한국어병학회지 제 15 권 제 2 호 (2002)
J. Fish Pathol., 15(2) : 83 ~ 92 (2002)

Screening of Potential Stress-Responsive and Immune-Related Genes by Expressed Sequence Tags in Mud Loach (*Misgurnus mizolepis*)

Yoon Kwon Nam† and Dong Soo Kim
Department of Aquaculture, Pukyong National University, Pusan 608-737, Korea

EST analysis was performed to identify stress-responsive and immune-related genes from mud loach (*Misgurnus mizolepis*). cDNA libraries were constructed with liver, intestine and kidney tissues and randomly chosen clones (216 for liver, 198 for intestine and 224 for kidney) were subjected to automated sequence analysis. Of 638 clones sequenced in total, approximately 25% of ESTs was novel sequences (no match to GenBank) or sequences with high homology to hypothetical/unknown genes. Several potential stress-responsive biomarker and/or immune-related genes were identified in all the tissues examined. It included lectin, MHC class I/II proteins, proteinase, proteinase inhibitors, superoxide dismutase, catalase, glutathione-S- transferase, heat-shock protein, warm temperature acclimation protein, complements, methyltransferase, zinc finger proteins, macrophage maturation associated protein, and others. This information will offer new possibilities as fundamental baseline data for the molecular genetics and breeding of this species with an emphasis on the development of stress (and disease)-resistant fish.

Key words: Expressed sequence tags, Mud loach, Stress, Immunity

Introduction

Expressed sequence tags, single pass sequences of randomly selected cDNA clones, can offer a fundamental basis for numerous genomic studies and other biological researches by providing powerful means to discover useful genes expressed in different tissues (Gong *et al.*, 1994). This techniques can also be of importance for illuminating the transcription profiling of an organism at a particular time: i.e. a snapshot of a given genome can be looked by ESTs (Douglas *et al.*, 1999). Recently numerous EST projects have been developed in many finfish and shellfish species in order to (1) find useful piscine genes (Hamilton *et al.*, 2000; Miyahara *et al.*, 2000), (2) develop an efficient genetic marker for genome mapping (Liu *et al.*, 1999) and (3) perform transcriptional profiling of different tissues under defined experimental stimuli (Kono *et al.*, 2000; Savan and Sakai, 2002). Also the genetic information achieved in EST project will be much helpful to extend the 'wish list' of transgenesis in fish (Nam and Kim, 2001). In present, over 13 million public entries exist in NCBI GenBank (http://www.ncbi.nlm.nih.gov/dbEST) including several fish dbESTs which correspond to around 3% of total ESTs.

Mud loach (*Misgurnus mizolepis*), an important freshwater food fish in Korea has many attractive merits as a model system for genomic studies. It includes (1) fast and transparent embryonic development (24 hours at 25℃), (2) short generation time (3-5 months), (3) small adult size (around 10 grams in adult), (4) high fecundity (several ten thousands of eggs per female simultaneously) and (5) well established breeding techniques allowing

†Corresponding Author

year-round spawning under controlled condition. Due to these advantageous traits, this species has been the subjects for numerous genetic manipulations including chromosome engineering and transgenesis (Kim *et al.*, 1994; Nam *et al.*, 2001*a*, 2002). Based on our long-term goal to develop this species as vertebrate model system for genomics and transgenesis, this study was aimed to identify stress-responsive and/or immune-related genes from mud loach using EST analysis, which will be important basis of further genetic manipulations to develop disease-resistant fish model with this species.

Materials and Methods

Experimental fish

Three-year-old female and male mud loaches that have been maintained at 25℃ in Fish Genetic Manipulation Laboratories (NRL), Pukyong National University were used for construction of cDNA libraries. Tissues sources for cDNA library constructions were liver, intestine and kidney. Tissues from three males (average BW 11.5 g) and females (average BW 17.5 g) each were pooled prior to RNA preparation.

RNA preparation and construction of cDNA libraries

Total RNA was prepared using Tripure Reagent (Roche Molecular Biochemicals, Germany) according to manufacturer's recommendations. Briefly, 10 *㎖* of reagent was added to 1 g of freshly obtained tissue and homogenization was performed using motor-driven tissue homogenizer. After homogenization, 2 *㎖* of chloroform was mixed with the homogenate and followed by centrifugation at 13,000 rpm for 10 min. Aqueous supernatant containing RNA fraction was mixed with equal volume of iosopropanol to precipitate RNA. Messenger RNAs containing poly (A) tails were isolated from the total RNA mix using mRNA Isolation Kit (Roche). The isolated mRNA was converted to cDNA with reverse-transcription using oligo d(T) primer according to the manufacturer's instruction of Lambda Zap cDNA Synthesis Kit (Stratagen Co. USA). The synthesized cDNA was subjected to size fraction and ligated to the UniZap XL vector. The ligate was packed with packaging extract supplied by manufacture (Stratagen). The primary library was amplified to 1 x 10^{10}pfu/*㎖* and the cDNAs were mass excised *in vivo* to plasmid vector (pBluescript SK).

EST analysis

Plasmid containing cDNA insert was isolated from the recombinant bacterial clones for sequencing reaction. Purified 0.5 *㎍* of plasmid DNA were used for a primer extension using BigDye terminator sequencing kit (Applied Biosystem Co., USA). The reaction mixture contained 2 pmoles (1 *㎕*) of SK primer (5' -CGCTCTAGA ACTAGTGGATC-3'), 4 *㎕* of BigDye terminator, 4 *㎕* of 2.5X dilution buffer, 2 *㎕* of plasmid DNA and 9 *㎕* of H_2O. The thermal cycling was carried out with iCycler PCR machine (BioRad, USA) as following thermal cycling conditions: 96℃ initial denauration for 3 min was followed by 25 cycles of 96℃ 10 sec., 50 ℃ 5 sec. and 65℃ 4 min. The extended product was purified with spin column (CHROMA-100, Clonetech Co. USA) to remove unincorporated ddNTPs and primers. Two microlitters of each product was run onto a sequencing gel and analyzed with ABI377 automatic sequencer (Applied Biosystem). Raw data of sequence were edited with an analysis software, Sequencher ver 3.01 (GeneCodes Co., USA) prior to BLAST search against GenBank. The sequence tags were clustered into several categories based on their annotated functions and/or

sequence structures.

Results and Discussion

cDNA library construction

Each primary cDNA library contained at least 1.3 x 10^6 pfu/ml, which was likely to be sufficient to represent expression mRNAs of that tissue when considering the genome size of the mud loach (2.8 pg/diploid cell, Nam *et al.*, 2001*b*). The average lengths of cDNA inserts in recombinant clones from libraries were ranged from 1.6 to 1.8 kb, which also indicated the possibilities that the cDNA libraries should contain full-length cDNA clones.

General EST profiles

In the present ESTs, less than 10% ESTs were represented more than once in these libraries, suggesting that a randomized collection should be made, and that constructed libraries would contain a rich source of genetic information. Most overlapping clones were housekeeping genes such as beta-actin and ribosomal transcripts. The average length of sequence reads was about 620 bp, which offers a high probability of a match with homologous in the database and is comparable with any previous reports (Douglas *et al.*, 1999).

Based on the BLAST search against the protein and dbEST databases at GenBank, the ESTs from

Table 1. Clustering of ESTs from liver, intestine and kidney of mud loach

Categories	Liver	Intestine	Kidney	Total
	no.(%)	no.(%)	no.(%)	no.(%)
Novel genes (no matches)	36 (16.7)	39 (19.7)	48 (21.4)	123 (19.3)
ESTs homologous to genes of unknown function (hypothetical or unknown)	9 (4.2)	13 (6.6)	18 (8.0)	40 (6.3)
Ribosomal RNA transcripts	14 (6.5)	16 (8.1)	19 (8.5)	49 (7.7)
Mitochondrial transcripts	17 (7.9)	11 (5.6)	25 (11.2)	53 (8.3)
DNA replication, splicing, chromatin, transcription and repair	27 (12.5)	27 (13.6)	21 (9.4)	75 (11.8)
Digestion	0 (0.0)	6 (3.0)	0 (0.0)	6 (0.9)
Reproduction and maturation	13 (6.0)	0 (0.0)	2 (0.9)	15 (2.4)
Cytoplasmic functions(cytoskeleton, inhibitors, kinases, phosphatases, translation factors etc)	34 (15.7)	35 (17.7)	31 (13.8)	100 (15.7)
Intracellular compartments(golgi apparatus, lysosomes, peroxisomes etc.)	35 (16.2)	31 (15.7)	25 (11.2)	91 (14.3)
Immune-related and/or stress-responsive)	15 (6.9)	6 (3.0)	18 (8.0)	39 (6.1)
Other micellaneous	16 (7.4)	14 (7.1)	17 (7.6)	47 (7.4)
Number of ESTs generated	216	198	224	638
Number of unique genes	198 (91.7)	179 (90.4)	205 (91.5)	582 (91.2)
Number of full-length clone	150 (69.4)	134 (67.7)	155 (69.2)	490 (68.8)

mud loach tissues could be clustered into 11 categories; (1) novel sequences yielding no matches or had little matches (e-value < 0.001), (2) ESTs with significant homology to genes of unknown function, (3) ribosomal RNA transcripts, (4) mitochondrial transcripts, (5) genes involved in nuclear process including DNA replication, transcription, RNA splicing and repair, (6) genes of digestion, (7) genes of reproduction and maturation, (8) genes involved in cytoplasmic functions such as cytoskeleton, inhibitors, kinases, phosphatases and translation factors etc., (9) genes intracellular compartments including of golgi apparatus, lysosomes and peroxisomes etc., (10) genes of known and potential immune functions including stress-responsive proteins, and finally (11) other miscellaneous sequences (Table 1).

In all the tissues, the genes related housekeeping genes in nuclear and cytoplasmic processes dominated, indicating that they are expressed in most tissues rather than tissue-specific expression. Sum of those genes are 44.4%, 47.0% and 34.4% in liver, intestine and kidney, respectively. Sequences of no match with the data in GenBank appeared at an average frequency of 19%, suggesting that those are novel genes found in the present study: kidney ESTs displayed the highest proportion (21.4%) of no match while liver sequences revealed the lowest (16.7%). ESTs matched to hypothetical or unknown genes at GenBank were found with the incidence ranging 4.2 - 8.0%. Liver ESTs are the most diverse covering numerous functions of fish, including reproduction, hormonal metabolism, lipid metabolism, immunity, oncogenic traits, and many housekeeping process. Intestine is the only tissues to generate ESTs falling into a category of digestive metabolism. On the other hand, many blood proteins such as erythorcyte membrane proteins and hemoglobin were identified in kidney tissue. Also kidney displayed many mitochondrial genes like cytochrome-c-oxidase polypeptides, NADHP-ubiquinone oxiso-reductase chain because this tissue has an extensive oxidative metabolism (Savan and Sakai, 2002). Interestingly, four microsatellite-like sequences of simple repeats (GA and CA) were found in kidney ESTs, indicating that those were the transcribable microsatellites that may have important implication as potential genetic marker in this species. Similar finding has been reported in shrimp ESTs (Lehnert *et al.*, 1999).

In all the three libraries, more than 90% ESTs were unique sequences and there was a linear relationship between the number of clones sequenced and the number of unique genes, indicating the richness and diversity of the libraries. Furthermore, about 68% ESTs out of 638 clones sequenced turned out to be potentially full-length cDNAs, when judged by Query-Subject alignment obtained by BLAST search against GenBank. It may facilitate the isolation of corresponding genomic copies of given cDNA clone using PCR technique.

Stress-responsive and/or immune-related genes

From the present ESTs, various putative genes were identified with respect to the stress-response and immunity. These putative sequences are shown in Table 2. Immune-related genes such as lectin, MHC class I/II proteins, heat-shock proteins, macrophage maturation associated protein, zinc finger protein, proteinase, complements, and methyltransferase were detected mostly in liver or kidney tissues. Genes responsive to stress, (especially the oxidative stresses caused by reactive oxygen species) were also detected, including superoxide dismutase (SOD), glutathione-S-transferase (GST), and catalase. The summary of these ESTs is shown in Table 2.

Lectins, an important molecules playing roles in

Table 2. Mud loach ESTs relating with potential immune and stress-responsive functions identified in liver (liv), intestine (int) and kidney (kid)

EST#	Putative match	Length (bp)*	Tissue sources	Closest species	e-value	Identity (%)	Accession no. of matched entry
ML012	C-type Lectin	649	liv	*Cyprinus carpio*	2e-35	46	BAA95671
ML098	MHC class I A	712	liv	*Oryzias latipes*	2e-24	40	BAA77557
MI112	MHC class I heavy chain	638	int	*Oncorhyn chus mykiss*	2e-48	46	AAB62232
MI171	MHC class I protein	598	int	*Cyprinus carpio*	1e-64	55	CAA62497
MK009	MHC class Ⅱ-associated protein	621	kid	*Danio rerios*	1e-89	70	AAD24542
MK048	MHC class Ⅱ invariant chain	620	kid	*Danio rerios*	9e-90	70	AAF01568
MI068	S-adenosyl-L-homocysteine hydrolase	599	int	*Xenopus laevis*	e-101	86	CAA07706
MK005	Ubiquitin specific protease	605	kid	*Homo sapiens*	6e-55	57	XP_02779
MK159	Proteasome subunit alpha 6A	665	kid	*Danio rerios*	e-119	94	AAK40122
MI160	Proteasome-macropain	647	int	*Rattus norvegicus*	e-123	91	NP_017238
MK201	Proteasome subunit beta 11	638	kid	*Danio rerios*	2e-93	85	AAD53516
ML049	Alpha-1-antiproteinase precursor	701	liv	*Rattus norvegicus*	2e-33	42	P17475
MI188	Alpha-1-antiproteinase	703	int	*Xenopus laevis*	9e-29	40	BAA36581
ML208	Inter-alpha-trypsin inhibitor family heavy chain-related protein	687	liv	*Homo sapiens*	9e-37	42	AAD05198
MI133	Serine proteinase inhibitor CP9	604	int	*Cyprinus carpio*	7e-25	36	150494
MK066	Superoxide dismutase	622	kid	*Danio rerios*	7e-72	86	CAA72925
ML210	Glutathione-S-transferase**	654	liv	*Sus scrofa*	7e-47	64	P79382

EST#	Putative match	Length (bp)*	Tissue sources	Closest species	e-value	Identity (%)	Accession no. of matched entry
ML085	Catalase	605	liv	*Rattus norvegicus*	e-102	78	P04762
ML037	Catalase	608	liv	*Canis familiaris*	3e-98	90	BAA36420
ML199	heat-shock protein	624	liv	*Danio rerios*	1e-81	69	BQ450553
ML200	Warm temperature acclimation-related 65-kDa protein, Wap65	631	liv	*Carassius auratus*	4e-93	73	150485
MK099	Complement C3-H1	589	kid	*Cyprinus carpio*	5e-87	73	BAA36618
ML204	Complement C3-Q1	622	liv	*Cyprinus carpio*	3e-79	68	BAA36622
ML078	Complement C3-S	615	liv	*Cyprinus carpio*	1e-88	70	BAA36621
ML009	Betaine--homocysteine s-methyltransferase	618	liv	*Sus scrofa*	9e-58	68	Q95332
ML040	Macrophage maturation-associated transcript dd3f protein	662	liv	*Homo sapiens*	8e-69	78	JC4503
MK209	Zinc finger protein 35	631	kid	*Mus musculus*	4e-86	51	NP_035885
ML007	Zinc finger protein	609	liv	*Gallus gallus*	3e-58	58	PC4161
ML155	Alpha-2-HS glycoprotein	618	liv	*Homo sapiens*	3e-19	42	NP_001613
MK158	Cell death regulator Mcl-la	615	kid	*Danio rerios*	5e-51	47	AAK00290
MK169	Ig mu chain C region precursor	624	kid	*Ictalurus punctatus*	4e-50	48	A45840
MK119	Rho GTPase Cdc42	591	kid	*Xenopus laevis*	1e-42	95	AAG36944
MK157	Ras GTPase activating protein	600	kid	*Homo sapiens*	1e-41	75	NP_004832
MK212	Ras-related Ran protein	624	kid	*Salmo salar*	1e-63	85	CAA10191

*Length of sequence read
**Mitochondrial transcripts

the cellular and tissue transport of carpohydrates, glycoproteins and calcium, are considered to be involved in mediation of pathogen recognition and neutralization of the invading organism during early stage of an infection (Vasta *et al.*, 1994), although their occurrence, structure, specificity, and natural functions have not fully understood. Recent works have claimed the possible role of lectins regarding non-self-recognition molecules in vertebrate and invertebrate immunity (Vasta *et al.*, 1999; Wilson *et al.*, 1999): it has been suggested that lectins have acquired similar immunoglobin like properties by being able to recognize and bind various microorganism that invade the host. Our EST clone, ML012 showed similarity to a wide range of C-type lectins with acceptable degree of homology to common carp, *Cyprinus carpio* C-lectin (46% identity) and in addition it shared similarities with the sequences of anti-freeze protein that is also lectin-based molecule.

EST clones ML098, MI112, MI171, MK009 and MK048 displayed significant homology either to MHC class I or II proteins from other fish species. Expression of MHC proteins is required for an efficient and complete presentation of antigens and immune response (Savan and Sakai, 2002). Proteinases (including proteosomes; MI068, MK005, MK159, MI160 and MK201) also are believed to have important implications in fish immunity by providing defensive way to invading pathogenic microorganims. Proteosome is a large multicatalytic proteinase (700,000 Mr) that is responsible for most of the nonlysosomal protein degradation in eukaryotic cells (Coux *et al.*, 1996): this proteinase degrades proteins into peptides, some of which are transported into the endoplasmic reticulum and loaded onto major histocmpatibility complex (MHC) class I molecules for presentation to the immune system. Various proteinase inhibitors (ML049, MI188, ML208 and MI133) also support host defense mechanism by affording protection from a variety of proteinases associated with pathogens.

High dose and/or inadequate removal of ROS, especially superoxide anion ($O_2^{\cdot}$), results in oxidative stress, which has been implicated in the pathogenesis of many diseases. Furthermore, such ROS have been involved in other physiological and immunological processes such as aging, infertility and nerodegenerative diseases (Mruk *et al.*, 2002). In teleost, the excessive ROS could cause also severe defects in performance of fish, including malformation during embryonic development, depressed performance in growth viability and oxidation of fatty acids (Bello *et al.*, 2000; Bols *et al.*, 2001; Klumpp *et al.*, 2002). Aerobic organisms including fish have both chemical and enzymatic defence systems against the toxicity caused by such reactive oxygen species (ROS) with various antioxidant enzymes. Cu -Zn superoxide dismutases (SOD), encoded by our EST clone (MK066) is metalloenzyme that catalyzes the dismutation of a charged substrate, the superoxide anion, at a diffusion-limited rate, while catalases, encoded by clones ML037 and ML085, are responsible for converting the resulted hydrogen peroxide to water molecules. Based on their enzymatic properties, these genes and enzymes have been given attention as biomarkers to monitor the health of fish and environmental pollutions (Lopes *et al.*, 2001; Orbea *et al.*, 2002). Another antioxidant enzyme expressed in mitochondria, glutathion-S- transferase (GST) was also identified in liver EST (ML210).

Clone ML199 encoded the putative heat shock protein, molecular chaperone that protects the cell and maintain homoestasis under stressful conditions. Many previous studies support their impor-

tant role and its validity as biomarkers for a variety of cellular stressors (Scofield *et al.*, 1999; Matranga *et al.*, 2000). The other EST clone (ML200) was annotated as a warm temperature-acclimation 65 kDa protein, which is also believed to represent similar roles with the heat shock proteins, although there has little information on the detailed function of the proteins in fish (Kikuch *et al.*, 1995; Kinoshita *et al.*, 2001).

In addition, various complements (encoded by MK099, ML078 and ML204) that are believed to be involved in cascade reaction of defense system in fish, were identified with the strong homologies to carp complements. Several other potential genes related with fish immunity (or defense) such as macrophage maturation associated protein (ML040), zinc finger protein (MK209 and ML007), cell death regulator (MK158) and Ig chain (MK169) were also detected in kidney and liver tissues. Finally, three kidney clones (MK119, MK157 and MK212) relating with GTPase-associated signal transduction were obtained, which is important in cell recognition, molecule transport and oncogenic activity (Quimby *et al.*, 2000; Nguyen *et al.*, 2002) (Table 2).

From this study, fundamental baseline data could be delivered with respect to (1) transcriptional profiling in different tissues of mud loach, (2) understanding host defense or immune mechanism at molecular level, (3) developing biomarkers for the monitoring of fish health, and finally (4) generation of novel fish strain resistant to various stressors and diseases. Based on the sequence data of cDNA clones, the isolation of corresponding genomic sequences followed by molecular manipulation would be facilitated. Also the RT-PCR analysis to trace the expression of target genes under experimentally designed stressful condition will provide a good possibility to bridge gaps in our knowledge on the genetic and cellular regulation of host defence mechanisms. Further study should be made to demonstrate *in vivo* and *in vitro* the biological functions of the proteins encoded by our present ESTs.

References

Bello, A.R.R, Fortes, E., Bello-Klein, A., Bello, A.A., Llesy, S.F., Robaldo, R.B., Bianchini, A. : Lipid peroxidation induced by *Clinostomum detruncatum* in muscle of the freshwater fish *Rhamdia quelen*. Dis. Aquatic Org., 42 : 233-236, 2000.

Bols, N.C., Brubacher, J.L., Ganassin, R.C. and Lee, L.E.J. : Ecotoxicology and innate immunity in fish. Dev. Comp. Immunol., 25 : 853-873, 2001.

Coux, O., Tanaka, K. and Goldberg, A.R. : Structure and functions of the 20S and 26S proteasomes. Annu. Rev. Biochem., 65 : 801-847, 1996.

Douglas, S.E., Gallant, J.W., Bullerwell, C.E., Wolff, C., Munholland J. and Reith, M.E. : Winter flounder expressed sequence tags: establishment of an EST database and identification of novel fish genes. Mar. Biotechnol., 1 : 458-464, 1999.

Gong, Z., Hu Z., Gong, Z.Q., Kitching, R. and Hew, C.L. : Bulk isolation and identification of fish genes by cDNA clone tagging. Mol. Mar. Biol. Biotechnol., 3 : 243-251, 1994.

Hamilton, L.C., Macpherson, G.R. and Wright J.M. : Expressed sequence tags derived from brain tissue of *Oreochromis niloticus*. J. Fish Biol., 56 : 219-222, 2000.

Kikuch, K., Yamashita, M., Watabe, S. and Aida, K. : The warm temperature acclimation 65 kDa protein, Wap65 in goldfish and its gene expression. J. Biol. Chem., 270 : 17087-

17092, 1995.

Kim, D.S., Jo, J.-Y. and Lee, T.Y. : Induction of triploidy in mud loach (*Misgurnus mizolepis*) and its effect on gonad development and growth. Aquaculture, 120 : 263-270, 1994.

Kinoshita, S., Itoi, S. and Watabe, S. : cDNA cloning and characterization of the warm-temperature-acclimation-associated protein Wap65 from carp, *Cyprinus carpio*. Fish Physiol. Biochem., 24 : 125-134, 2001.

Kono, T., Sakai, M. and LaPatra, S.E. : Expressed sequence tag analysis of kidney and gill tissues from rainbow trout (*Oncorhynchus mykiss*) infected with infectious hematopoietic necrosis virus. Mar. Biotechnol., 2 : 493-498, 2000.

Klumpp, D.W., Humphrey, C., Huasheng, H., Tao, F. : Toxic contaminants and their biological effects in costal waters of Xiamen, China. II. Biomarkers and embryo malformation rates as indicators of pollution stress in fish. Mar. Poll. Bull., 44 : 761-769, 2002.

Lehnert, S.A., Wilson, K.J., Byrne, K. and Moore, S.S. : Tissue-specific expressed sequence tags from the black tiger shrimp *Penaeus monodon*. Mar. Biotechnol., 1 : 465-476, 1999.

Liu, Z., Karsi, A. and Dunham, R.A. : Development of polymorphic EST markers suitable for genetic linkage mapping of catfish. Mar. Biotechnol., 1 : 437-447, 1999.

Lopes, P.A., Pinheiro, T., Santosm M.C., Mathiasm M.L., Collares-Pereira, M.J., Viegas-Crespo, A.M. : Response of antioxidant enzymes in freshwater fish populations (*Leuciscus alburnoides* complex) to inorganic pollutant exposure. Sci. Total Environ., 280 : 151-163, 2001.

Matranga, V., Toia, G., Bonaventura, R. and Muller, W.E.G. : Cellular and biochemical responses to environmental and experimentally induced stress in sea urchin coelomocytes. Cell Stress Chaperones 5 : 113-120, 2000.

Miyahara, T., Hirono, I. and Aoki, T. : Analysis of expressed sequence tags from a Japanese eel *Anguilla japonica* spleen cDNA library. Fish. Sci., 66 : 257-260, 2000

Mruk, D.D., Silvestrini, B., Mo, M.-Y. and Cheng C.Y. : Antioxidant superoxide dismutase - a review: its function, regulation in the testis, and role in male fertility. Contraception, 65 : 305-311, 2002.

Nam, Y.K. and Kim, D.S. : Bioinformatics in fish: its present status and perspectives with particular emphasis on expressed sequence tags. *J. Aquacult.*, 14 : 9-16, 2001.

Nam, Y.K., Noh, J.K., Cho, Y.S., Cho, H.J., Cho, K.N., Kim, C.G., Kim, D.S. : Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach (*Misgurnus mizolepis*). Transgenic Res., 10 : 353-362, 2001*a*.

Nam, Y.K., Park, I.-S., Park, D.J. and Kim, D.S. : Survival and growth of induced tetraploid mud loach, *Misgurnus mizolepis*. Aquaculture Intl., 9 : 61-71, 2001*b*.

Nam, Y.K., Cho, Y.S., Cho, H.J. and Kim, D.S. : Accelerated growth performance and stable germ line transmission in androgenetically derived homozygous transgenic mud loach, *Misgurnus mizolepis*. Aquaculture, 209 : 257-270, 2002.

Nguyen, K.T., Zong, C.S., Uttamsingh, S., Sachdev, P., Bhanot, M., Le, M., Chan, L.K. and Wang, L. : The role of phosphatidylinositol 3-kinase, Rho family GTPases, and STAT3 in Ros-induced cell transformation. J. Biol. Chem., 277 : 11107-11115, 2002.

Orbea A., Ortiz-Zarragoita, M., Sole, M., Porte, C.

and Cajaraville, M.P. : Antioxidant enzymes and peroxisome proliferation in relation to contaminant body burdens of PAHs and PCBs in bivalve molluscs, crabs and fish from the Urdaibai and Plentzia estuaries (Bay of Biscay). Aquatic Toxicol., 58 : 75-98, 2002.

Quimby, B.B., Lamitina, T., L'Hernault, S.W. and Corbett, A.H. : The mechanism of Ran import into the nucleus by nuclear transport factor 2. J. Biol. Chem., 275 : 28575-28582, 2000.

Savan, R. and Sakai, M. : Analysis of expressed sequence tags (EST) obtained from common carp, *Cyprinus carpio* L., head kidney cells after stimulation by two mitogens, lipopolysaccharide and concanavalin-A. Comp. Biochem. Physiol., 131 : 71-82, 2002

Scofield; E., Bowyer, R.T., and Duffy, L.K. : Baseline levels of Hsp 70, a stress protein and biomarker, in halibut from Cook Inlet region of Alaska. Sci. Total Environ., 226 : 85-88, 1999.

Vasta, G.R., Ahmed, H., Fink, N.E., Elola, M.T., Marsh, A.G., Snowden, A. and Odom, E.W. : Animal lectins as self/non-self recognition molecules. Biochemical and genetic approaches to understanding their biological roles and evolution. Ann. N.Y. Acad. Sci. 712 : 77-73, 1994.

Vasta, G.R., Quesenberry, M., Ahmed, H. and O'Leary, N. : C-type lectins and alectins mediate innate and adaptive immune functions: their roles in the complement activation pathway. Dev. Comp. Immunol., 23 : 401-420, 1999.

Wilson, R., Chen, C. and Ratcliffe, N.A. : Innate immunity in insects: the role of multiple, endogenous serum lectins in the recognition of foreign invaders in the cockroach, *Blaberus discoidalis*. J. Immunol., 162 : 1509-1596, 1999.