare* infection (Fig. 7; Supplementary Table 2S). Similar to post-ESC responses, a general pattern of RBL up-regulation was observed in gill. At 4 h, IpRBL1c and IpRBL3a were induced. By 24 h, IpRBL1a and IpRBL1b rose together over 100-fold, with a small, significant up-regulation of IpRBL3a. At 2 d post-infection, only IpRBL1b remained significantly up-regulated. However, by 2.5 d, IpRBL1a and IpRBL1b had again reached 24 h levels (Fig. 7A).

RBL gene expression in the skin of catfish exposed to *F. columnare* was relatively modest compared with the gill. There was no consistent pattern across the six IpRBLs with some up-regulated and some down-regulated. IpRBL1a was induced at 24 h and 2.5 d. IpRBL5a was significantly repressed starting at 24 h in a pattern similar to that observed in the gill. Expression of IpRBL3b was induced in the final sampled timepoint (Fig. 7B).

4. Discussion

Rhamnose-binding lectins are a poorly-characterized family of lectins isolated predominantly from invertebrates and fishes. Early research attention focused on their presence and role in fish eggs (Hosono et al., 1993a,b; Tateno et al., 1998), with later work identifying their capability to bind bacterial lipopolysaccharides and lipoteichoic acid (Tateno et al., 2002b). Few studies have examined the expression of RBLs either basally or following infection, but several of those have reported high expression in a variety of lymphoid populations and in goblet cells of the gill and intestine (Okamoto et al., 2005; Tateno et al., 2002c). Our previous research revealed a major role for a RBL in early host responses to *F. columnare* in the catfish gill (Beck et al., 2012; Peatman et al., 2013; Sun et al., 2012). Unlike beneficial roles described for RBLs elsewhere (e.g. Bah et al., 2011), our results indicate that a catfish RBL is enhancing *F. columnare* adhesion to the gill surface. Given this newly-described role for RBLs in mucosal immunity, we sought here to understand better the diversity of RBLs in channel catfish and their expression patterns following infection in tissues known to facilitate pathogen entry.

We utilized multiple RNA-seq data sets developed from previous studies, together with ESTs and a draft catfish genome to mine for RBL sequences in channel catfish. The RBL sequence previously described (Beck et al., 2012; Sun et al., 2012) was subsequently named IpRBL1a. Unexpectedly, a highly similar (94.2% identity) sequence was identified, named here as IpRBL1b. QPCR primers utilized in previous studies only amplified IpRBL1a, but their high identity with one another could result in mis-assignment of short RNA-seq reads in some cases. In total, 6 RBLs were identified based on cDNA sequences, with 5 of those matching genomic sequence within a single scaffold. Four additional "orphan" partial-length RBLs derived from cDNA and/or genomic sequence evidence were discovered in our searches (data not shown), including some showing evidence of alternative splicing and exon shuffling. As we could not classify them completely at this time, they were not included in the subsequent analysis. The presence of at least 6 RBLs in catfish, differs from the situation in white-spotted charr (*S. leucomaenis*), chum salmon (*O. keta*), rainbow trout (*O. mykiss*), and amur

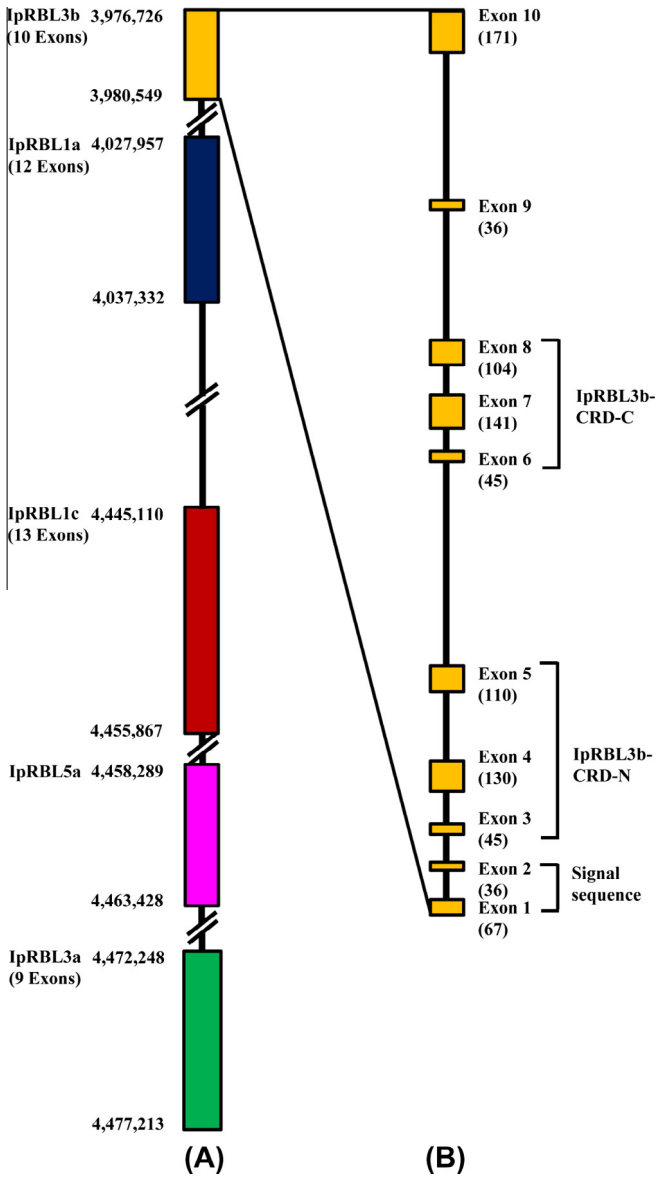

Fig. 4. Genome schematic depiction of IpRBL genes. This schematic represents (A) five IpRBLs locate on the same scaffold and (B) IpRBL3b genomic organization. (A) The location of each IpRBL gene on scaffold ID jcf7180003676198 is indicated using the starting and the ending point of each gene. The number of exons composed of gene is shown in the parenthesis. (B) IpRBL3b genomic organization where shaded boxes represent exons while string represent introns. The number of nucleotide contained in each exon is denoted in the parenthesis.

catfish (*S. asotus*) (Hosono et al., 1999; Shiina et al., 2002; Tateno et al., 1998, 2001, 2002a) where only 1–3 RBLs have been identified. On the other hand, there may be as many as 16 RBLs in zebrafish (*D. rerio*), although they remain unstudied (Ogawa et al., 2011). While zebrafish (*D. rerio*) may present a special case due to rapid tandem gene duplication in that species (Lu et al., 2012), it is likely

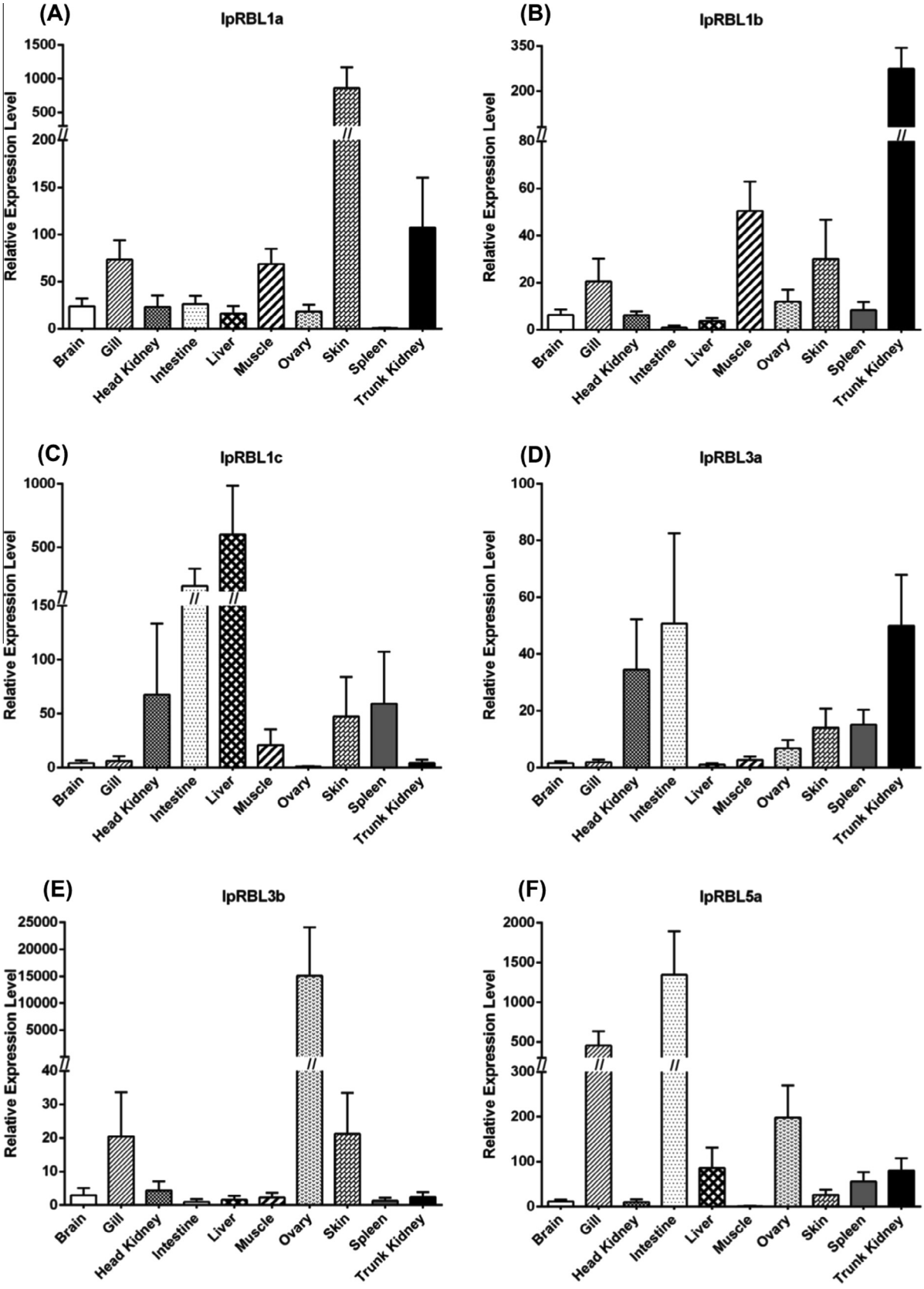

Fig. 5. Basal tissue expression of RBLs in healthy channel catfish by qRT-PCR. Expression values were normalized to that of the 18S rRNA. Data are expressed as the mean fold change relative to the tissue with lowest level expression of each gene (set at 1).

that there are additional RBL in the salmonids and amur catfish which remain to be identified.

Phylogenetic analysis of both constituent CRD and full-length amino acid sequences revealed several points touching on the evolutionary relationships of invertebrate and fish RBLs. As shown in Figs. 1 and 2 and Table 3, highest amino acid similarities are shared among CRD of the same type and domain position. For example, the middle (M) CRD of IpRBL1a shares 70.8% amino acid identity with IpRBL1c-M CRD, but only 54.8% identity with IpRBL1c-C CRD, even though in all cases these domains belong to CRD Group 3. This phenomenon also extends across similar species as seen in the comparison of channel catfish with amur catfish SAL (Table 3). Other evidence can be seen in Fig. 1 where salmonid CRD of the same group are more closely clustered based on domain position (N, M, C). For example, white-spotted charr (*S. leucomaenis*) WCL3-C (CRD4) forms a well-supported clade with chum salmon (*O. keta*) CSL3-C (CRD4) and rainbow trout (*O. mykiss*) STL3-C (CRD4). This phenomenon has been previously noted by Jia et al. (2010). They suggested that RBLs may share a common ancestor gene and evolved in tandem during the evolutionary process. They also posit that the ancestral gene of RBL diverged and evolved by exon shuffling and gene duplication (Jia et al., 2010). While we agree that there is indeed evidence supporting this theory, the relatively small number of RBLs identified in a given species at this date makes comprehensive phylogenetic analyses impossible. It is clear that groups of similar species have evolved RBL repertoires

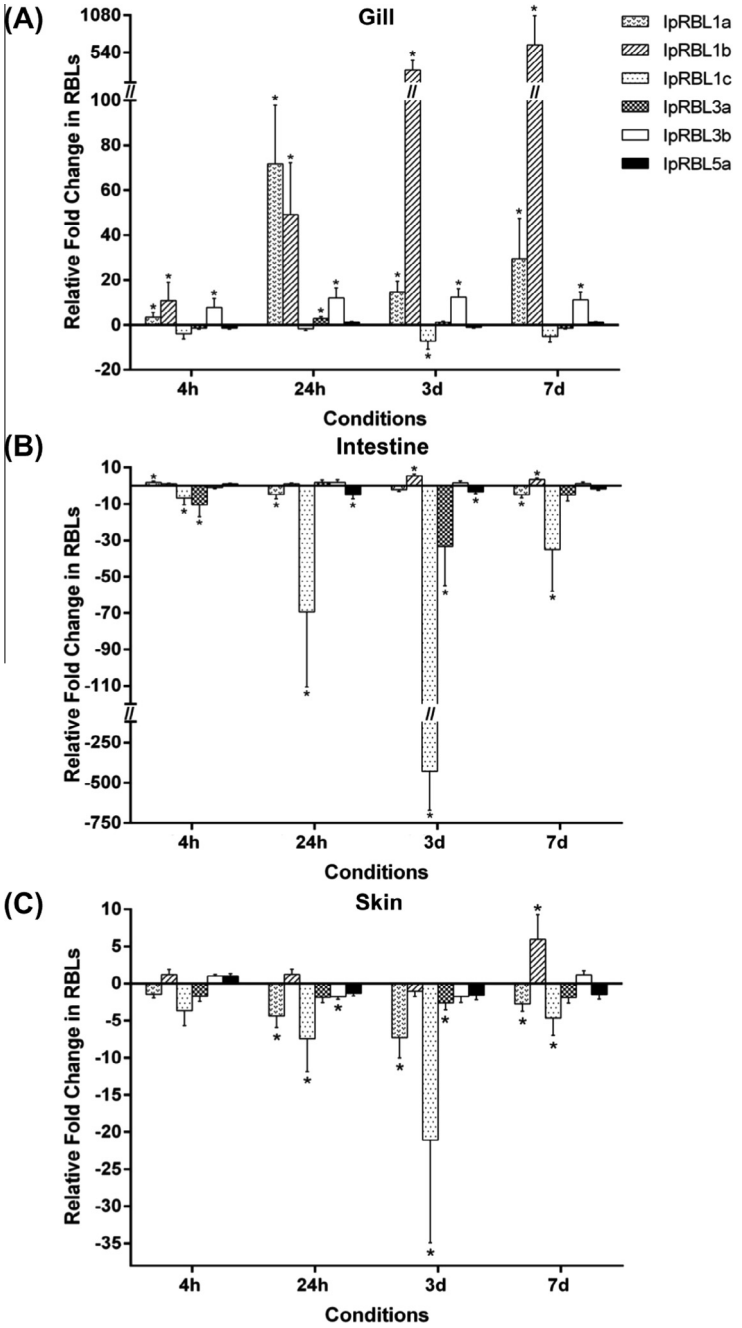

Fig. 6. Real time qRT-PCR analysis for IpRBL expression following *Edwardsiella ictaluri* infection. The IpRBL expression was measured in the mucosal tissues including (A) Gill, (B) Intestine, and (C) Skin at the timepoints of 4 h, 24 h, 3 d, and 7 d post-infection. Fold change was calculated by the change in expression at a given timepoint relative to the untreated control and normalized by changes in the 18S housekeeping gene. The results are presented as mean ± SE of fold changes and the asterisk indicates statistical significance at $P < 0.05$.

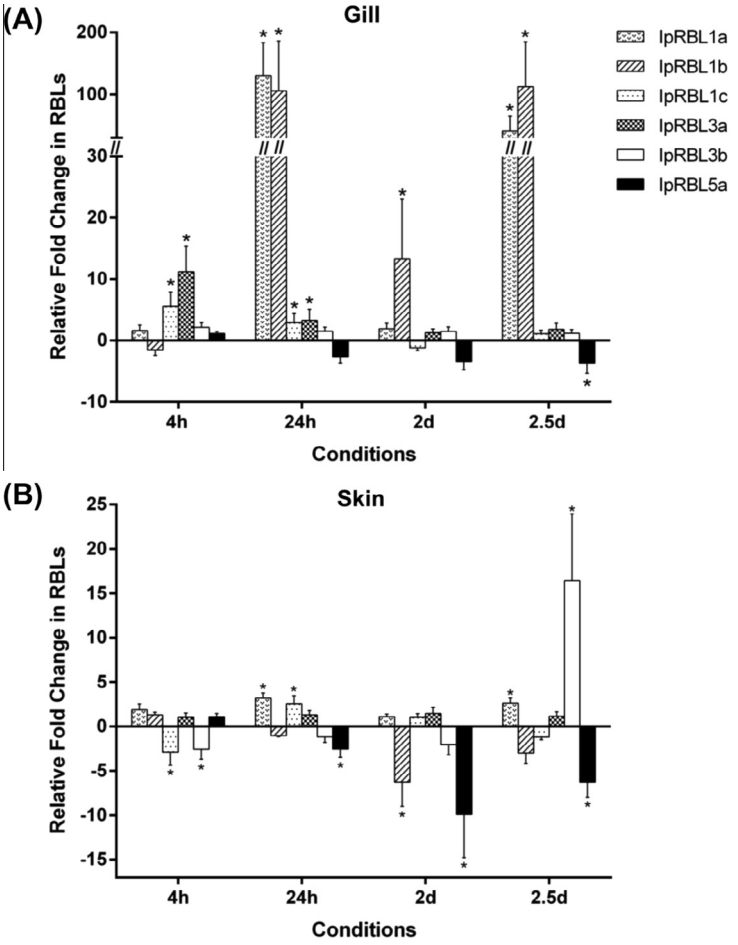

Fig. 7. Real time qRT-PCR analyses for IpRBL expressions following *Flavobacterium columnare* infection. The IpRBL expression was measured in the mucosal tissues including (A) Gill and (B) Skin at the timepoints of 4 h, 24 h, 2 d, and 2.5 d post-infection. Fold change was calculated by the change in expression at a given timepoint relative to the untreated control and normalized by changes in the 18S housekeeping gene. The results are presented as mean ± SE of fold changes and the asterisk indicates statistical significance at $P < 0.05$.

which differ significantly with more distant groups (Fig. 3 and Table 3). For example, the pattern of high identity among CRDs of shared CRD group and domain position, breaks down when IpRBL CRDs are compared with the STL CRDs of rainbow trout (*O. mykiss*) (Table 3). This pattern is immediately evident when examining the full-length sequence phylogenetic tree (Fig. 3). RBLs from species with more than one RBL are usually in clades with RBLs from closely related species. Often times the topology connecting these clades to those of other species groups is only weakly supported (e.g. catfish and zebrafish, *D. rerio*). As seen in the catfish and salmonid clades, RBLs can be well conserved between closely related species. This pattern suggests that much of RBL diversity emerged following the divergence of ray-finned fishes, rather than each species evolving a species-specific repertoire from a shared single ancestral gene. However, exceptions abound, potentially due to the incomplete nature of the RBL repertoire from any given species. Clearly, more comparative genomic analyses and functional studies of shared RBL in multiple species are needed to better elucidate the history of the structural and functional diversification of fish RBLs.

To begin to explore the potential for catfish RBL function in mucosal immune responses, we conducted two bacterial challenges, *F. columnare* and *E. ictaluri*, and examined expression in key tissues for bacterial entry. Skin and gill are the primary routes of *F. columnare* infection (Arias et al., 2012). While many studies have focused on *E. ictaluri* entry through the intestine, other recent studies have also showed entry through gill and skin (Li et al., 2012; Menanteau-Ledouble et al., 2011; Shoemaker et al., 2012; Skirpstunas and Baldwin, 2002). We observed substantial differences in the RBL expression profiles between the two Gram-negative pathogens. One commonality between both pathogens was the strong response of the closely-related IpRBL1a and IpRBL1b in the gill of catfish (Figs. 6A, and 7A). We had previously reported on the expression of IpRBL1a (Beck et al., 2012), but found here the presence of a likely gene duplicate, IpRBL1b, whose expression was often highly induced along with IpRBL1a in gill. Expression changes in these two IpRBLs were of smaller magnitude and often differed in the direction of expression change in other tissues (e.g. 7d ESC skin; Fig. 6C). IpRBL1a and IpRBL1b induction in gill, in fact, was the only example of the catfish RBLs being consistently up-regulated across several timepoints following challenge.

By contrast, IpRBL1c was down-regulated at all ESC post-challenge timepoints in both intestine and skin. More broadly, both tissues showed a pattern of down-regulation across multiple RBLs following ESC challenge (Fig. 6B and C). Only IpRBL1b was significantly up-regulated relative to control greater than 5-fold at any timepoint in intestine and skin. The IpRBLs appear to play distinct roles in gill mucosal responses to ESC infection, while skin and intestine have similar patterns of suppressed IpRBL expression. The similarity between IpRBL responses in intestine and skin following ESC challenge are striking in the light of recent studies describing the remarkable morphological and functional parallels

between fish gut and skin (Landeira-Dabarca et al., 2013; Liu et al., 2013; Xu et al., 2013). Further studies are needed to determine whether these RBL responses are protective or, as is the case with IpRBL1a and *F. columnare* infection (Beck et al., 2012), they are the result of pathogen manipulation of expression and aid in pathogen adhesion.

Focusing on IpRBL expression in *F. columnare*-challenged gill and skin, again expression profiles differed dramatically between tissues. The strong induction of IpRBL1a and IpRBL1b seen in gill was absent in skin, and a variety of RBLs there including IpRBL1b, IpRBL3a, and IpRBL5a were down-regulated. Only IpRBL3b was strongly induced in skin. Again, our results suggest that mucosal tissues, although shared targets of pathogen adhesion, can have substantially different RBL responses to infection. IpRBL1a and IpRBL1b, highly induced in gill following two distinct bacterial challenges, deserve future cell-level localization of expression and characterization of function.

The functional roles identified for the diverse fish and invertebrate RBL repertoire are quite broad. They include the regulation of carbohydrate metabolism, control of fertilization, cytotoxicity, and immunity (Ballarin et al., 2013). RBLs from chinook salmon (*O. tshawytscha*) eggs play an important role in prevention of polyspermy during fertilization (Murata et al., 2007; Yasumasu, 2000). RBLs from sea urchin (*A. crassispina*) eggs act as extraembryonic support by crosslinking hyaline components to embryonic cells so that these extraembryonic matrixes may alter the interaction between cells and substrates and play a role in development and morphogenesis (Ozeki et al., 1995). RBLs have been suggested to have protective functions in the cortex of teleost eggs as well as in the skin mucus. RBL from amur catfish (*S. asotus*) and chum salmon (*O. keta*) are reported to have anti-proliferative effects on a variety of cancer cells (Nitta et al., 2007). We described here six new RBLs from channel catfish, the largest number described from a species to-date, and examined their responses to bacterial challenge in mucosal tissues. Further studies are needed, however, to expand functional characterization and examine whether IpRBLs may also play additional physiological roles in areas such as egg protection and carbohydrate metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2014.01.018.

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