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The catfish liver-expressed antimicrobial peptide 2 (LEAP-2) gene is expressed in a wide range of tissues and developmentally regulated

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Abstract

Antimicrobial peptides (AMPs) are important components of the host's innate immune response against microbial invasion. The cysteine-rich AMPs such as defensin and hepcidin have been extensively studied, but the recently identified cysteine-rich liver-expressed antimicrobial peptide 2 (LEAP-2) has been characterized from only a few organisms. Here we cloned and sequenced the LEAP-2 cDNAs from both Channel catfish and Blue catfish. The LEAP-2 gene from Channel catfish was also sequenced and characterized. Channel catfish LEAP-2 gene consists of two introns and three exons that encode a peptide of 94 amino acids with a 28 amino acid signal peptide and a mature peptide of 41 amino acids. The amino acid sequences and gene organization were conserved between catfish and other organisms. The Channel catfish LEAP-2 gene is expressed in a wide range of tissues except brain and stomach. Its expression is developmentally regulated with no detection of mature mRNA in early stages of development. It appears that the catfish LEAP-2 gene is regulated at the level of splicing; it is constitutively transcribed, but remains unspliced until 6 days after hatching. The expression of LEAP-2 was induced in a tissue-specific manner. Its expression was upregulated in the spleen, but not in the liver and head kidney, after challenge with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC).

Keywords: LEAP-2; Antimicrobial peptide; Fish; Catfish; Disease; Infection; Innate immunity; Gene; cDNA

1. Introduction

Antimicrobial peptides (AMPs) are essential components of the host's innate defense system in a wide range of organisms from plants to mammals. They are believed to act as antimicrobial agents by disrupting the membrane integrity of invading microbes (Kagan et al., 1990; Satchell et al., 2003), but the specific mechanisms involved are not yet completely understood (Hancock and Scott, 2000; Chinchar et al., 2004). With the emergence of antibiotic-resistant bacteria, and the scarcity of new classes of useful antibiotics, there is an increasing need to identify novel antimicrobial peptides from various species for the development of alternative therapeutants (for review, see Patrzykat and Douglas, 2003). Several characteristics of AMPs make them particularly appealing as potential therapeutants. First, they display a broad spectrum of activity against a variety of bacteria, viruses, fungi, and protozoa; second, they kill rapidly (99.9% within 20 min, Piers et al., 1994) and exhibit synergy with conventional antibiotics (Hancock and Diamond, 2000; Yan and Hancock, 2001); third, they are effective

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against antibiotic-resistant strains of bacteria; fourth, they do not tend to select for resistant mutants; and finally, unlike many antibiotics that can indirectly cause sepsis because of the release of endotoxin from dead bacterial cells, cationic antimicrobial peptides bind endotoxin and reduce septic shock (Patrzykat and Douglas, 2003). Insect-derived cecropin B was shown to be effective against fish pathogenic viruses (Chiou et al., 2002). Over 800 AMP sequences have been deposited in the Antimicrobial Peptide Database (http://bbcm1.univ.trieste.it/~tossi/pag1.htm). However, sequences have been reported from only a minimal number of fish species (Douglas et al., 2003a, 2003b; Table 1), the largest vertebrate group containing over 23,000 species (Helfman et al., 1997).

Most AMPs are small in size and positively charged. They can be broadly classified into three categories: alpha helical, beta-sheets, and extended structures with a predominance of a single amino acid, often tryptophan, proline, or histidine (Zasloff, 2002). Two cysteine-rich AMPs recently identified from vertebrate species, liver-expressed antimicrobial peptide-1 (LEAP-1, or hepcidin) and LEAP-2 are of particular interest because they are considered counterparts of defensins, AMPs identified from invertebrates that have been extensively studied.

Table 1
A summary of previously reported fish antimicrobial peptides (AMPs)

Species	AMP	References
Rainbow trout	Oncorhyncin II	Fernandes et al., 2004
	Oncorhyncin III	Fernandes et al., 2003
	LEAP-2	Zhang et al., 2004
Atlantic salmon	Hepcidin	Douglas et al., 2003a
	Histone H1	Richards et al., 2001
Hybrid striped bass	Moronecidin	Lauth et al., 2002
	Hepcidin	Shike et al., 2002
	Piscidins	Noga and Silphaduang, 2003
Winter flounder	Pleurocidin	Cole et al., 1997; Douglas et al., 2001; Douglas et al., 2003b
	Hepcidin	Douglas et al., 2003a
Channel catfish	Innate antimicrobial factor	Ourth and Chung, 2004
	Histone-like protein	Robinette et al., 1998
Loach	Misgurnin	Park et al., 1997
	MAPP	Dong et al., 2002
Zebrafish	Hepcidin	Shike et al., 2004
Carp	Apoliproteins A-I and AII	Concha et al., 2004
Atlantic halibut	Hipposin	Birkemo et al., 2003
Hagfish	HFIAP-1, -2, and -3	Uzzell et al., 2003
Catfish (*Parasilurus asotus*)	Parasin	Park et al., 1998
Rockfish	Unnamed protein	Nagashima et al., 2003
Soapfish	Grammistins	Shiomi et al., 2000
Red sea bream	Chrysophsin	Iijima et al., 2003
Shark	Squalamine	Moore et al., 1993
Red Sea Moses sole	Pardaxin	Oren and Shai, 1996

LEAP-2 is the second blood-derived peptide from humans that is predominantly expressed in the liver and exhibits antimicrobial activity (Krause et al., 2003). It was initially identified by using mass spectrometry-based techniques in searching for cysteine-rich peptides, as these often display important biological activities (Sillard et al., 1993). Using conserved PCR primers, Krause et al., (2003) was able to amplify LEAP-2 cDNAs from pig, guinea pig, rhesus monkey, and bovine, indicating that the LEAP-2 genes were highly conserved among mammals. Recently, LEAP-2 cDNAs have also been identified from chicken (Lynn et al., 2003) and Rainbow trout (Zhang et al., 2004) using bioinformatics analysis of the Expressed Sequence Tag (EST) databases. While a large number of *LEAP-1* (hepcidin) genes have been identified from fish including White bass, Medaka, Rainbow trout, Japanese flounder, Winter flounder, Long-jawed mudsucker, Atlantic salmon, Zebrafish and Channel catfish (Inoue et al., 1997; Shike et al., 2002; Pigeon et al., 2001; Douglas et al., 2003a; Shike et al., 2004; Bao et al., 2005), LEAP-2 has been reported only from rainbow trout (Zhang et al., 2004). In this work, we cloned and sequenced LEAP-2 cDNAs from both Channel catfish and Blue catfish, characterized the gene encoding LEAP-2 of Channel catfish including its upstream and downstream sequences, assigned it to bacterial artificial chromosome (BAC) clones for comparative genome analysis, analyzed its genomic organization, and investigated gene expression in various tissues during development and after bacterial infection.

2. Materials and methods

2.1. Fish rearing and bacterial challenge

Channel catfish larvae were reared at the hatchery of the Auburn University Fish Genetics Research Unit. Challenge experiments were conducted as previously described (Dunham et al., 1993) with modifications (Baoprasertkul et al., 2004). Briefly, the catfish were challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared culture of ESC bacteria, *E. ictaluri*. One single colony of *E. ictaluri* was isolated from a natural outbreak in Alabama (outbreak number ALG-02-414) and inoculated into brain heart infusion (BHI) medium and incubated in a shaker incubator at 28 °C overnight. The bacterial concentration was determined using colony forming unit (CFU) per ml by plating 10 μl of 10-fold serial dilutions onto BHI agar plates. At the time of challenge, the bacterial culture was added to the tank to a concentration of 3×10^7 CFU/ml. During challenge, an oxygen tank was used to ensure a dissolved oxygen concentration above 5 mg/ml. After 2 h of immersion exposure, 15 fishes were randomly taken and placed into a rectangular trough containing pond water with constant water flow through. Replicates of troughs were used to provide one

trough for each sampling time point. For the control fish, 15 fishes were incubated in a separate rectangular tank with the same fish density as the challenge tanks. The only difference was that ESC bacteria were not added. After 2 h, these control fish were incubated in a separate trough at the same density as the challenged fish.

2.2. Tissue sampling and RNA extraction

Eleven tissues were collected from healthy Channel catfish including brain, gill, head kidney, intestine, liver, muscle, ovary, skin, spleen, stomach and trunk kidney. Three tissues, liver, head kidney and spleen were collected from challenged fish. Samples were collected from 10 fishes at each time point including control (before challenge), and 4 h, 24 h, 3 days, and 7 days after challenge. Samples of each tissue from 10 fishes were pooled. The experimental fishes were euthanized with tricaine methanesulfonate (MS 222) at 100 mg per liter before tissues were collected. Tissues were quick frozen in liquid nitrogen and kept in a −80 °C ultra-low freezer until preparation of RNA. In order to obtain samples representing the average of the 10 fishes, the pooled tissue samples were ground with a mortar/pestle to fine powders and thoroughly mixed. A fraction of the mixed tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method (Chomczynski and Sacchi, 1987) using the Trizol reagents kit from Invitrogen (Carlsbad, CA) following manufacturer's instructions. Extracted RNA was stored in a −70 °C freezer until used as template for reverse transcriptase PCR (RT-PCR).

2.3. RT-PCR

RT-PCR reactions were conducted using SuperScript™ III One Step RT-PCR System (Invitrogen). The system contained a mixture of SuperScript™ III reverse transcriptase and the Platinum *Taq* DNA polymerase in an optimized buffer. Detailed procedures followed the instructions of the manufacturer. Briefly, the following was added to a reaction of 50 μl:25 μl 2× reaction mix, 1 μl total RNA (∼200 ng), 2 μl (100 ng) each of the upper and lower primer (Table 2), 2 μl SuperScript III RT/Platinum *Taq* polymerase mix, and water to bring the reaction volume to 50 μl. The reaction also included the primers of β-actin (Table 2), serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: 45 °C for 15 min for one cycle (reverse transcription reaction), the samples were pre-denatured at 94 °C for 2 min, then the samples were amplified for 40 cycles at 94 °C for 15 s, 53 °C for 30 s, 72 °C for 30 s. Upon the completion of PCR, the reaction was incubated at 72 °C for an additional 5 min. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel and documented with a Gel Documentation System. The RT-PCR products were quantified by using GelExpert software package, Version 3.5 (Nucleotech Corp., San Mateo, CA).

2.4. Assigning LEAP-2 gene(s) to BAC clones

A cDNA clone for Blue catfish LEAP-2 was obtained during analysis of expressed sequence tags (EST) (He et al., 2004; Karsi et al., 2002; Kocabas et al., 2002). The Blue catfish cDNA sequences were used to design overgo probes (Peatman et al., 2004) for hybridization to BAC filters. High-density filters of Channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA). Each set of filters contained 10-genome coverage of the Channel catfish BAC clones from BAC library CHORI 212 (http://bacpac.chori.org/library.php?id=103). Sequences of the overgo primers are shown in Table 2 and their positions within the LEAP-2 cDNA is shown in Fig. 1. Overgo hybridization method was adapted from a web protocol (http://www.tree.caltech.edu/). Briefly, overgo primers were selected following a BLAST search against GenBank to screen out repeated sequences and then purchased from Sigma Genosys (Woodlands, TX). Two hundred nanograms of overgo primers each were labelled with 40 μl of a freshly prepared master mix composed of 14.0 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.02 mM dGTP, 0.02 mM dTTP, 20 μCi [α-^{33}P]dCTP, 20 μCi [α-^{33}P]dATP (3000 Ci/mmol, Amersham, Piscataway, NJ), and 5 units of Klenow enzyme (Invitrogen). Labelling reactions were carried out at room temperature for 2 h. After removal of unincorporated nucleotides using a Sephadex G50 spin column, probes were denatured at 95 °C for 10 min and added to the hybridization tubes containing high-density BAC filters. Hybridization was performed at 54 °C for 18 h in hybridization solution (50 ml of 1% BSA, 1 mM EDTA at pH 8.0, 7% SDS, 0.5 mM sodium phosphate, pH 7.2). BAC filters were washed with 2× SSC at room temperature for 15 min, and exposed to X-ray film at −80 °C for 2 days. Positive clones were identified according to the clone

Table 2
Primers and their sequences used in this study

Primers	Sequences (5′–3′)	Utilization
AU75080	GTAATGCCTGGATGCTGCACAAGTAG	Sequencing BAC 138M8
Overgo 1	AGGAGATCAGAGGTCACTCAAGAG	Overgo probes for hybridization to BAC filters and sequencing BAC 138M8
Overgo 2	TGTCATACGGGCCATTCTCTTGAG	Overgo probes for hybridization to BAC filters and RT-PCR, and sequencing BAC 138M8
AU75053	GTTGCAGCCTGGTGCTTGGTAC	RT-PCR and Sequencing BAC 138M8
Actin upper	AGAGAGAAATTGTCCGTGACATC	RT-PCR internal control
Actin lower	CTCCGATCCAGACAGAGTATTTG	RT-PCR internal control

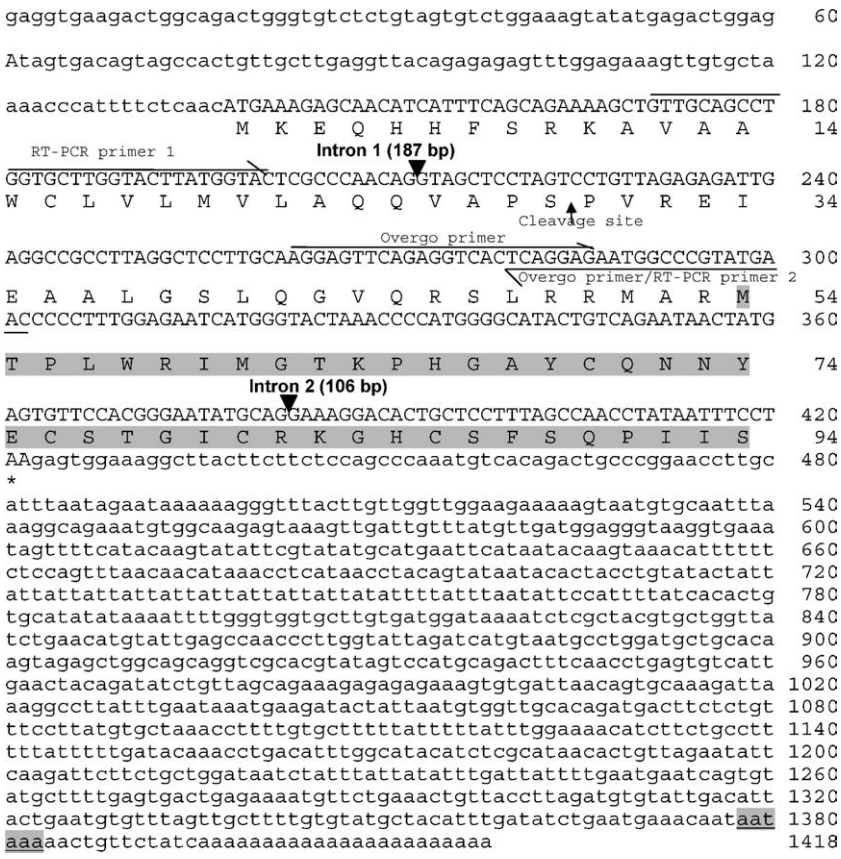

Fig. 1. cDNA sequence of Channel catfish LEAP-2 gene. The translation start codon ATG and termination codon TAA are both underlined. The polyadenylation signal sequences AATAAA is underlined and in bold font. The primer positions and orientations are indicated by semi-arrowed lines. Intron positions are indicated by solid triangles.

distribution instructions from CHORI, and picked out from Channel catfish BAC library.

2.5. Preparation of BAC DNA and Southern blot analysis

After assigning of LEAP-2 to BACs and being confirmed by hybridization of BAC filters using entire cDNA probes, the LEAP-2 positive BAC DNA was isolated with the Perfectprep® BAC 96 BAC DNA isolation kit (Brinkmann Instruments Inc., Westbury, NY). The isolated BAC DNA was subjected to Southern blot analysis to determine genomic copy numbers. The rationale was that if the clones represented a single copy, the same hybridization pattern would be expected. Alternatively, if the clones were from different genomic copies (more than one gene), then variation in the hybridization pattern would be expected. Briefly, BAC DNA was first digested with restriction endonuclease *Eco*RI and *Pst*I separately and electrophoresed on 0.8% agarose gel. The DNA was transferred to a piece of Immobilon nylon membrane (Millipore, Bedford, MA) by capillary transfer with 0.4 M NaOH overnight (Reed and Mann, 1985). DNA was fixed to the membrane by UV cross linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) with the auto crosslink function. The membrane was washed in 0.5% SDS (w/v) at 65 °C for 15 min and then pre-hybridized in 50% formamide, 5× SSC, 0.1% SDS (w/v), 5× Denhardt's and 100 μg/ml sonicated and denatured Atlantic salmon sperm DNA (100 μg/ml) overnight. Hybridization was conducted overnight at 42 °C in the same solution with probes added. The LEAP-2 cDNA was used as the probe. The probe was prepared using the random primer labelling method (Sambrook et al., 1989) with a labelling kit from Roche Diagnostics (Indianapolis, IN). The nylon membranes were washed first in 500 ml of 2× SSC for 10 min, followed by three washes in 0.2× SSC with SDS at 0.2% (w/v) at 65 °C for 15 min each. The membranes were then wrapped in Saran wrap and exposed to Kodak BioMax MS film for autoradiography.

2.6. DNA and sequencing and sequence analysis

One BAC was sequenced using gene-specific primers and primer-walking methods (Kim et al., 2000). Fourteen picomoles of each primer were used for sequencing reactions. BAC sequencing was performed in a 10 μl reaction using the BigDye Terminator v3.0 Ready Reaction kit (Applied Biosystems, Foster City, CA) following manufacturer's instructions.

The plasmid clone containing the Blue catfish LEAP-2 was obtained through EST analysis. After sequence anal-

ysis of the Channel catfish LEAP-2 genomic DNA, a pair of primers was designed, based on the start and the end of the Blue catfish cDNA, for the amplification of complete Channel catfish LEAP-2 cDNA. Plasmid DNA containing the LEAP-2 cDNA was prepared using Qiagen Spin Column Mini-plasmid kits (Qiagen, Valencia, CA). Three microliters of plasmid DNA (about 0.5–1.0 μg) was used in sequencing reactions. Chain termination sequencing was performed using Thermosequenase (Amersham, Piscataway, NJ). The PCR profiles were: 95 °C for 30 s, 55 °C for 40 s and 72 °C for 45 s for 30 cycles. An initial 2 min denaturation at 96 °C and a 5 min extension at 72 °C were used. Sequences were analyzed on an automatic LI-COR DNA Sequencer Long ReadIR 4200 or ABI Prism 3100 sequencer.

BLAST searches (Zhang and Madden, 1997) were conducted to determine gene identities. DNASTAR software package was used for sequence analysis. MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTAL W (Serapion et al., 2004). For the analysis of potential cleavage site(s) of the signal peptide, SignalP (http://www.cbs.dtu.dk/services/SignalP/) software was used (Bendtsen et al., 2004). Transcriptional factor binding sites were determined by searching the upstream sequences against the TRANSFAC database using TFSEARCH software (http://www.cbrc.jp/research/db/TFSEARCH.html) (Heinemeyer et al., 1998). The "vertebrate" database was used, and the cut off value was set at 90. After obtaining the results, the putative transcriptional factor binding sites were tabulated using the transcription start site as +1, and all upstream sequences were given a negative number.

2.7. Phylogenetic analysis

The relevant sequences were retrieved from GenBank for multiple sequence alignments using Clustal W (Thompson et al., 1994). Percentage of amino acid identities were recorded after all multiple alignments. Phylogenetic trees were drawn by the neighbor-joining method (Saitou and Nei, 1987) with amino acid sequence p-distances, using the PAUP program (Swofford, 2002) using the heuristic algorithm. The topological stability of the neighbor-joining trees was evaluated by 1000 bootstrapping replications. The human hepcidin (LEAP-1) sequences were used to root the phylogenetic tree.

3. Results

3.1. Channel catfish and Blue catfish LEAP-2 cDNA sequences

Both the Channel catfish and Blue catfish LEAP-2 cDNAs were sequenced and their sequences have been deposited to GenBank with accession numbers AY845141 and AY845142, respectively. The Channel catfish LEAP-2 cDNA contained a 137 bp 5′-untranslated region (UTR), an open reading frame of 285 bp encoding a protein of 94 amino acids, and a 973 bp 3′-UTR (Fig. 1). A typical AATAAA polyadenylation signal exists 12 bp upstream of poly $(A)^+$ tails. The 94 amino acid peptide contained a signal peptide, a prodomain region and the mature peptide. Analysis using SignalP indicated that the cleavage site of signal peptide was located between amino acid 29 and 30 using both the neural networks model and the hidden Markov model (Bendtsen et al., 2004; Fig. 1). The mature peptide was predicted to be 41 amino acids in length.

The Blue catfish LEAP-2 cDNA shared similar features with the Channel catfish LEAP-2 cDNA (Table 3). It also encodes a peptide of 94 amino acids that differ only in two amino acids from the deduced amino acid sequences of the Channel catfish LEAP-2. At amino acid position 28, the Channel catfish LEAP-2 has a proline residue while the Blue catfish LEAP-2 has a serine residue; at position 32 the Channel catfish LEAP-2 has an arginine residue while the Blue catfish LEAP-2 has a proline residue. Variation of a microsatellite sequence $(TTA)_n$ within the 3′-UTR made the sequenced Blue catfish cDNA shorter by 12 bp. The two LEAP-2 cDNAs had 25 single nucleotide polymorphisms (1.79%) (Table 3), slightly greater than the average SNP rate of 1.32% within cDNAs of the two species as surveyed by EST analysis (He et al., 2003).

The Channel catfish and Blue catfish LEAP-2 share 98% amino acid identities. The Channel catfish LEAP-2 had identities ranging from the lowest with chicken (26.3%) to the

Table 3
Similarities between the Channel catfish LEAP-2 cDNA and Blue catfish LEAP-2 cDNA

	Channel catfish LEAP-2	Blue catfish LEAP-2
Accession number		
cDNA length	1395 bp	1395 bp
5′-UTR	137 bp	137 bp
Open reading frame	285 bp	285 bp
3′-UTR	973 (contained microsatellites $[TTA]_{10}$)	961 (contained microsatellites $[TTA]_6$)
Poly A signals	12 bp upstream of poly A tails	12 bp upstream of poly A tails
SNP sites	37t/a, 63a/c, 70g/a, 97g/t, 104t/c, 136a/g, 219c/t, 231a/c, 232g/c, 438c/t, 578t/g, 593a/g, 709c/a, 721a/g, 724a/g, 759a/t, 765c/t, 782g/a, 832t/a, 849t/c, 876t/c, 1057t/c, 1070g/t, 1077c/a, 1362a/g	

The numbers in the category of SNP sites refers to the positions of SNP sites using the first base of the channel cDNA as 1 with Channel catfish base in front of the slash (e.g., 58g/a indicates that a SNP site at position 58 with the Channel catfish cDNA having a "G" and the Blue catfish having an "A".

highest with zebrafish (partial sequence, CD284721, 67.4%) and a carp sequence in the EST database (CF662655), with 65.2% amino acid identity (Table 4). Phylogenetic analysis properly placed the catfish genes into the clade of LEAP-2 (Fig. 2). The catfish LEAP-2 was much more similar to LEAP-2A from rainbow trout than to LEAP-2B, indicating that the gene duplication for the rainbow trout LEAP-2A and -2B genes were quite ancient. All fish LEAP-2 genes were clustered in the same clade while the mammalian genes were clustered into another, indicating that gene duplication of LEAP-2 in the teleost species occurred after the divergence of teleosts from other species.

3.2. Assigning LEAP-2 to BACs and copy number of Channel catfish LEAP-2 gene

Comparative genome analysis demands information about gene locations on genetic linkage maps and physical maps. In catfish, gene marker-based linkage mapping (Waldbieser et al., 2001; Liu et al., 2003) and BAC-based physical mapping (Liu, unpublished) is underway. Assigning genes to both linkage maps and physical maps should allow map alignment for the development of integrated gene maps. We, therefore, used overgo probes designed based on the cDNA sequences of LEAP-2 (Table 2) to assign LEAP-2 genes

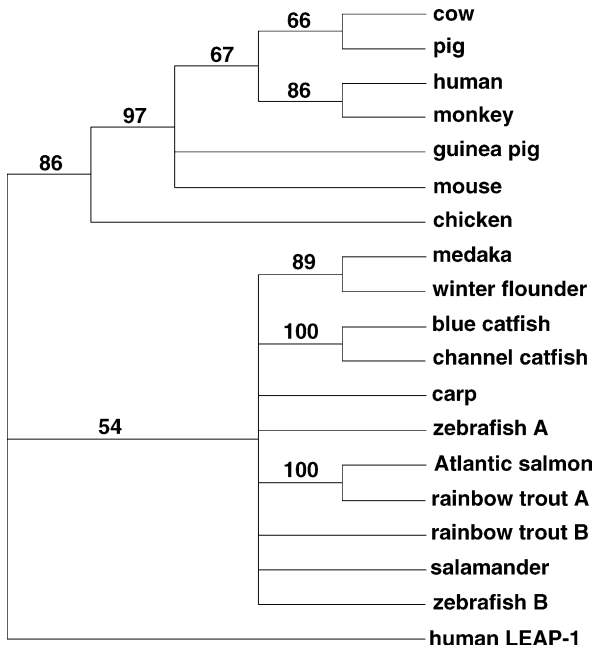

Fig. 2. Phylogenetic analysis of LEAP-2. Phylogenetic trees were drawn by the neighbor-joining method as detailed in Section 2. All sequences except the catfish LEAP-2 sequences were retrieved from GenBank with accession numbers as the following: bovine (CF368628), pig (CN155555), human (CF368628), monkey (CF368628), guinea pig (AJ409054), mouse (NM_153069), chicken (BU397418), medaka (AU179996), winter flounder (AW013446), Blue catfish (AY845142), Channel catfish (AY845141),carp (CF662655), zebrafish A (CD284721), Atlantic salmon (BM414176), rainbow trout A (BX889970), rainbow trout B (BX879371), salamander (CN057710), zebrafish B (AL918619), human LEAP-1 (CO580258).

Table 4
Pair wise similarities of 19 LEAP-2 genes

Channel catfish	Blue catfish	Atlantic salmon	Carp	Chicken	Cow	Guinea pig	Human	Medaka	Monkey	Mouse	Pig	Rainbow trout A	Rainbow trout B	Sala-mander	Winter flounder	Zebrafish A	Zebrafish B	
***	97.9	51.1	65.2	26.3	33.8	38.0	39.0	43.6	39.0	36.8	35.1	54.3	47.3	38.3	46.8	67.4	39.6	Channel catfish
	***	52.1	66.3	32.9	35.1	39.4	40.3	43.6	40.3	39.5	36.4	55.3	47.3	39.4	45.7	67.4	44.0	Blue catfish
		***	54.3	30.3	32.5	39.4	33.8	36.5	33.8	38.2	31.2	94.8	45.1	30.2	33.3	63.0	39.6	Atlantic salmon
			***	35.5	36.4	39.4	35.1	45.7	35.1	42.1	35.1	56.5	48.4	37.0	44.6	73.9	38.5	Carp
				***	57.9	57.7	57.9	39.5	57.9	57.9	52.6	30.3	34.2	31.6	30.3	34.8	28.9	Chicken
					***	83.1	90.9	35.1	90.9	84.2	89.6	33.8	39.0	28.6	31.2	32.6	33.8	Cow
						***	84.5	32.4	84.5	85.9	78.9	38.0	39.4	28.2	29.6	30.4	36.6	Guinea pig
							***	36.4	98.7	85.5	85.7	35.1	37.7	28.6	31.2	32.6	35.1	Human
								17.9	23.4	21.1	20.8	15.5	16.7	8.3	13.1	6.5	10.7	Human LEAP-1
								***	36.4	35.5	32.5	38.5	40.7	31.7	54.9	50.0	28.6	Medaka
									***	85.5	85.7	35.1	37.7	28.6	31.2	32.6	31.2	Monkey
										***	81.6	38.2	39.5	30.3	27.6	32.6	28.9	Mouse
											***	32.5	37.7	37.7	29.9	30.4	23.4	Pig
												***	45.1	31.3	35.4	65.2	38.5	Rainbow trout A
													***	33.0	38.5	52.2	44.0	Rainbow trout B
														***	22.5	47.8	33.0	Salamander
															***	50.0	31.9	Winter flounder
																***	50.0	Zebrafish A
																	***	Zebrafish B

Amino acid sequences were used for alignments using Clustal W. The sequences and their accession numbers were the same as specified in Fig. 2.

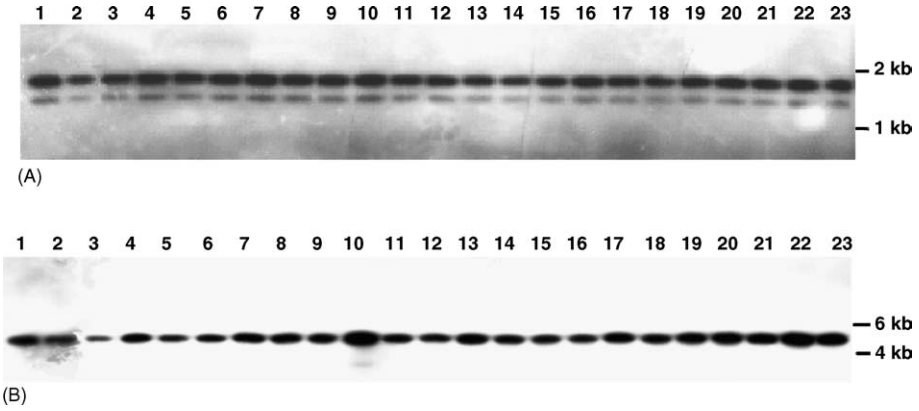

Fig. 3. Southern blot analysis using BAC clones. BAC DNAs were digested with *Eco*R1 (A) and *Pst*I (B), electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to hepcidin cDNA probes. Lanes 1–23 contained BAC DNA from 23 different BAC clones. Molecular weight is indicated on the right margin.

on BACs. Hybridization of LEAP-2 overgo probes resulted in identification of 23 positive BAC clones: 7H23, 9M6, 23M1, 27D13, 33J3, 35K16, 63J8, 63L8, 66H8, 76G8, 80C7, 87D12, 101F23, 113F12, 113G4, 134A9, 138M8, 154A10, 160O20, 171K12, 172B18, 178J23, and 178O21.

Although the number of LEAP-2 positive clones suggested the possibility of more than one copy of the LEAP-2 in the Channel catfish genome, analysis by BAC-based Southern blot hybridization revealed the presence of only a single copy of the LEAP-2 gene in the catfish genome. BAC DNA from the positive clones were isolated and subjected to Southern blot analysis after digest with *Eco*R1 and *Pst*I. As shown in Fig. 3, both restriction enzymes produced identical restriction patterns with all 23 BAC clones. In consideration that the number of LEAP-2 positive clones is significantly larger than that expected from screening of filters with 10× genome coverage, and that zebrafish has two copies of LEAP-2 gene, we also conducted restriction fingerprinting using four sets of 6 bp cutters along with a 4 bp cutter (Luo et al., 2003). All the clones fall within a single contig (data not shown), confirming the presence of only a single copy of LEAP-2 in the catfish genome.

3.3. Channel catfish LEAP-2 gene

The Channel catfish LEAP-2 gene was sequenced from a BAC 138M8. The segment spanning the entire LEAP-2 gene was sequenced. The LEAP-2 gene sequences of 2712 bp were deposited to GenBank with the accession number of AY845143.

The Channel catfish LEAP-2 gene has three exons and two introns, with a very similar genomic organization as LEAP-2 genes reported from human (Krause et al., 2003), chicken (Lynn et al., 2003), and Zebrafish (Zhang et al., 2004).

A search of transcriptional regulatory sequences within the 637 bp upstream sequences against the TRANSFAC database indicated the presence of various regulatory elements within the upstream sequences of the Channel catfish LEAP-2 gene (Table 5). Particularly of interest is the presence of five CdxA binding sites that appear to be similar to those found in LEAP-1 gene of Channel catfish (Bao et al., in press), suggesting roles of LEAP-2 during development.

Table 5
Putative transcription factor binding sites within the upstream sequences of the Channel catfish LEAP-2 gene

Transcription factor	Positions
AML-1α	−545
CdxA	−616, −512, −493, −409, −180
C-Rel	−584
Delta E	−353
Evi-1	−253
GATA-1	−29
GATA-3	−28
Max	−67
Pbx-1	−429
SRY	−426
S8	−416
USF	−64, −67

3.4. Channel catfish LEAP-2 gene expression

Analysis of gene expression using RT-PCR indicated that the Channel catfish LEAP-2 gene was expressed in a wide range of tissues (Fig. 4). Its RNA was detected in all tested tissues, but brain and stomach, including liver, gill, head kidney, intestine, muscle, ovary, skin, spleen, and trunk kidney. In the brain and stomach, a band with the size of genomic segment

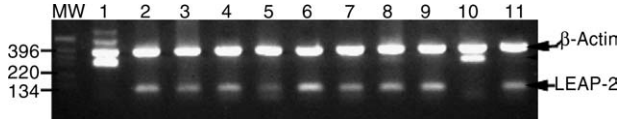

Fig. 4. RT-PCR analysis of LEAP-2 gene expression in various tissues. Total RNA isolated from various tissues was used to carry out RT-PCR reactions, as detailed in Section 2. MW, 1 kb molecular weight standard; lanes 1–11 were RT-PCR products from various tissues with (1) brain; (2) gill; (3) head kidney; (4) intestine; (5) liver; (6) muscle; (7) ovary; (8) skin; (9) spleen; (10) stomach; and (11) trunk kidney.

or unspliced transcript was prominent. Although the nature of this band needs to be further studied to determine if it was derived from amplification of DNA template or unspliced RNA template, it is clear that no mature RNA was produced from the brain and stomach. Surprisingly, it appeared that the so-called liver-expressed antimicrobial peptide-2 was expressed in lowest amount in the liver while it is expressed in nearly equal amounts in all other tested tissues (Fig. 4).

The Channel catfish LEAP-2 gene is regulated during the development. The mature mRNA of the Channel catfish LEAP-2 was not detected until 6 days after hatching (Fig. 5). After that, it was expressed at almost constant levels at 10, 13, and 17 days after hatching. The mature LEAP-2 mRNA was not detected at 48 h after fertilization, at the time of hatching (approximately 4 days after fertilization), and 3 days after hatching. However, an unexpected band was detected at early stages of development until 6 days after hatching when both the mature mRNA and the unexpected band were detected (Fig. 5). This band was of the same size as the unspliced transcript or genomic DNA (approximately 330 bp using the RT-PCR primers) as observed from the brain and the stomach tissues (see above). Using only Taq polymerase without reverse transcriptase did not produce this band while the intron-specific probes specifically hybridize to this band (Fig. 6A and B), indicating that this band represented PCR products from unspliced transcripts, rather than from contaminated DNA templates. Therefore, it appeared that the LEAP-2 gene was transcribed at all time points during early development, but the transcripts were not spliced until 6 days after hatching.

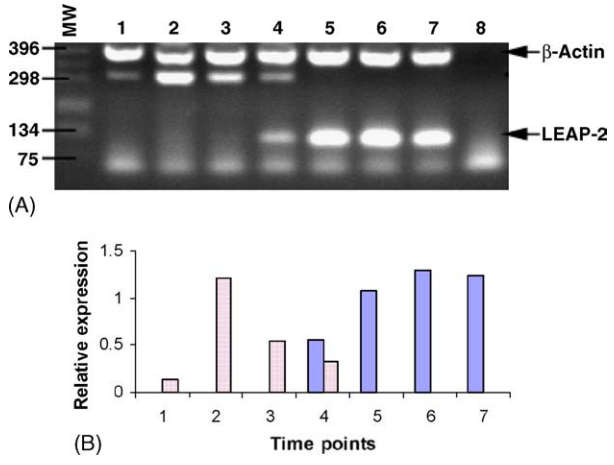

Fig. 5. RT-PCR analysis of LEAP-2 transcripts during early development of Channel catfish. A. RT-PCR reaction was conducted as detailed in Section 2. The products of RT-PCR were separated on an agarose gel by electrophoresis. MW, molecular weight standards of 1 kb ladder with sizes of interest indicated on the left margin; (1) embryos at 48 h after fertilization; (2) embryos at the time of hatching (5 days after fertilization); (3) three days after hatching; 4, 6 days after hatching; 5, 10 days after hatching; 6, 13 days after hatching; and 7, 17 days after hatching; and 8, no RNA control. Positions for the expected size of LEAP-2 transcript and β-actin are indicated by the arrows on the right margin. B. Quantitation of LEAP-2 transcripts using GelExpert software. Levels of expression were expressed as the ratio of LEAP-2 and β-actin. Solid bar, LEAP-2 transcripts; sketched bars, an unexpected RT-PCR products with approximately the same size of unspliced LEAP-2.

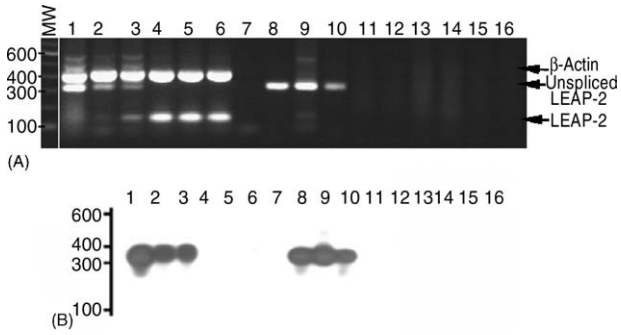

Fig. 6. Characterization of the unknown band positioned at the expected size of unspliced LEAP-2. A. RT-PCR products were analyzed on an agarose gel. MW, molecular weight standards of 1 kb ladder with sizes of interest indicated on the left margin; lanes 1–7, RT-PCR products with RNA isolated from: 1, embryos at the time of hatching (5 days after fertilization); 2, 3 days after hatching; 3, 6 days after hatching; 4, 10 days after hatching; 5, 13 days after hatching; 6, 17 days after hatching; 7, no RNA control; 8 and 9, PCR product using BAC DNA containing the LEAP-2 gene as templates; 10, PCR products using genomic DNA as templates; lanes 11–16 are the same as lanes 1–6, but no reverse transcriptase was used; only *Taq* polymerase was used. Positions for the expected size of LEAP-2 transcript, unspliced LEAP-2 transcript, and β-actin are indicated by the arrows on the right margin. Note that no PCR products were generated without reverse transcriptase (lanes 11–16), and that the unexpected band of RT-PCR products in lanes 1–3 was of the same size as the PCR product produced by using DNA template. B. The agarose gel in A was transferred to nylon membrane and subjected to Southern blot analysis using intron 1 probes. Molecular weight standards are indicated on the left margin. The samples and their order of loading were the same as in A. Note that the unexpected RT-PCR band was positive to the intron probe.

The Channel catfish LEAP-2 gene was only modestly upregulated in the spleen after the infection with *E. ictaluri*, the causative agent of enteric septicemia of catfish (ESC), but not in the head kidney and liver (Fig. 7). Although the upregulation in the spleen appeared to be rapid with an increase of the LEAP-2 mRNA 4 h after challenge, the extent of upregulation was less than two folds.

4. Discussion

Teleost fish, as the most abundant and diverse group of vertebrate containing over 23,000 species, holds a wealth of information with regard to the study of comparative immunology. Their tremendous diversity of habitats and life history strategies ensure a wide range of defensive strategies to microbial assaults. Channel catfish *(Ictalurus punctatus)*, the predominant aquaculture species in the United States, faces almost continuous microbial assaults in their densely stocked pond environments. Channel catfish shows particular susceptibility to ESC. Under artificial challenge conditions, ESC is responsible for high mortality levels well before the adaptive immune system of catfish can mount a response (Wolters and Johnson, 1994; Wolters et al., 1996). The innate immune response of Channel catfish to ESC and other

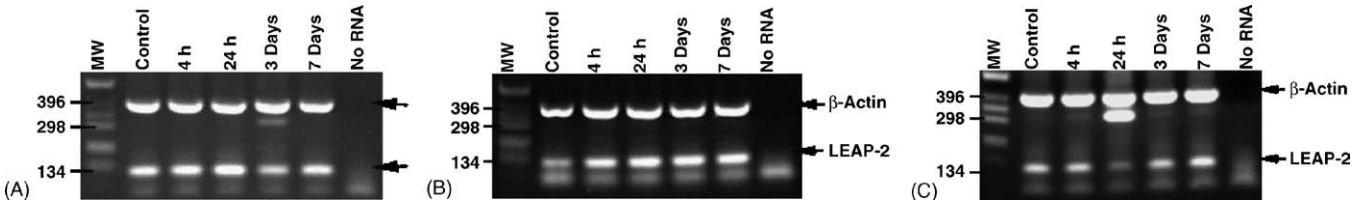

Fig. 7. RT-PCR analysis of LEAP-2 gene expression after bacterial challenge with *Edwardsiella ictaluri*. Samples of head kidney (A); spleen (B); and liver (C) were collected at 0 h, 4 h, 24 h, 3 days, and 7 days after challenge. RT-PCR products were analyzed on agarose gels (A–C) along with no RNA control (CK). The expression levels were quantified and expressed as the ratio of hepcidin expression over that of beta-actin for head kidney (D); spleen (E); and liver (F).

bacterial infections is critical, therefore, in preventing the onset of infection. Research to understand components of innate immunity such as AMPs is essential in order to comprehend disease resistance, better control disease outbreaks, and select for resistant strains of Channel catfish in the future. This research, for the first time, reports the entire locus of the LEAP-2 gene from this economically important fish species. While the antimicrobial activities of catfish LEAP-2 remain to be determined, its gene features such as the conservation of the cysteine residues, size and sequence similarities to the mammalian LEAP-2 genes, suggest their potency against microbial organisms.

This research indicated that Channel catfish harbors only a single copy of the LEAP-2 gene, a situation unique among the fish species from which LEAP-2 has been analyzed. Rainbow trout has two copies of LEAP-2 (Zhang et al., 2004). Although only partial sequences are available, two LEAP-2 related sequences were identified from zebrafish. In a phylogenetic analysis, all fish LEAP-2 were placed into a single clade (Fig. 3). One of the two zebrafish sequences CD284721 was more similar to the catfish LEAP-2 genes, and the rainbow trout LEAP-2A and the other was more similar to rainbow trout LEAP-2B. This indicated that the recent LEAP-2 gene duplication in rainbow trout and in zebrafish did not happen in Channel catfish. Taken together, it appears that the gene duplication events of LEAP-2 gene in catfish occurred after the lineage branches off between catfish and zebrafish.

The Channel catfish LEAP-2 gene was expressed in a wide range of tissues. Out of 11 tissues tested, mature LEAP-2 mRNA was only not detected in the brain and stomach, although a band was produced corresponding to the size produced from using genomic DNA as templates or using the unspliced RNA as templates. The possibility of this band being amplified from an unspliced transcript could not be excluded because Taq polymerase also has reverse transcriptase activity. In spite of the fact that we treated the RNA samples with DNases, one can always argue that the DNase treatment was incomplete. Therefore, the true nature of the templates represented by the PCR amplified products in the brain and stomach awaits further studies. The widespread expression of LEAP-2 in catfish differs from what was found from rainbow trout where LEAP-2 RNA was detected only from the liver and intestine of uninfected fish, but was not detected in the other four tested tissues including gill, head kidney, skin, and spleen. Since RT-PCR was used in both situations, the sensitivity of detection methods was not the cause for the difference. Using real time PCR, Krause et al. (2003) found that LEAP-2 was expressed in many tissues in human including liver, kidney, jejunum, duodenum, antrum, brain, bladder, testis, heart, lung, colon, trachea, prostate, salivary gland, adrenal gland, and thyroid gland. Taken together, these results indicate that LEAP-2 may exhibit different tissue expression profiles in different organisms.

The catfish LEAP-2 appears to be developmentally regulated. There was no mature mRNA until 6 days after hatching when the catfish fry can swim well. While developmental regulation is often observed, it is particularly interesting that LEAP-2 appeared to be regulated post-transcriptionally at the level of splicing. Unspliced transcripts could be detected as early as 48 h after fertilization, the first sampling time point in this study, throughout till 6 days after hatching. At 6 days after hatching, it was evident that both the spliced and unspliced transcripts coexist at about the same levels. This raises an important question as to what factors keep the LEAP-2 transcripts unspliced at early stages of development and suggests a potential role for LEAP-2 during development. One possible explanation is that LEAP-2 is not required at early stages of development, since the unspliced transcripts have only a coding capacity of 42 amino acids with the first 25 amino acids of LEAP-2 (Fig. 1). It appears that aberrant splicing is quite common for LEAP-2 gene as unspliced transcripts and partially spliced products were observed in the human LEAP-2 gene (Krause et al., 2003). It is possible that splicing is used as a major mechanism of gene regulation for LEAP-2.

Investigation of antimicrobial peptides in fish represents a part of ongoing efforts to understand all aspects of teleost innate immunity. Antimicrobial peptides hold further potential for aquaculture species such as Channel catfish as novel therapeutants against the widespread disease outbreaks still prevalent in intensive fish culture. The sequencing, characterization, and extensive expression analysis of the LEAP-2 gene in catfish described here, advances our knowledge of teleost immunity and represents another step towards the eventual control of fish disease.

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