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– Review –

Bioinformatics in Fish: its Present Status and Perspectives with Particular Emphasis on Expressed Sequence Tags

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Characterization of a single pass of cDNA sequence, an expressed sequence tag (EST) has been a fast growing activity in fish genomics. Despite its relatively short history, fish EST databases (dbESTs) have already begun to play a significant role in bridging the gaps in our knowledge on the gene expression in fish genome. This review provides a brief description of the technology for establishing fish dbESTs, its current status, and implication of the ESTs to aquaculture and fisheries science with particular emphasis on the discovery of novel genes for transgenic application, the use of polymorphic EST markers in genetic linkage mapping and the evaluation of signal-responsive gene expression.

Key words : Expressed sequence tags, Fish dbEST, Implication to aquaculture

Introduction

Expressed sequence tags (ESTs), i.e. the single-pass sequences of randomly selected cDNA clones, represent the transcribed fraction of an organism's genome. Consequently, characterization of such ESTs can provide a 'snapshot' of a given genome at a particular time (Douglas et al., 1999a). It has been considered as one of the most powerful tools to identify numerous novel genes expressed in different tissues of many plants e.g. rice, *Oryza sativa* (Aliyeva et al., 1996) and animals e.g. *Caenorhabditis elegans* (Waterston et al., 1992) including humans (Adams et al., 1991).

Currently the dbEST is the fastest growing division of GenBank (Wolfsberg and Landsman, 1997). For instance, the human ESTs, which were around 415,000 in middle 1990s, have grown to over 3 millions public entries in 2001. The total number of public entries from a variety of organisms in GenBank (http://www.ncbi.nlm.nih.gov/dbEST) was more than 7 millions by January 2001. The EST databases (called dbESTs) are believed to offer basic information to address the questions arising from a variety of biological researches to enable us (1) to understand what kinds of genes are expressed in different tissues, (2) to develop informative polymorphic markers that are needed for the analysis of gene mapping, and (3) to estimate the differential regulation of transcripts in an organism exposed to a specific environmental or biological stimulus.

Several dbESTs of fish species such as medaka, *Oryzias latipes* (Hirono and Aoki, 1997), Japanese flounder, *Paralichthys olivaceus* (Inoue et al., 1997), zebrafish, *Danio rerio* (Gong et al., 1997), channel catfish, *Ictalurus punctatus* (Liu et al., 1999) and winter flounder, *Pleuronectes americanus* (Douglas et al., 1999a) have been established; besides, a number of fish EST projects are in progress. Although the ESTs of fish still account for only less than 2% of the total ESTs

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released by GenBank database, they have already begun to play a significant role in expanding our knowledge on fish genes and their expression. This review provides a brief description of the current status of bioinformatics of fish dbESTs and its prospects to aquaculture and fisheries science.

Milestone Events of EST Database

Expressed sequence tags are relatively short sequences of complementary DNAs from arbitrarily selected clones. As an attractive tool, they have led to the rapid genetic characterization of an organism. Complementary DNA libraries are generated, using poly(A+) mRNA purified from tissues (e.g. Venugopal et al., 2001; Vikas et al., 2001). Generally, cDNA is directionally cloned into vectors like UniZAP (Stratagenes) and the resultant libraries usually contain more than 1.0×10^6 pfu/ml. Primary libraries are amplified to more than 1,000-fold, excised into plasmid vectors. Most important advance in the analysis of the ESTs is the automation of the process. A simple method for the preparation of sequencing templates has also been developed (Chen et al., 1996; Sasho, 2000), and even several automated robots for a template preparation are now commercially available. Detection of sequences can be performed using fully automated instruments. Currently the most advanced sequencing machine can deal with samples based on 96-well and capillary formats. It has a theoretical capacity to analyze a maximum of more than 500-600 clones a day. When the readable length of the ESTs is considered as around 700 bp, researchers can obtain 1-2 megabase information of nucleotides every week, using only one such machine. Genomes of most aquatic bacteria are believed to be less than 10 mega bp in size; hence single-pass sequencing of their whole genomes can be accomplished within 1-2 weeks. Likewise, complete sequencing of mitochondrial genomes of fish, and viral genomes responsible for fish disease can be completed within a couple of days. Such an advance, based on automation, is not limited to a sequencing reaction. With the establishment of the EST databases including homology, searches are also presently computerized via a Web-browser interface. The ESTs can be automatically identified by BLAST searches (Altschul et al., 1997) against protein, nucleotides and dbEST at GenBank. The use of an automated Web-based search script makes it more user-friendly, and efficient to obtain database matches in an organized fashion. For instance Douglas et al. (1999a). They have developed an HTML page, which enables its users to carry out a BLAST search repeatedly on each sequence, and the results of each tissue source are compiled in summary pages with the links between sequences and results.

Status of Fish EST Database

The current dbESTs of fish released from the database of GenBank are summarized in Table 1. The latest release January 19, 2001 includes 17 commercially important (such as flounders, tilapia, catfish and salmonid) and experimental model (such as medaka and zebrafish) for species. At present, most fish ESTs (more than 90%) have been generated from the experimental model species, especially from zebrafish: of over 88,000 total fish ESTs, the rate of public entries from zebrafish (77,338) is 88%. Of the commercially important species, three (channel catfish, Japanese flounder and winter flounder) have sufficient numbers of ESTs (ranging from 915 to 3,593) to provide a representative sample of expressed cDNA.

In view of diverse tissue resources, zebrafish and winter flounder dbESTs are the most informative with representative expression profiles in 10 and 7 kinds of tissues (or organs), respectively. Of various tissues examined in other fish species, liver is the organ used most frequently for the generation of ESTs. Zebrafish dbEST also contained ESTs in regenerating fins and embryos at different developmental stages, which may provide valuable information on cell cycle, cell propagation, and developmental biology of vertebrates (Gong et al., 1997). On the

Table 1. Current status of fish ESTs in the release of dbEST

Species	ESTs in public entry[1] (No)	Type of tissue or organ
Experimental model fish species		
Zebrafish (*Danio rerio*)	77,338	Regenerating fin, Embryonic heart, Adult heart, Liver, Brain, Kidney, Olfactory rosetles, Retina, Whole embryos & Whole adult fish
Medaka (*Oryzias latipes*)	3,018	Whole body of 1-month-old fish, Liver, Fin & Embryo
Puffer fish (*Tetraodon fluviatilis*)	24	Whole adult
Commercially important species (freshwater)		
Channel catfish (*Ictalurus punctatus*)	3,593	Pituitary
Common carp (*Cyprinus carpio*)	326	Liver, Leukocyte & Peritoneum
Nile tilapia (*Oreochromis niloticus*)	294	Brain
Rainbow trout (*Oncorhynchus mykiss*)	273	Liver, Gill infected with IHN virus, & Kidney infected with IHN virus
Carp (*Lebeo rohita*)	2	Spleen
Mud loach[2] (*Misgurnus mizolepis*)	224	Liver & Intestine
Commercially important species (marine)		
Japanese flounder (*Paralichthys olivaceus*)	2,091	Liver, Spleen, Kidney injected with peptoglycan & Leukocyte infected with hirame rhabdovirus
	268	Skin[2]
Winter flounder (*Pleuronectes americanus*)	915	Liver, Spleen, Ovary, Pyloric cecae, Stomach, Intestine & Pituitary
Arctic charr (*Salvelinus alpinus*)	63	Whole body of juvenile
Atlantic halibut (*Hippoglossus hippoglossus*)	32	Pituitary
Atlantic salmon (*Salmo salar*)	9	Liver & Muscle
Miscellaneous or primitive species		
Ray, Yellowtail (*Seriola quinqueradiata*)	75	Kidney
Marbled electric ray (*Torpedo marmorata*)	41	Electric lobe
Sea lamprey (*Petromyzon marinus*)	14	Foregut
Lumpfish (*Cyclopterus lumpus*)	12	Pituitary
Tiger shark[2] (*Scyliorhinus torazame*)	832	Brain, Kidney, Liver & Pituitary
Hagfish[2] (*Eptatretus burgeri*)	342	Brain, Kidney, Liver & Intestine
Sum	88,120	(total number of fish ESTs released in dbEST)

[1]dbEST release on January 19, 2001 (http://www.ncbi.nlm.nih.gov/dbEST)
[2]Unpublished data of Fish Genetic Manipulation Laboratory, Dept. of Aquaculture, Pukyong National University. The number of ESTs from these sources didn't included in the total number of fish EST released.

other hand, winter flounder ESTs from stomach and intestine libraries represent numerous important genes related with digestive metabolism (Douglas et al., 1999a), which can offer key information on the domestication of flounder species, including larval nutrition and weaning onto artificial diets (Douglas et al., 1999b, 2000).

Several dbESTs provide information on gene expression of phylogenetically lower species like as sea lamprey, *Petromyzon marinus,* which may offer new possibilities both for addressing the evolution of vertebrate genes and for discovering novel genes that have lost in higher vertebrates during evolution. However, the number of ESTs and tissue resources in current dbEST are too insufficient and moreover, sequences are relatively short with minimal annotations. Our recent ESTs generated from the evolutionary ancient species such as tiger shark, *Scyliorhinus torazame,* and hagfish, *Eptatretus burgeri* increase not only the total number of EST but also substantially the number of tissue-specific ESTs with many novel genes which have not been detected in fish and even in vertebrates (unpublished data).

In most fish dbESTs, about half of the ESTs have identifiable functions predicted by search of GenBank for homology, and about 20-30% of these ESTs are homologous to each other; representative genes responsible for actin, tubulin, transferrin, mitochondrial proteins, ribosomal proteins and complement components are involved in basic housekeeping functions. Most fish ESTs fall in putative categories involved in digestion, reproduction, immune function, metabolism, development, nuclear process, intracellular compartments, cytoplasmic processes. Of unidentified ESTs, many were unique cDNA, suggesting identification of novel genes, providing new genetic markers, and creating the basis for a genome mapping project.

Fish dbEST for Aquaculture and Fisheries Science

The dbEST can be applied in numerous fields, including developmental biology, transgenics, nutrition, physiology, and selection. However, this review is to highlight only their three-way application to (1) transgenic research by providing the genes, which encode novel functions, (2) genetic linkage mapping using polymorphic EST markers and (3) analyzing the gene expression responsive to environmental or biological stimuli. Bioinformative database based on ESTs also can be used to build a network with other biological data (Fig. 1).

Novel genes in transgenic technology

The most unique power of EST is the rapid discovery of novel genes expressed. As already mentioned, information on several hundreds of sequences can be obtained in a day under fully automated condition and this makes it possible to establish tissue-specific expression profiles. More importantly, the EST study has provided clones useful for the isolation of full-length cDNAs (Douglas and Gallant, 1998). The full-length of an isolated cDNA can be directly used as a transgene, ligated to an appropriate expression vector. The most serious bottleneck of transgenic technology in aquaculture is the inadequate information on function of genes. At present the transgenic application of fish phenotypes can be regarded only as one of the items on the limited 'wish' list, as the responsible genes have yet to be isolated and identified. Besides the transgenes used in most transgenic studies of commercially important fish species have been limited to growth hormone and anti-freeze protein genes only (Dunham and

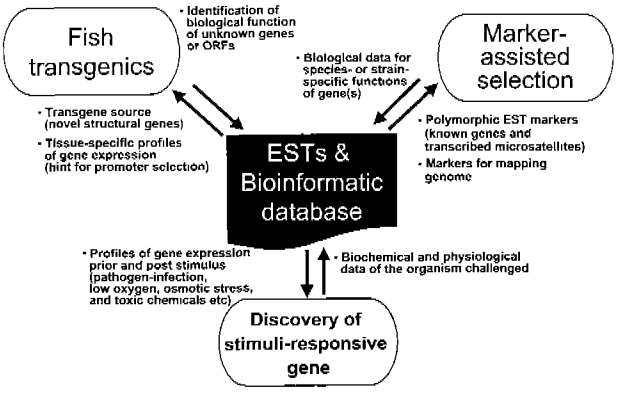

Fig. 1. A representative diagram showing the possible application of bioinformatic database into fisheries sciences.

Devlin, 1999). To isolate expressed genes in bulk by the EST project can provide a useful method to list the potential transgenes. Numerous genes, listed in the fish dbESTs can be used as candidates in transgenic application. Pituitary cDNA libraries contain lots of clones related with growth regulation (growth hormones, prolactin and somatolactin) and reproduction (gonadotropins) (Gong et al., 1994). Also many tissue-specific ESTs can provide opportunity to initiate new transgenic projects (Moav, 2000). Molecular manipulation of ascorbic acid pathway using gulonolactone oxidase EST clone obtained from shark kidney (Nam et al., 2001) is an example for targeted metabolic engineering. Keeping a similar concept, active research for discovering genes useful in transgenic technology is in progress. For instance, steroid-metabolizing genes for sex differentiation, gonadotropin subunit genes for maturation and fertility, lipid metabolizing genes for flesh character, pigment synthesizing genes for flesh quality, glucose transport genes for nutritional capabilities, and antibiotic protein (including immunoglobulins) genes for enhanced immunity with disease-resistance are rapidly being made available in fish dbESTs (McLean and Devlin, 2000). As dbEST can offer tissue-specific profiles of gene expression, they provide information, on which gene(s) is actively expressed in a given tissue, and this information, in turn, can be used for selecting candidate promoters and regulatory regions.

Genetic linkage mapping using polymorphic EST markers

The EST approach is useful not only for the identification of new genes but also for genome mapping. The polymorphic types of markers associated with a gene of known function are useful as anchorage points for comparative gene mapping, and consequently such maps can be important for the basis for marker-assisted selection (MAS). The generation of human ESTs has played a crucial role in making progress in a human genome project (Boguski and Schuler, 1995). In a syntenic group, gene organization is conserved, therefore genes can be mapped by establishing syntenic groups between "map-rich" species and "map-poor" species (Liu et al., 1999). Thus the development of such markers should facilitate the location of mapped markers for a fish species and the map should in turn facilitate mapping of other fish species. Currently, a study of genetic mapping in fish using polymorphic EST markers was published in channel catfish, *Ictalurus punctatus* (Liu et al., 1999). Liu et al. (1999) performed EST experiment with 100 randomly selected pituitary clones of channel catfish for designing PCR primers, and detected length polymorphism between channel catfish, *I. punctatus* and blue catfish, *I. furcatus*, in 11 PCR products amplified from genomic DNAs. Five of 11 EST markers were from known genes (gonadotropin I-b, propiomelanocortin and three ribosomal proteins) (Liu et al., 1999). They also suggested potential three-way application of EST polymorphic markers to the genetics of channel catfish: (1) genetic linkage mapping involving the interspecific reference/resource families, (2) physical linkage mapping using radiation hybrid panels and (3) identifying and mapping single nucleotide polymorphism (sNuP) markers in this species.

Besides, distinct clones contained simple sequence repeats (SSR or microsatellites) can be detected in ESTs; interestingly, it indicates that many microsatellites may be transcribed and should be exploited for the analysis of gene mapping. Although microsatellites are commonly believed to occur primarily in non-coding DNA, analysis of currently available cDNA libraries and dbEST has indicated that probably several clones may contain microsatellite-like sequences (Ruyter-Spira et al., 1996; Lehnert et al., 1999; Liu et al., 1999). The use of transcribed microsatellite may be more advantageous in serving as better anchorage points for comparative gene mapping and allowing easier PCR optimization with unique gene sequences flanking microsatellites. As difference in the length of microsatellites is believed to be caused by a difference in repeat numbers due to the slippage of DNA polymerase (Tautz and Schlotterer, 1994), mutation in repeats can occur at frequencies as high as 10^{-2} per generation leading to a good possibility of

creating highly polymorphic markers.

Gene expression profiles and environmental stimuli

Living organisms respond to a variety of stress or stimuli with expressive genes in order to protect themselves or to maintain their homeostasis. However, little is now known of the molecular mechanisms of such genes in fish. Lack of knowledge of gene expression related to a signal-responsive pathway may hinder not only the domestication of many marine fish species but also hamper our efforts to improve the yield of domesticated species. Due to technical simplicity, the characterization of ESTs enables to obtain abundant information on gene expression at a particular time, thus it can be an useful tool to evaluate the gene expression profile of an organism subjected to various environmental or biological stimuli. Comparative EST profiles before and after stimuli can offer valuable information on gene expression involved in a stress-responsive pathway. Experimentally designed stress such as exposure to pathogenic microbes, toxic chemicals, low oxygen level, and different levels of osmotic pressure might be good strategies to obtain basic data for addressing how the fish reacts to these stimuli. Special attention must first be directed to the interaction between the fish and its pathogenic bacteria. Several research groups, including Institute for Marine Biosciences (IMB), NRC, Canada have initiated a project to understand the gene expression both pathogen and a host under infection using Atlantic salmon, *Salmo salar* and *Aeromonas salmonicida*, as experimental model (Douglas, 2000). This research team in IMB is carrying out a combination of complete genome sequencing of the pathogen with survey of EST profiles in infected fish.

EST profiles have also been surveyed in Japanese flounder leukocytes infected with *Hirame rhabdovirus* (Aoki et al., 1999). Aoki et al, 1999 have sequenced 596 cDNA clones corresponding to 566 kb in total, and found gelatinase b and ribosomal protein L23 are most frequently identified genes. However, the reason for prevalence of these two genes has not been understood yet. In a similar search, EST study has been reported in gills and kidneys of rainbow trout, *Oncorhynchus mykiss*, infected with infectious hematopoietic necrosis virus (IHNV) (Kono et al., 2000). Interestingly, the authors have observed that IHNV-resistant fish (i.e. low-susceptibility strain) specifically expressed octin, attractin and vasoactive cardiac hormone genes based on RT-PCR assay, which have not been detected in high-susceptibility rainbow trout (Kono et al., 2000). All of such research groups have been trying to identify genes coding infection-specific proteins and defensive proteins by examining the changes that take place in both fish and pathogen upon infection.

Conclusion and Perspective

As non-coding DNA such as introns and intergenic spacers, which comprise the bulk of a genome, is not included in ESTs, dbEST provide an 'information-rich' source of data that are relatively easily interpreted and managed. Based on this merit of ESTs, which are characterized as rapid accumulation of data, there has been speedy and substantial progresses in fish EST projects for purposes of both gene discovery and comparison with other organisms, despite their relatively short history. In addition to the ESTs deposited in the GenBank database, numerous activities are being made to develop dbESTs in fish. Recent accumulation of several thousands of ESTs combined with other marker databases is producing genetic linkage maps encompassing the whole genomes in model species (Shima, 2000), which ultimately helps further elucidation of molecular mechanisms underlying a variety of biological questions. Moreover, progress made in a commercially important species, channel catfish, based on over 7,000 ESTs representing 4,000 genes expressed in different tissues (Dunham and Liu, 2000) might be believed to contribute to the genetic improvement program of this species by providing the invaluable basis for marker-assisted selection (MAS) with useful quantitative trait loci (QTL) data. Consequently, it will

be certainly helpful to understand key factors for developing the best catfish genotype for future aquaculture.

EST projects in other aquatic animals including shrimps and oysters have now actively triggered off by the advance in fish ESTs. Since the first step of EST projects in black tiger shrimp, *Penaeus monodon*, was reported by Australian research group (Lehnert et al., 1999), much effort has been focused on several other commercially important shrimp species using genomic approaches. The baseline of ESTs is being enlarged for the penaeoid shrimp and used as a database to examine differential gene expression in shrimp, encountering environmental stimuli and diseases with particular emphasis on viral diseases (Alciva-Warren, 2000; Warr, 2000). Recently an EST project has also been initiated in Pacific oyster, *C. gigas*, for purposes of searching genes involved in sex-determination and growth regulation (Shimizu et al., 2000).

Clearly the EST databases will make a significant contribution to understand the biology of fish, associated with basic molecular biology, transgenic studies, developmental biology and physiology, by providing novel informations on expressed genes in fish genome. Besides, these dbESTs will also play an important role in mapping fish genomes and understanding signal-responsive pathway in fish. In addition, the rapid promotion of automated instruments ensures the extensive use and speedy extension of aquatic dbESTs. Future efforts should ideally be made towards their combination with other biological databases of genomic sequencing, microarray research (DNA chip) and proteome analysis, in order to open the new frontiers of functional genomics using fish system.

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