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https://doi.org/10.5109/1434399

出版情報：九州大学大学院農学研究院紀要. 59 (1), pp.109-116, 2014-02-28. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **59** (1), 109–116 (2014)

Identification of Immune Relevant Genes Using Expressed Sequence Tags (ESTs) in Common carp (*Cyprinus carpio*) Gills and Intestine

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(*Received October 31, 2013 and accepted November 11, 2013*)

Identification of immune related molecules that function in fish organs is important for better understanding of the host defense mechanisms in fish. Expressed sequence tags (ESTs) represent the expressed portion of a genome, so they have proven to be useful tool for gene identification and confirmation of gene predictions. In the present study, a transcriptome analysis of carp gill and intestine EST libraries have been done as an attempt to identify the immune relevant genes expressed in those organs. A total of 2148 EST clones were generated from the two libraries: 1099 clones from gill library in which 632 clones were matched with functional proteins and 1049 clones from intestine library in which 559 clones were matched with functional proteins. The results of gill EST library has more frequency of innate immune–molecules containing MHC class I and MHC class Ⅱ, which showed 18.3% of the immune molecules than those in intestine library which represents 10.8% only. The gill library also showed higher frequency of the cytokines and chemokine molecules and/or their receptors compared to the intestine library.

Key words: Common carp, expressed sequence tags (EST), gills, intestine, immune relevant genes.

INTRODUCTION

Expressed sequence tags (ESTs) are single–pass reads of approximately 200–800 base pairs (bp) generated from randomly selected cDNA clones. They represent the expressed portion of a genome, so they have proven to be useful for gene identification and confirmation of gene predictions. They therefore represent a low–cost substitute to the full genome sequencing (Parkinson and Blaxter, 2009). Currently, the number of fish–related ESTs in the public databases is still small compared with mammalian sequences and there are relatively few tissue–specific cDNA libraries (Ton *et al*., 2000). Large–scale EST studies have been conducted on zebrafish (Gong *et al*., 1997; Gong, 1999; Kelly *et al*., 2000; Borchardt *et al*., 2010). In addition to other commercially important species as fugu (Aparicio *et al*., 2002); Japanese flounder (Nam *et al*., 2000; Kono and Sakai, 2001; Nam *et al*., 2003); channel catfish (Ju *et al*., 2000); rainbow trout (Kono *et al*., 2000); Atlantic salmon (Davey *et al*., 2001) and common carp (Savan and Sakai, 2002; Kono *et al*., 2004; Ji *et al*., 2012; Liao *et al*., 2013). Still, comprehensive information on mRNA levels at steady state is not known for the most known fish transcripts (Virlon *et al*., 1999). Actually, EST–based approaches have been especially effective in fish for identification of cytokines, a wide range of small proteins that are secreted from various cells of the immune system to act on immune–related cells, functioning as a signal messenger for immune responses (Bird *et al*., 2006; Savan and Sakai, 2006; Wang *et al*., 2009). This was largely because many of the cytokines including interleukins, interferons, and chemokines of fish have poorly conserved primary structure compared with mammalian homologues, inhibiting application of conventional homology cloning techniques dependent on high degree of sequence conservation.

The common carp, *Cyprinus carpio*, belonging to the family Cyprinidae is one of the most widely cultured fish species in aquaculture and considered to be an important source of animal protein (FAO, 2009). Despite the importance in global aquaculture, genomic information in the cyprinid is still very limited, except for zebrafish (*Danio rerio*) (Christoffels *et al*., 2006), partly due to the polyploidy that represent a characteristic feature of several members of this family (Larhammar and Risinger, 1994; David *et al*., 2003). Since the species, *Cyprinus carpio*, is believed to have been established as a result of allotetraploidization about 50 million years ago, the pseudotetraploid nature of the carp genome has been implicated to have an impact to allow diversification of various innate immune–related genes in this species (David *et al*., 2003, 2007; Christoffels *et al*., 2006).

To date, identification of immune–related genes have gained considerable successes in the common carp, resulting from EST analysis of immune–related organs such as kidney (Savan and Sakai 2002; Kono *et al*., 2004), testes (Christoffels *et al*., 2006), thymus (Huang *et al*., 2009), peritoneal cell exudate (Fujiki *et al*., 1999, 2000), in addition to skin, which is an immediate interface between fish body and environmental water (Gonzalez *et al*., 2007). The present study was started as further attempts to unveil novel immune–related genes by applying the EST approaches to the gill and intestine, which

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are considered to be important interfaces for outer environment and potential sites of microbial infections.

In fish gill, intestine and skin are organs exposed to the outer environment. They are considered the first line of defense against any invading pathogen or infection. Therefore identification of immune related molecules that function in those organs is important for better understanding of the host defense mechanisms in fish. In the present study, a recent transcriptome analysis of carp gill and intestine EST libraries have been done as an attempt to identify the immune relevant genes expressed in those organs. The obtained data will be a good tool for establishment of prevention of infectious diseases, which damage the cultured fish stock.

MATERIALS AND METHODS

Reagents

Scleroglucan (a fungal β–1,3–glucan from *Sclerotium glucanicum*) and *Edwardsiella tarda* NG8104 were obtained as described elsewhere (Yano *et al*., 1991). *E. tarda* was cultured using the heart infusion agar and broth (Eiken Chemical Co., Tokyo, Japan) and harvested during the logarithmic phase. ISOGEN reagent was purchased from Nippon Gene (Tokyo, Japan). Quick Prep Micro mRNA kit was from GE Healthcare (Tokyo, Japan). cDNA Synthesis Kit, Uni–ZAP XR vector and Gigapack Ⅲ Gold were obtained from Stratagene (USA), PCR Super Mix HF (Invitrogen), Exo SAP–IT kit was from GE Healthcare (Tokyo, Japan).

Fish

Mature common carp (*Cyprinus carpio*) weighing about 1 kg purchased from a local fish farm were maintained in a recirculation tank system kept at 25ºC with daily feeding of commercial pellets for a month before experiments.

Construction of cDNA libraries for EST sequence analysis of carp gill and intestine

The carp was stimulated by oral administration of scleroglucan for 3 days at a dose of 5 mg/kg–body weight/day, and by an immersion in a live *E. tarda* suspension (3×10^7 CFU/ml) for five minutes in the third day. Then the fish was kept without feeding for two more days until sacrificed for RNA isolation.

The fish was anesthetized in 25 mg/L quinaldine and subjected to cannulation to the bulbus arteriosus in the thoracic cavity by surgical operation. Then the circulating blood was removed as completely as possible by perfusion with freshwater fish physiological saline (0.75% NaCl, 0.02% KCl, 0.02% $CaCl_2$ and 0.002% $NaHCO_3$; Kawamoto, 1970) supplied from the cannula. After the color of the gill became almost white, the gill lamellae and the intestine (the mid gut part or the second segment) were excised and immediately homogenized in ISOGEN reagent for total RNA purification according to the manufacturer's instructions. Poly $(A)^+$ RNA was purified using Quick Prep Micro mRNA kit. Double stranded cDNA was synthesized with the cDNA Synthesis Kit, and after ligation with EcoRI adaptor and digestion with XhoI, unidirectional cDNA libraries for gill and intestine were constructed in Uni–ZAP XR vector. In vitro packaging was performed using Gigapack Ⅲ Gold. Titers of 3.6×10^7 pfu and 2×10^7 pfu were obtained for the gill and intestine cDNA libraries, respectively. The libraries were amplified once, aliquoted, and stored at –80°C.

Nucleotide sequencing

Phages of an aliquot of the once amplified ZAP library were converted to pBluescript SK (–) plasmids by *mass excision* with ExAssist helper phage and XLOLR bacterial host strain, according to the manufacturer's instructions. Resultant recombinant colonies were randomly selected for colony–direct PCR of the insert using PCR Super Mix HF with a set of M13 forward and reverse primers under the following conditions: 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec, and 68˚C for 7 min. After analysis of the amplicon size by agarose gel electrophoresis, templates for sequencing reactions were purified from the amplified product using Exo SAP–IT kit.

Nucleotide sequences were determined from the 5'–end of each cDNA insert with the PCR–amplified template and the sequencing primer was ACAAAAGCTGGAGCTCCACCG. ABI Prism Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.0 was used for ABI310 and ABI377 sequencers (Applied Biosystems), and GenomeLab™ DTCS– Quick Start Kit was for analysis on CEQ8800 sequencer (Beckman). In addition, mass sequencing was also conducted in Takara Bio Co. (Shiga, Japan) using ABI3700 sequencer with the same primer.

Sequence analysis

Nucleotide sequences were clustered and cleared from vector–derived sequences by using ATGC software (GENETYX), and the non–redundant insert sequences were subjected to a BlastX homology search against non–redundant set (nr) of amino acid sequence on the NCBI database (http://www.ncbi.nlm.nih.gov/blast/). The homology was determined to be significant when the E–value was less than 10^{-8}. The sequences that showed homology to known proteins were classified based on KEGG orthology (KO) System (http://www.genome.jp/kegg/ko.html). The EST frequency (percentage) was calculated from the number of clone in the number of total clones.

RESULTS

Annotation analysis of gill and intestine EST clones

As shown in Table 1, the database analysis of a total number of gill EST 1099 random clones yields 632 clones that were matched with functional proteins and 134 clones with unknown function while 333 clones showed non–significant similarity. In case of intestine cDNA library, the database analysis of EST 1049 random clones yields 559 clones that were matched with functional proteins and 223 clones were matched with unknown func-

Table 1. Annotation analysis of the carp gill and intestine EST clones

Group	Gills EST clones		Intestine EST clones	
	Clones number	Percentage (%)	Clones number	Percentage (%)
Functional	632	57.5	559	53.3
Unknown function	134	12.2	223	21.3
No significant similarity	333	30.3	267	25.4
Total	1099	100	1049	100

tion while 267 clones showed non–significant similarity.

Clustering and functional classification of the EST clones

The sequenced clones that showed homology to known proteins were classified based on KEGG Orthology (KO) System. The summary of the results is shown in Table 2. For gill library, out of 143 clones (13.0%) classified into metabolism category, 185 clones (17.0%) classified into genetic information processing category, 80 clones (7.3%) classified into environmental information processing category, 212 clones (19.3%) classified into cellular processing category, 12 clones (1.1%) classified into diseases category while unknown function represents 134 clones (12.0%) and the non–significant similarity represents 333 clones (30.3%). For intestine library, out of 163 clones (15.5%) classified into metabolism category, 144 clones (13.7%) classified into genetic information processing category, 87 clones (8.3%) classified into environmental information processing category, 161 clones (15.4%) classified into cellular processing category, 4 clones (0.4%) classified into disease category while unknown function represents 223 clones (21.3%) and the non–significant similarity represents 267 clones (25.4%).

Clusters of immune related genes of the gill and intestine EST clones

All the EST sequences have been deposited in DDBJ/EMBL/GenBank databases under the accession numbers from DC995888 to DC997897. Of the many transcripts that had homology to known genes in the gill and intestine libraries, a number appeared to be related to the host defense mechanisms were occupied 104 clones (9.5%) and 93 clones (8.9%) from gill and intestine EST clones respectively. In addition, the gill library has more frequency of innate immune–molecules containing MHC class I, MHC class Ⅱ which showed 18.3% of the immune molecules than those in intestine library which represents 10.8% only. Gill library also showed higher frequency of the cytokines and chemokine molecules and/or their receptors compared to cytokines expressed from intestine library (Tables 3 and 4).

DISCUSSION

In fish, gills, intestine and skin are important immune organs (Gonzalnez *et al*., 2007). In order to identify the different immune molecules inside carp gills and intestine, cDNA libraries have been constructed after stimulation with *Edwardsiella tarda*. In this paper, stimulation of carp gill and intestine leads to expression of newly discovered molecules in carp such as XCR1, CysLTR2 receptors, and melanin–inhibiting protein. Others showed higher similarity to mammalian homologues rather than to fish homologues such as IL–8, an interleukin–8 (IL–8–like CXC–chemokine) encoded by EST clone accession number (DC996606) obtained from gill library shows significantly higher sequence similarity to sheep (*Ovis aries*) than to chemokines from other fish species. This clone was designated CaIL–8 and now considered the second legend of IL–8 family in carp fish (IL–8L2) (Abdelkhalek *et al*., 2009). Many other immune related molecules have been identified either related to innate immunity such as complement factors, cathepsins, lectins and fucolectin or acquired immunity such as CD2, CD3, CD83, T–cells receptor, MHC complex and immunoglobulin heavy chain and immunoglobulin light chain. In addition, chemokine CK–1, chemokine CXCL2, CC chemokine SCYA117, monocyte chemo–attractant protein, NF–kappa

Table 2. Functional classification of the carp gill and intestine EST clones

Group	Gills EST clones			Intestine EST clones		
	Clone frequency	immune–related clone frequency	Percentage (%)	Clone frequency	immune–related clone frequency	Percentage (%)
Metabolism	143	0	13.0	163	0	15.5
Genetic information processing	185	0	17.0	144	4	13.7
Environmental information processing	80	21	7.2	87	14	8.3
Cellular processing	212	83	19.2	161	75	15.4
Diseases	12	0	1.1	4	0	0.4
Total	632	104	57.5	559	93	53.3

Table 3. Common carp gill EST clones encoding for possible immunologically–relevant proteins

Hit name	Species	Identified genes accession numbers	AA region/ AA–length (%)	E–value	Clone accession numbers
Adenosine A1 receptor	*Xenopus laevis*	CAB62281	434–667/ 326 (65%)	4.00E–21	DC996583
Art2a protein	*Mus musculus*	AAI20754	25–174 / 287 (34%)	2.00E–13	DC996184
B–cell translocation gene 2	*Danio rerio*	NP_570997	182–670/ 165 (89%)	3.00E–80	DC996623
Beta–2 microglobulin	*Danio rerio*	NP_998291	17–116/ 116 (77%)	3.00E–42	DC996032
Beta–2–microglobulin precursor	*Cyprinus carpio*	Q03422	25–116 /116 (97%)	7.00E–50	DC995975
Bone morphogenetic protein 4	*Danio rerio*	NP_571417	381–638/ 400 (96%)	1.00E–39	DC996563
C–type lectin	*Cyprinus carpio*	BAA95671	1–151/ 163 (70%)	1.00E–61	DC996096
Carcinoembryonic antigen–cell adhesion molecule 1	*Rattus norvegicus*	NP_001029032	155–329/519 (30%)	6.00E–18	DC996273
Cathepsin B preproprotein	*Cyprinus carpio*	BAE44111	1–195/ 330 (92%)	2.00E–112	DC996115
Cathepsin L precursor	*Sus scrofa*	Q28944	139–666/ 334 (39%)	2.00E–26	DC996691
CC chemokine SCYA117	*Ictalurus punctatus*	ABA54964	67–261/ 96 (48%)	6.00E–09	DC996712
CD2–associated protein	*Xenopus tropicalis*	CAJ83986	119–406/ 482 (80%)	5.00E–23	DC996668
CD3 gamma delta–A	*Salmo salar*	ABO10198	90–452/ 181 (35%)	2.00E–10	DC996356
CD83	*Ginglymostoma cirratum*	AAO62993	26–127/194 (33%)	3.00E–10	DC996062
Chemokine CK–1	*Oncorhynchus kisutch*	AAF23861	154–378/100 (45%)	4.00E–15, 2.00E–11	DC996582, DC996601
Chemokine receptor	*Oncorhynchus mykiss*	CAA05917	17–94/368 (66%)	4.00E–20	DC995899
Chemokine C–X–C motif receptor 3	*Ctenopharyngodon idella*	AAW69766	10–136/341 (76%)	2.00E–27	DC995896
Claudin e	*Danio rerio*	NP_571840	87–656/ 209 (84%), 102–653/ 209 (80%), 100–654209 (85%)	5.00E–70, 2.00E–67, 7.00E–55	DC996343, DC996511, DC996304
Claudin h	*Danio rerio*	NP_571842	189–656/214 (88%)	7.00E–71	DC996449
Connective tissue growth factor	*Danio rerio*	NP_00101501	69–662/ 345 (85%)	2.00E–102	DC996521
Coagulation factor IX	*Gallus gallus*	NP_989674	1–107/471 (47%)	4.00E–23	DC996171
CXC chemokine receptor–1	*Cyprinus carpio*	AB010468	318–414/ 2852 (81%)	1.00E–14	DC996593
CXCL2–like chemokine	*Ictalurus punctatus*	AAX40735	15–112/131 (42%)	2.00E–09	DC996211
Cysteinyl leukotriene receptor 2(CysLTR2)	*Sus scrofa*	Q95N03	254–646/ 345 (31%)	5.00E–11	DC996646
Fc fragment of IgG binding protein	*Homo sapiens*	EAW56926	62–658/ 3004 (41%)	3.00E–30	DC996806
Fish–egg lectin	*Cyprinus carpio*	P68512	186–662/ 238(62%), 186–669/ 238(79%)	2.00E–46, 4.00E–72	DC996679, DC996706
Fucolectin	*Fundulus heteroclitus*	AAU21486	98–670/ 227 (40%)	7.00E–27, 2.00E–26	DC996431, DC996804
G–protein signaling modulator 2	*Danio rerio*	NP_956732	299–661/ 649 (93%)	1.00E–60	DC996665
Glucocorticoid receptor	*Oncorhynchus mykiss*	P49843	58–648/ 758 (82%)	2.00E–94	DC996525
Granulin	*Carassius auratus*	ABD03952	114–509/ 158 (67%)	4.00E–54	DC996408
Growth hormone protein gene	*Catla catla*	AY053360	557–650/ 11576 (84%)	2.00E–18	DC996592
Immunoglobulin mu heavy chain	*Oncorhynchus mykiss*	ABH09025	142–669/ 198 (62%)	2.00E–56	DC996512
Immunoglobulin heavy chain	*Cyprinus carpio*	BAA3471	252–445/445 (100%)	8.00E–104	DC996158
Immunoglobulin light chain	*Cyprinus carpio*	BAB91004	133–666/ 204 (92%)	5.00E–69	DC996338
Inhibitor of DNA binding 2	*Danio rerio*	AAH56303	123–473/ 137 (97%)	6.00E–57	DC996526
Interferon induced protein 2	*Ictalurus punctatus*	AAN04880	359–57792 (47%)	2.00E–10	DC996736
Interferon regulatory factor 5 isoform b	*Bos taurus*	AAX46454	300–426/499 (70%)	5.00E–44	DC995900
Interleukin 8	*Ovis aries*	NP_001009401	116–409/ 101 (36%)	4.00E–08	DC996606

Continue to the next page

Invariant chain like protein 2	*Cyprinus carpio*	BAC53768	57–350/ 198 (100%), 121–654/ 198 (85%), 173–655198 (99%), 155–673/ 198 (86%)	5.00E–54, 1.00E–74, 6.00E–89, 4.00E–74	DC996412, DC996658, DC996688, DC996801
Invariant chain like protein 1	*Cyprinus carpio*	BAC53767	53–664/ 234 (97%)	3.00E–101	DC996826
Legumain (lgmn), mRNA	*Danio rerio*	NM_214759	57–295/ 2340 (77%)	2.00E–43	DC996814
LY6D protein	*Homo sapiens*	AAH22806	56–352/ 129 (26%)	2.00E–05	DC996282
Lymphocyte cytosolic plastin 1	*Danio rerio*	NP_571395	117–656/ 624 (93%)	3.00E–92	DC996365
Macrophage inflammatory protein–1–alpha(XCR1)	*Homo sapiens*	AAA36543	70–657/ 355 (36%)	2.00E–26	DC996427
MHC class I antigen	*Barbus intermedius*	CAD12381	108–641/ 418 (85%)	1.00E–87	DC996562
MHC class I antigen	*Ctenopharyngodon idella*	BAD01529, BAD01666	168 –635/ 254 (61%), 1–200 / 254 (65%), 146–295 /329 (77%)	3.00E–50, 1.00E–72, 2.00E–64	DC996370, DC996006, DC996193
MHC class I UFA gene	*Danio rerio*	AAH66754	1–239 / 350 (64%)	4.00E–87	DC995993
MHC class II–associated invariant chain	*Danio rerio*	AAD24542	123–608/234 (33%), 83–676/ 234 (72%), 86–646/ 234 (65%), 360–451/ 234 (66%), 4–145/234 (44%), 7–224/234 (33%)	5.00E–24, 1.00E–80, 2.00E–64, 1.00E–29, 1.00E–53, 7.00E–24	DC996452, DC996614, DC996673, DC996345, DC996157, DC996025
MHC class II beta chain	*Cyprinus carpio*	CAA64709	71–664/ 249 (81%), 19–435/ 246 (89%)	2.00E–89, 2.00E–53	DC996478, DC996581
MHC class II antigen	*Barbus intermedius*	CAD44962	86–652/ 224 (87%), 91–675/ 224 (61%), 1–183 / 224 (87%)	6.00E–94, 1.00E–67, 6.00E–91	DC996700, DC996813, DC996239
MHC class I protein Zr2	*Cyprinus carpio*	CAB96993	104–661/ 244 (93%)	8.00E–100	DC996744
Metastasis suppressor homolog	*Rattus norvegicus*	AAC05159	123–647/ 266 (54%)	7.00E–34	DC996628
Mucolipin 3	*Xenopus tropicalis*	NP_001016804	162–410/ 553 (67%)	2.00E–43	DC996570
Melanin–inhibiting protein	*Drosophila melanogaster*	AB205184	9–54/ 1208 (100%), 8–52/ 1208 (97%), 8–64/ 1208 (93%)	6.00E–13, 3.00E–10, 6.00E–13	DC996707, DC996600, DC996508
Nuclear factor (erythroid–derived 2)–like 2	*Danio rerio*	AAH45852	165–659/ 586 (78%)	1.00E–50	DC996663
Nuclear NF–kappaB activating protein	*Siniperca chuatsi*	AAY79251	506–670/147 (75%)	7.00E–16	DC996664
Protein kinase C binding protein 1	*Danio rerio*	NP_955935	50–532/ 1111 (69%)	1.00E–39	DC996751
RAN, member RAS oncogene family, isoform CRA_a	*Homo sapiens*	EAW98515	34–669/ 236 (88%)	2E–106	DC996595
RAR–related orphan receptor A isoform a	*Homo sapiens*	NP_599023	272–658/ 523 (87%)	6.00E–62	DC996349
Ras–related protein Rab–7A	*Bos taurus*	Q3T0F5	318–656/ 207 (100%)	9/E–59	DC996754
Regulator of G–protein signalling 3, isoform CRA_c	*Homo sapiens*	EAW87389	216–602/ 1088 (57%)	1.00E–38	DC996711
Serine/threonine–protein phosphatase 2A (PP2A–alpha)	*Sus scrofa*	P67776	228–656/ 309 (97%)	2.00E–77	DC996578
Serine/threonine kinase A	*Danio rerio*	NP_997731 XP_700037	57–656/ 320 (94%)	4.00E–110	DC996735
Suppressor of typt1	*Danio rerio*	NP_878281	46–675/ 632 (97%)	4.00E–112	DC996803
Tumor–associated calcium signal transducer	*Danio rerio*	NP_99834, AAH66716	213–662/ 302 (74%), 223–660/ 302 (73%), 175–302 /302 (76%),	2.00E–62, 3.00E–60, 4.00E–38,	DC996591, DC996533, DC996185
Tumor suppressor candidate 3	*Mus musculus*	NP_084530	110–640/ 347 (70%)	4.00E–73	DC996441
Transmembrane 9 superfamily member 2	*Danio rerio*	NP_997893	568–658 / 658 (100%)	3.00E–39	DC996261
Tyrosine 3–monooxygenase / tryptophan 5–monooxygenase activation protein	*Danio rerio*	NP_958892, NP_998310	55–657/245 (96%), 127–654/ 242 (92%)	9.00E–106, 1.00E–85	DC996321, DC996501

Table 4. Common carp intestine EST clones encoding for possible immunologically–relevant proteins

Hit name	Species	Identified genes accession numbers	AA region/ AA–length (%)	E–value	Clone accession number
Angiopoietin–like 2	*Danio rerio*	NP_001012502	12–213/510 (97%)	1E–105	DC997336
Attractin	*Mus musculus*	NP_033860	1037–1228/1428 (84%)	1E–103	DC997227, DC997212
Attractin–like 1 (Atrnl1) protein	*Mus musculus*	AAH50020	655–787/787 (73%)	3E–43	DC997188
Beta–catenin	*Carassius auratus*	AAP94282	670–780/780 (97%)	4E–61	DC996928
Beta–2–microglobulin precursor	*Cyprinus carpio*	Q03422	1–116/116(98%)	9E–64	DC997091
Beta–2–microglobulin	*Barbus intermedius*	CAD44965	1–116/116 (91%)	1E–59, 4E–60	DC997439, DC997085
C–type lectin domain family 3. member B	*Homo sapiens*	NP_003269	12–199/202 (53%)	1E–55	DC997874
Cathepsin L	*Sus scrofa*	NP_999057	26–204/334 (53%)	2E–44	DC997269, DC997391, DC997140
CD2 antigen (cytoplasmic tail) binding protein 2	*Danio rerio*	NP_957255 XP_001341245	172–364/378 (65%)	5E–66	DC997518
CD80–like protein	*Oncorhynchus mykiss*	CAG25516	201–349/372 (35%)	2E–16	DC997516
Cell division cycle 42 (GTP binding protein 25 kDa)	*Xenopustropicalis*	NP_001017070	1–190/191 (99%)	1E–106	DC997410
Class I helical cytokine receptor number 1	*Tetraodon nigroviridis*	AAR25664	110–313/394 (78%)	4E–92	DC997764
Claudin 14b	*Takifugu rubripes*	AAT64039	2–131/246 (49%)	6E–29	DC996974
Cytokine inducible SH2–containing protein	*Gallus gallus*	NP_989957	1–168/249 (46%)	2E–32	DC997371
Dipeptidyl peptidase 4	*Rattus norvegicus*	NP_036921	411–590/ 767 (57%)	1E–56	DC996958
Leukocyte immune–type receptor TS32.15 L1.2a1	*Ictalurus punctatus*	ABI16039	128–296/ 483 (32%)	1E–15	DC997165
Lymphocyte antigen 75	*Mus musculus*	NP_038853	959–1130/ 1723 (39%)	7E–23	DC997698
Lymphocyte antigen 75 variant	*Homo sapiens*	BAD92152	918–1115/ 1340 (36%)	1E–26	DC997636
Glutamyl amino–peptidase (EAP) CD249	*Homo sapiens*	Q07075	95–288/ 957 (65%)	1E–68	DC997775
GTPase activating protein (SH3 domain) binding protein 1	*Danio rerio*	AAH65323	1–169/477 (89%)	6E–81	DC997689
Heat shock 70 kDa protein 5 (glucose–regulated protein)	*Danio rerio*	AAH52971	1–141/650(97%)	1E–74	DC997149
HGF–regulated tyrosine kinase substrate	*Mus musculus*	CAM27049	615–741/ 767 (47%)	3E–19	DC996991
Human immunodeficiency virus type I enhancer binding protein 1	*Homosapiens*	NP_002105	2087–2227/ 2718 (56%)	6E–40	DC997885
Inositol polyphosphate–5–phosphatase, isoform CRA_d	*Homo sapiens*	EAX07310	195–375/913 (60%)	1E–56	DC996986
Insulin receptor substrate 2–B (IRS–2–B)	*Xenopus laevis*	Q5RJW5	688–898/ 1077 (30%)	2E–17	DC997048
Interferon gamma inducible protein 30	*Danio rerio*	AAH83267	1–205/255(75%)	8E–91	DC997060
Interferon regulatory factor 1	*Danio rerio*	NP_991310	1–176/ 287 (70%)	3E–62	DC99768, DC997685
Interferon regulatory factor 2	*Channa argus*	ABK63484	228–328/ 328(45%)	6E–10	DC996873
Invariant chain like protein 2	*Cyprinus carpio*	BAC53768	1–197/ 198 (84%)	4E–85	DC99764, DC997504
Invariant chain like protein 2	*Cyprinus carpio*	BAC53768	1–181/198 (99%)	1E–100	DC997791
Legumain	*Danio rerio*	NP_999924	295–437/438 (84%)	7E–62	DC997010
Legumain	*Bos taurus*	NP_776526	19–199/ 433(78%)	2E–75	DC997151
Peptide transporter PEPT2	*Danio rerio*	NP_001034917	1–186/719(90%), 521–670/719(83%)	3E–94, 1E–65	DC99717, DC997095

Continue to the next page

Platelet–derived growth factor alpha polypeptide	*Danio rerio*	NP_919407	1–106/195 (90%)	4E–48	DC996918
Plexin C1	*Mus musculus*	NP_061267 XP_290070	1364-1405/1574 (76%)	1E-15	DC997384
Protein tyrosine kinase 2 beta	*Danio rerio*	NP_997735	701–906/1004 (80%), 1–133/1004 (88%)	1E–89, 2E–64	DC997593, DC997534
RAS–related C3 botulinum substrate 1	*Danio rerio*	NP_956065	1–129/192 (100%)	1E–71	DC997846
Similar to Contactin 3 precursor	*Danio rerio*	XP_695624	62–263/ 697(37%)	5E–23	DC997089
Similar to membrane–Toll–like receptor 5	*Danio rerio*	XP_001343149	357–519/881 (40%)	2E–18	DC996968
Small inducible cytokine SCYA105	*Paralabidochromis chilotes*	AAO21207	1–87/105 (45%)	3E–19	DC996970
Solute carrier family 3, member 2	*Danio rerio*	AAH53236	1–180/180 (89%), 1–173/485 (90%)	1E–90, 2E–87	DC996849, DC997378
Syndecan 4	*Danio rerio*	NP_001041614	16–50/201 (80%)	7E–10	DC997001
Transforming growth factor beta–1 precursor (TGF–beta–1)	*Cyprinus carpio*	Q9PTQ2	1–59/376 (81%)	3E–17	DC997847
Transmembrane 4 superfamily member protein	*Homo sapiens*	EAW78874	1–163/202 (41%)	4E–24	DC996876
Transmembrane and immunoglobulin domain 1	*Homo sapiens*	NP_996663 XP_371037	60-184/262 (37%)	5E-18	DC997621
TRPC4–associated protein A	*Danio rerio*	NP_571644	271–465/774(98%)	2E–93	DC997129
Tumor necrosis factor receptor superfamily member8	*Gallus gallus*	NP_989775	1–201/467(33%)	3E–09	DC997192
Tumor necrosis factor, alpha–induced protein 8–like 3	*Homo sapiens*	NP_997264	89–223/292 (70%)	2E–47	DC996858
X–box binding protein 1	*Danio rerio*	AAH66493	1–158/263 (94%)	3E–65	DC997144

B activating protein, interferon induced protein and cytokine receptors such as CXCR3, CXCR1, CRFB5 and TNFR member19 that were more frequent in gill library than in intestine library, which suggests the important role of gills as a first line of defense relying on innate and acquired immunity. This may be because the gill is very fragile and weak organ acting as a physical barrier easily to enter the forging substance from the surrounding environment. In addition, the outer environment surrounding gills is full of many kinds of pathogen, so gills become an important place for the antigen presenting molecules compared to intestine or other studies in carp ESTs libraries (Savan and Sakai 2002; Kono *et al*., 2004; Christoffels *et al*., 2006; Gonzalez *et al*., 2007). The results of gill and intestine cDNA libraries indicating the useful role of EST for identifying immune–related genes of fish as well as identifying novel genes.

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