J. Fish. Sci. Tech., 6(3), 110-115, 2003

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

Expressed Sequence Tag Analysis of Olive Flounder (*Paralichthys olivaceus*): Genes and Expression Profile from the Liver

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Expressed sequence tag (EST) analysis was conducted using a cDNA library made from the liver mRNA of olive flounder (*Paralichthys olivaceus*). In the survey of 421 ESTs, 362 showed significant homology to previously described genes while 59 were unidentified or novel. Comparative analysis of the identified ESTs showed that 69 (19.0%) clones were identified as homologous to the previously reported olive flounder ESTs, and 279 (77.1%) clones were identified as orthologs of known genes from other organisms. The remaining 14 (3.9%) clones were identified as orthologs of known sequences with unknown functions. These tagged cDNA clones, identified and unidentified, could provide fundamental baseline data for genomic studies of this species.

Key words: *Paralichthys olivaceus*, Olive flounder, Expressed sequence tags (ESTs), Expression profile

Introduction

Identification of genes expressed in cells of a tissue is a basic step to understand gene function and tissue physiology. An efficient approach to characterize transcripts of genes is to partially sequence cDNA clones from cDNA libraries obtaining expressed sequence tags or ESTs (Adams et al., 1991). EST analysis not only identifies genes transcribed in specific tissues, but also reveals expression profiles of the tissue from which the cDNA library was made. With the advancement of sequencing technology, it is now possible to produce large numbers of ESTs representing a large proportion of the overall transcriptional activity of an organism.

Molecular biology of fish largely remains unexplored and the number of fish genes that have been identified is limited. Moreover, comprehensive information on steady state mRNA levels has not been known for most known fish transcripts (Virlon et al., 1999). This lack of knowledge may represent one obstacle to the effective use of genetics in aiding both fish aquaculture and conservation activities. Currently, the greatest effort so far has been made in zebrafish (Gong, 1999), winter flounder (Douglas et al., 1999), olive flounder (Inoue et al., 1997; Aoki et al., 1999; Nam et al., 2000), medaka (Hirono and Aoki, 1997), channel catfish (Ju et al., 2000; Cao et al., 2001; Karsi et al., 2002a), salmon (Davey et al., 2001; Martin et al., 2002), American oyster (Jenny et al., 2002), and shrimp (Gross et al., 2001). In such aquatic organisms with economical interest, the access to genomic data may provide new insight into the management of aquaculture activities.

Olive flounder (*Paralichthys olivaceus*) is one of the most widely cultured fish species and considered to be an important source of protein, and investigations into the molecular mechanisms are required for the establishment of new methods for determination of performance traits such as feed conversion and behavioral traits. Therefore, as part of our long-term genome analysis of olive flounder, here we extend our expressed sequence tags analysis of olive flounder by analyzing 421 ESTs from the liver tissue.

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Materials and Methods

Tissue preparation and RNA isolation

Olive flounders were obtained from Koje Marine Hatchery of National Fisheries Research and Development Institute (NFRDI) and maintained in 6 tons flow-through tank at 12 ± 1℃ under a natural photoperiod. Liver tissues from 10 fish were collected and cut into as small pieces as possible. Pooled liver tissues were rapidly frozen with liquid nitrogen and were ground with a mortar/pestle, and then homogenized with a hand-held tissue tearor in RNA extraction buffer following the guanidium thiocyanate method (Chomczynski and Sacchi, 1987).

Construction of liver cDNA library

Total RNA was extracted using the TRIzol reagent (Gibco BRL Life Technologies Ltd., Renfrewshire, UK), and mRNA was enriched by oligo-(dT) cellulose chromatography using the PolyA Tract mRNA isolation kit (Promega, WI, USA). mRNA (7.5 μg) was reverse transcribed and directionally cloned in the Uni-ZAP XR vector (Stratagene). The primary library was amplified to a titer of 1.0×10^7 pfu/mL. Mass excision was performed, and the cDNA inserts from the amplified Uni-ZAP XR library were rescued as pBluescript phagemids in SOLR *Escherichia coli*. A total of ,000 colonies were randomly picked and rearranged in 96-well plates

Plasmid preparation and sequencing

The plasmid cDNA library was plated to a density appropriate for picking individual colonies. Random clones were grown in 1.5-mL LB medium overnight in 12×75-mm culture tubes. Plasmid DNA was prepared by the alkaline lysis method using the Qiagen Spin Column Mini-plasmid kits. Three microliters of plasmid DNA (about 0.5-1.0 μg) were used in a sequencing reaction. Single-pass sequencing of the 5'-termini of selected liver cDNA clones in phagemid form was performed using the ABI 3100 automatic DNA sequencer (PE Applied Biosystems, CA, USA) and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

Bioinformatic analysis

Bioinformatic analysis was conducted to determine gene identities using GeneMaster software (Ensoltek, Korea). Briefly, vector sequences were removed and database search were limited to ESTs >100 bp in length. ESTs were then assembled in clusters of contigous sequences (contig) using ICAtools program (Parsons, 1995). Gene annotation procedures and homology searches of the sequenced ESTs have been locally done by BLASTX for amino acid similarity comparisons (Altschul et al., 1997). Matches with the Expect value (E) less than $1.0\times e^{-3}$ were considered to be significant. After the BLAST searches, a visual inspection was made to determine if the significant similarity was caused by simple sequences. ESTs with significant similarities in searches were considered orthologs of known genes only when the similarities were not caused by simple sequences. All ESTs that were not identified as orthologs of known genes were consideredas unknown EST clones.

Results and Discussion

EST sequencing and general characteristics

A total of 421 randomly selected clones, in phagemid form, were single-pass sequenced from the 5' end, resulting in the characterization of cDNA clones that were longer than 100 bp after elimination of vector sequence. The average insert size was estimated to be 2.0 kb ± 0.5 by PCR amplification of inserts from 20 randomly selected clones (Table 1). Using the assembly program, the 421 liver ESTs coalesced into 46 contigs and 192 singletons, suggesting that the overall redundancy of the library was 43.5%. Gene annotation procedures and homology searches of the sequenced ESTs have been locally done by BLASTX for amino acid similarity com-

Table 1. General characteristics of the liver ESTs of olive flounder (*Paralichthys olivaceus*). [1]Prior to sequence comparison, the clones with inserts less than 100 bp were excluded. [2]The average insert size was calculated with 20 randomly selected cDNA clones using PCR. [3]ESTs with 90% or greater identity over a 100 bp region were clustered together forming 46 EST clusters. [4]192 sequences did not sufficiently match any sequence in the data set to allow assembly. [5]Redundancy = The number of genes / Total number of cDNA sequenced.

Total number of cDNA sequenced[1]	421
Average insert size[2]	2.0 kb ± 0.5
Average EST length	532 bp
EST clusters[3]	46
Singletons[4]	192
Redundancy[5]	43.5%

Table 2. List of identified ESTs from the liver cDNA of olive flounder (*Paralichthys olivaceus*). ESTs with the Expect value (E) less than $1.0\times e^{-50}$ were represented. [1]Accession No. of closest species. [2]Identity (%). [3]Frequency of the clones in the sequenced pool.

Clone No.	Putative identification	Closest species	Accession No.[1]	E-value	I[2]	F[3]
Liver-1-B10	alpha-1-microglobulin	*Pleuronectes platessa*	P36992	0.00E+00	76	6
Liver-1-C11	CCT epsilon subunit	*Carassius auratus*	BAA89277	0.00E+00	91	1
Liver-1-C8	elastase 1 precursor	*Paralichthys olivaceus*	BAA82367	0.00E+00	92	3
Liver-2-C7	chymotrypsinogen 2	*Paralichthys olivaceus*	BAA82366	0.00E+00	94	5
Liver-2-C5	receptor for activated protein kinase C	*Oreochromis niloticus*	AAB81618	0.00E+00	95	1
HEPA1G09	elastase 3 precursor	*Paralichthys olivaceus*	BAA82369	0.00E+00	96	4
Liver-3-D2	ribosomal protein S7	*Takifugu rubripes*	CAA64412	0.00E+00	98	1
HEPA2C08	transferrin	*Paralichthys olivaceus*	AAF33233	0.00E+00	98	11
Liver-2-B5	trypsinogen 2	*Paralichthys olivaceus*	BAA82363	0.00E+00	100	7
Liver-2-G2	complement component C9	*Paralichthys olivaceus*	BAA86878	3.00E-93	99	1
HEPA1D04	cytochrome b	*Paralichthys olivaceus*	NP_037594	5.00E-93	93	3
Liver-1-A7	cytosolic inorganic pyrophosphatase	*Homo sapiens*	AAD24964	2.00E-92	80	1
Liver-2-C9	NADH dehydrogenase subunit 5	*Paralichthys olivaceus*	NP_037592	3.00E-91	76	1
Liver-3-D1	brain acidic ribosomal phosphoprotein P0	*Rana sylvatica*	AAG09233	5.00E-90	90	4
Liver-3-H9	elastase 4 precursor	*Paralichthys olivaceus*	BAA82370	6.00E-90	88	1
Liver-2-D8	transketolase	*Xenopus laevis*	AAF76194	3.00E-89	79	1
Liver-2-A7	cytochrome c oxidase subunit II	*Paralichthys olivaceus*	NP_037585	7.00E-88	83	2
Liver-1-D12	complement component C3	*Paralichthys olivaceus*	BAA88901	5.00E-87	92	8
Liver-1-G1	cytochrome c oxidase subunit I	*Paralichthys olivaceus*	NP_037584	1.00E-85	94	6
Liver-2-F1	IGFALS	*Sphoeroides nephelus*	AAL73046	2.00E-84	73	1
Liver-1-A2	complement component C7	*Paralichthys olivaceus*	BAA88899	2.00E-84	90	1
Liver-3-G9	carboxypeptidase A2 (pancreatic)	*Homo sapiens*	AAH07009	2.00E-83	65	4
Liver-2-B4	alanine:glyoxylate aminotransferase	*Xenopus laevis*	CAC17015	2.00E-83	70	1
HEPA1F09	trypsinogen 1 precursor	*Pseudopleuronectes americanus*	AAC32751	5.00E-83	82	9
Liver-2-G3	elastase 2 precursor	*Paralichthys olivaceus*	BAA82368	1.00E-82	97	1
HEPA1H10	F-actin capping protein alpha-1 subunit	*Homo sapiens*	NP_006126	4.00E-81	85	1
HEPA1H09	ribosomal protein L18	*Oreochromis mossambicus*	AAF64457	1.00E-80	87	1
HEPA1B05	fumarylacetoacetase (AA 1-349)	*Homo sapiens*	CAA36016	2.00E-80	78	1
HEPA1F08	oxysterol binding protein-like 9	*Mus musculus*	AAH21507	2.00E-80	80	1
Liver-2-D4	TBT-binding protein	*Paralichthys olivaceus*	BAB83525	2.00E-80	100	8
Liver-3-G4	plasminogen	*Mus musculus*	AAH14773	3.00E-80	55	1
Liver-2-E9	eukaryotic translation elongation factor 1 gamma	*Danio rerio*	AAM21716	6.00E-79	83	2
HEPA1E02	unnamed protein product	*Homo sapiens*	BAB71331	1.00E-78	80	1
Liver-1-D3	betaine-homocysteine methyltransferase	*Homo sapiens*	XP_003920	1.00E-77	79	1
Liver-2-A8	hypoxanthine guanine phosphoribosyl transferase	*Gallus gallus*	CAB46657	3.00E-77	86	1
Liver-3-E10	ribosomal protein L12	*Ictalurus punctatus*	AAK95138	2.00E-76	88	1
HEPA1D01	aldolase B	*Salmo salar*	AAD11573	6.00E-76	90	1
Liver-2-H1	serine-pyruvate aminotransferase	*Gillichthys mirabilis*	AAG13347	5.00E-75	85	1
Liver-3-B1	alpha 4 subunit of 20S proteasome	*Carassius auratus*	BAA89276	3.00E-74	99	1
Liver-2-G8	dihyrolipoamide acetyl transferase	*Homo sapiens*	AAB50223	1.00E-73	71	1
Liver-1-C6	apolipoprotein E	*Scophthalmus maximus*	CAB65356	6.00E-73	80	1
HEPA1C04	fibrinogen	*Gallus gallus*	AAA48770	3.00E-72	67	1
Liver-2-E12	alpha-2-macroglobulin-1	*Cyprinus carpio*	BAA85038	6.00E-72	64	2
Liver-3-E9	trypsinogen II	*Engraulis japonicus*	BAB40330	2.00E-71	66	1
Liver-1-D2	cathepsin F	*Homo sapiens*	AAC78838	4.00E-71	70	1
Liver-2-A6	high choriolytic enzyme 1 precursor	*Takifugu rubripes*	AAL40376	2.00E-70	71	1
HEPA1G10	fucosidase, alpha-L- 1, tissue	*Homo sapiens*	XP_086355	2.00E-70	71	1
HEPA1F07	eukaryotic translation initiation factor 3	*Mus musculus*	NP_061219	5.00E-70	93	1
HEPA1H04	pancreatic carboxypeptidase A1 precursor	*Takifugu rubripes*	AAL40361	2.00E-68	70	2
HEPA1A08	cyclophilin 1	*Drosophila subobscura*	AAB87889	4.00E-68	84	1
Liver-3-B4	aldehyde dehydrogenase	*Danio rerio*	AAK49120	1.00E-67	69	1
Liver-3-D11	enolase	*Gallus gallus*	BAA07132	3.00E-67	88	1
Liver-1-E9	RIKEN cDNA 4931406C07	*Mus musculus*	NP_598493	4.00E-67	67	1
Liver-1-E5	40S ribosomal protein S18	*Ictalurus punctatus*	AAK95201	5.00E-67	93	1
HEPA1B10	medaka complement	*Oryzias latipes*	BAA12207	2.00E-66	63	6
Liver-1-F9	ferritin middle subunit; ferritin M	*Salmo salar*	AAB34576	6.00E-66	85	1

Table 2. continued.

Clone No.	Putative identification	Closest species	Accession No.[1]	E-value	I[2]	F[3]
Liver-1-C5	triglyceride lipase	*Anguilla japonica*	BAB85636	2.00E-63	49	1
Liver-2-B2	protein for MGC:28547	*Mus musculus*	AAH27189	2.00E-63	73	1
Liver-1-B12	cryptochrome 1b	*Danio rerio*	NP_571865	2.00E-63	87	1
Liver-2-G5	mannose-specific lectin	*Homo sapiens*	NP_005561	2.00E-61	60	1
HEPA1C08	major histocompatibility class I receptor	*Stizostedion vitreum*	AAL11413	2.00E-61	68	2
Liver-2-F11	cellular retinol-binding protein	*Danio rerio*	AAL38648	4.00E-61	82	1
Liver-3-F5	ribosomal protein L13a	*Ictalurus punctatus*	AAK95140	7.00E-61	82	1
Liver-2-F3	carboxypeptidase A	*Bos taurus*	CAA83955	1.00E-60	52	1
Liver-1-H11	tax-responsive element binding protein 107	*Gallus gallus*	AAK52090	2.00E-60	69	2
Liver-3-H11	hypothetical protein YR-29	*Homo sapiens*	NP_055701	2.00E-59	79	1
HEPA1G03	RIKEN cDNA 2310020H20 gene	*Mus musculus*	AAH28800	5.00E-59	74	1
Liver-3-H1	ferritin, heavy subunit (ferritin H)	*Salmo salar*	P49946	5.00E-58	93	2
HEPA2D02	kidney-specific protein 32	*Rattus norvegicus*	AAK00767	2.00E-57	70	1
Liver-3-E8	elastase A precursor	*Gadus morhua*	AAB58351	2.00E-57	73	7
HEPA1D08	4-hydroxyphenylpyruvate dioxygenase	*Homo sapiens*	XP_012192	6.00E-56	74	1
Liver-1-H10	complement C1r/s-A	*Cyprinus carpio*	BAB17845	1.00E-55	59	1
HEPA1D10	complement regulatory plasma protein SB1	*Paralabrax nebulifer*	S46199	2.00E-55	61	5
Liver-1-B2	cathepsin L precursor	*Sarcophaga peregrina*	A53810	2.00E-55	63	1
Liver-2-E2	antithrombin III	*Takifugu rubripes*	BAA77461	8.00E-55	76	1
HEPA1A02	heparin cofactor II	*Gallus gallus*	AAC16324	1.00E-54	59	1
HEPA1A04	25-hydroxyvitamin D3-24-hydroxylase	*Sus scrofa*	AAF81204	2.00E-54	64	1
HEPA1H03	plasminogen	*Papio hamadryas*	AAB97887	5.00E-54	58	1
Liver-1-A8	TB2	*Homo sapiens*	AAA66351	5.00E-54	60	1
Liver-3-A10	carboxypeptidase homolog	*Bothrops jararaca*	AAF01344	6.00E-54	71	1
HEPA2C09	ribophorin I	*Sus scrofa*	CAC04096	8.00E-54	70	1
Liver-3- A10	carboxypeptidase B precursor	*Rattus norvegicus*	NP_036665	2.00E-53	68	1
Liver-2-A3	protein for MGC:37818	*Mus musculus*	AAH22133	4.00E-53	76	1
Liver-3-C1	similar to unnamed protein product	*Homo sapiens*	XP_059600	2.00E-52	85	1
Liver-3-C12	RIKEN cDNA 0610040H15	*Mus musculus*	NP_080581	2.00E-52	85	1
Liver-1-D9	ATP synthase subunit B	*Xenopus laevis*	AAF31360	2.00E-51	76	1
Liver-2-D12	annexin max4	*Oryzias latipes*	CAA72125	2.00E-51	81	1
HEPA2C12	inter-alpha-trypsin inhibitor heavy chain3	*Oryctolagus cuniculus*	BAB17302	7.00E-51	52	4
Liver-2-E6	hypothetical protein MGC4248	*Homo sapiens*	NP_115709	7.00E-51	68	1

parisons. The ESTs with significant similarities ($E<1\times e^{-3}$) to known proteins were evaluated to determine if the significant similarities were caused by simple amino acid matches.

BLASTX comparisons established 362 (86.0%) clones as orthologs of known genes, representing 182 unique transcripts. The remaining 59 (14.0%) clones could not be identified by similarity comparisons ($E \geq 1\times e^{-3}$). Among the 362 EST clones, 69 (19.0%) clones represented 17 unique genes identified as homologous to the previously reported olive flounder ESTs, and 279 (77.1%) clones were matched with 151 unique genes identified as orthologs of known genes from other organisms. Orthologs were established for 14 (3.9%) clones representing 14 genes of known sequences with unknown functions (Table 2). A large number of clones showed significant similarities to known sequences of unknown function from model systems such as human, mouse, zebrafish, *Drosophila* and *Xenopus laevis*. Although functions are not yet known, their conservation in fish demonstrated the existence of many gene families through evolution. Once a gene is characterized in any one of these species, comparative functional genomics will allow efficient annotation to these orthologous genes.

Expression profile and gene identification

Expression profiles of the EST clones identified from the liver of olive flounder is shown in Fig. 1. As shown in Fig. 1, the percentage of singletons might be acceptable (45.6%), although redundancy will increase as the number of sequenced clones increases. Among 362 identified distinct known genes, 139 genes (38.4%) were sequenced only once; 83 genes (22.9%) 2-5 times; 140 genes (38.7%) over 5 times. In spite of the fact that the substantial portion of known genes were sequenced only once, gene expression in the liver of olive flounder is highly

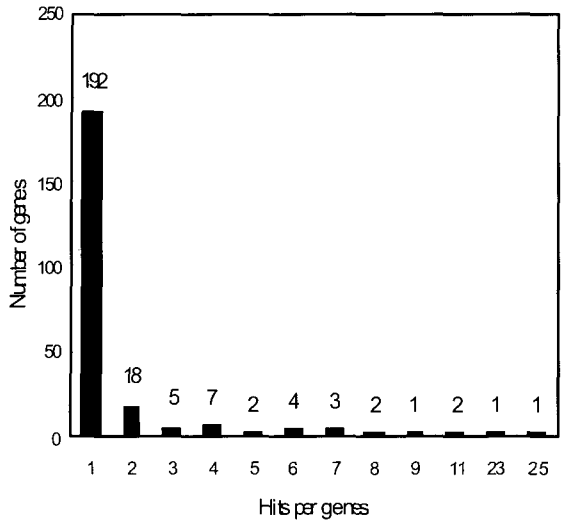

Fig. 1. Expression profiles and sequencing redundancy in the analysis of ESTs from the liver of olive flounder (*Paralichthys olivaceus*). While 192 genes were singletons, the remaining clones were sequenced 2-25 times.

polarized: a small number of genes accounted for a large proportion of transcripts in the liver.

An interesting group of genes showed a significant level of similarities to apolipoprotein from various fish including sea bream (*Sparus aurata*), eel (*Anguilla japonica*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), European flounder (*Platichthys flesus*) and turbot (*Scophthalmus maximus*). In this study, 66 clones representing 7 unique genes were identified for apolipoprotein. These genes included five types of fish apolipoprotein ; apolipoprotein A-I (25 and 23 clones), B (1 clone), C-II (4 clones), E (1 clones), 4 kDa apolipoprotein (11 clones) and 14 kDa apolipoprotein (1 clones). They accounted for almost 16% of the 421 EST clones sequenced. This expression profile in the liver of olive flounder is more polarized than in other fish tissues, where the most abundantly expressed gene accounted for less than 5% (Ju et al., 2000; Cao et al., 2001; Karsi et al., 2002a). Therefore, part of this polarization was attributed to the high levels of expression of apolipoprotein genes in the liver, presumably due to higher levels of translational activities (Karsi et al., 2002b). The high levels of expression of these genes indicated that either high copy numbers of these genes existed in the olive flounder genome, or their promoters were highly active.

We have also observed a high percentage of clones containing repetitive sequences. Twenty-one out of the 421 clones have obvious repetitive sequences, including di-, tri- and penta-nucleotide repetitives. These microsatellites can be potentially useful for genomic mapping if they are polymorphic. We found that targeting microsatellite regions within cDNAs is an efficient way to develop type I molecular markers representing genes of known functions (O'Brien, 1991). Because of the evolutionary conservation, mutation rates within gene-coding sequences are lower than those in non-coding genomic sequences. As a result, type I polymorphic markers are often more difficult to be identified. By tagging the highly polymorphic microsatellites to known genes, efficiency for the development of type I markers can be dramatically enhanced.

The expansion of the olive flounder ESTs from the present study should provide 1) a good spectrum of useful data for selection of tissue-specific or cell type specific markers, 2) isolation of full-length clones and gene promoters, and 3) analysis of the gene expression pattern and gene function.

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(Received March 2003, Accepted September 2003)