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GENOMICS PROTEOMICS & BIOINFORMATICS

Article

EST-Based Identification of Genes Expressed in Skeletal Muscle of the Mandarin Fish (*Siniperca chuatsi*)

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Genomics Proteomics Bioinformatics 2011 Apr; 9(1-2): 30-36 DOI: 10.1016/S1672-0229(11)60005-3

Received: Jun 25, 2010; Accepted: Oct 22, 2010

Abstract

To enrich the genomic information of the commercially important fish species, we obtained 5,063 high-quality expressed sequence tags (ESTs) from the muscle cDNA database of the mandarin fish (*Siniperca chuatsi*). Clustering analysis yielded 1,625 unique sequences including 443 contigs (from 3,881 EST sequences) and 1,182 singletons. BLASTX searches showed that 959 unique sequences shared homology to proteins in the NCBI non-redundant database. A total of 740 unique sequences were functionally annotated using Gene Ontology. The 1,625 unique sequences were assigned to Kyoto Encyclopedia of Genes and Genomes reference pathways, and the results indicated that transcripts participating in nucleotide metabolism and amino acid metabolism are relatively abundant in *S. chuatsi*. Meanwhile, we identified 15 genes to be abundantly expressed in muscle of the mandarin fish. These genes are involved in muscle structural formation and regulation of muscle differentiation and development. The most remarkable gene in *S. chuatsi* is nuclease diphosphate kinase B, which is represented by 449 EST sequences accounting for 8.86% of the total EST sequences. Our work provides a transcript profile expressed in the white muscle of the mandarin fish, laying down a foundation in better understanding of fish genomics.

Key words: mandarin fish, cDNA library, EST, muscle

Introduction

With gradual decline in natural fish resources, aquaculture has developed fast in the world food economy in recent years. The consumption and demand for fish is on the increase due to the world's growing population. Asia produces roughly 90% of global aquaculture output, and China is one of the major contributors in aquaculture products (*1*). The most popular aquaculture species in China are Chinese carp varieties including black carp, grass carp, silver carp, and bighead carp. The other freshwater species, such as salmon and mandarin fish, have also been becoming major aquaculture species in China. The nutritional attribute of fish is dependent on the amount of digestible proteins, lipids, vitamins and mineral contents (*2*). Fish meat is also an excellent source of essential amino acids and polyunsaturated fatty acids (*2, 3*). Our previous work confirmed that the amounts of both the essential and flavor-enhancing amino acids in mandarin fish (*Siniperca chuatsi*) are much higher

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than those in silver carp (*Hypophthalmichthys molitrix*) (*4*).

There are nine species in the mandarin fish genus. *S. chuatsi* and *S. kneri* are the two most commercially important species of them in aquaculture. High nutritional value, high protein content as well as the appealing taste of the mandarin fish stimulate a large-scale culture and commercial exploitation (*4*). Therefore, we hypothesize that there may exist unique genes or metabolic pathways in control of the fish's protein expression, or a functional transcription profile in the fish's muscle (*5, 6*). Expressed sequence tag (EST) analysis is an efficient and reliable method for gene discovery and annotation (*7*). Nowadays, large-scale EST assays have been carried out in several fish species and provide us valuable information about fish genomic characterization (*8-10*). Recently we have reported an EST resource of muscle tissue for the mandarin fish (*11*). To progress towards a better understanding of the genetic basis of meat quality and expression pattern of muscle in this fish species, we downloaded 5,191 sequences from the mandarin fish EST database and finally obtained 5,063 high-quality EST sequences; clustering analysis yielded 1,625 unique sequences, including 443 contiguous sequences (contigs) (from 3,881 EST sequences) and 1,182 singletons. BLASTX searches showed that 959 of the 1,625 unique sequences shared homology to proteins in the NCBI non-redundant (nr) database. A total of 740 unique sequences were functionally annotated using Gene Ontology (GO). We identified 15 genes to be abundantly expressed in muscle of the mandarin fish, which function in muscle structural formation and regulation of muscle differentiation and development. Our work detected the gene expression pattern from *S. chuatsi* muscle, and highlighted a number of candidate genes for further investigation of fish muscle differentiation and development.

Results

EST statistics and gene annotation

After removal of clones with no insert or short inserts (<100 bp) among the 5,191 EST sequences, we finally obtained 5,063 high-quality ESTs. The average read length was 587 bp. The 5,063 high-quality ESTs were assembled into 443 contigs and 1,182 singletons, resulting in a total of 1,625 unique sequences (**Table 1**). The annotation of the unique sequences of *S. chuatsi* was achieved through BLASTX similarity searches. Of the 1,625 unique sequences, 959 shared homology with proteins in the nr database, where a cut-off E-value of 1e-05 was used, while the remaining 666 unique sequences failed to match proteins in the nr database and therefore represented potentially novel sequences or untranslated regions of known genes. Of the 959 annotated sequences, 71 (7.4%) have an E-value of 1e-100 or less, therefore they are considered true orthologs. 671 (70%) unique sequences have a hit with an E-value between 1e-20 and 1e-99 and are assigned significant orthologs. The remaining 217 (22.6%) unique sequences were assigned weak homology (E-value between 1e-05 and 1e-19) (*12*). The unique sequences were annotated using GO for their function analysis (*13*). Among 1,625 unique sequences, 740 were assigned with one or more GO terms. **Figure 1** shows the percentage distributions of GO terms according to the GO consortium. Among them, 494 unique sequences were assigned to "biological process" and "metabolic process" is the most dominant term (77%); 675 unique sequences were assigned to "molecular function"; and 405 unique sequences were annotated to "cellular component".

The most abundant ESTs

Using the clustering method described in Materials and Methods, we totally identified 835 nr clusters. As shown in **Table 2**, 558 clusters were represented by a single EST, and 277 clusters were represented by ≥2 ESTs. Particularly, 132 (47.6%) of the 277 clusters

Table 1 Overview of the results from the *S. chuatsi* cDNA library

EST sequences	5,191
High-quality sequences	5,063
Short inserts (<100 bp)	128
Unique sequences	1,625
Contigs	443
Singletons	1,182
Annotated sequences (nr)	959
Unigenes	835

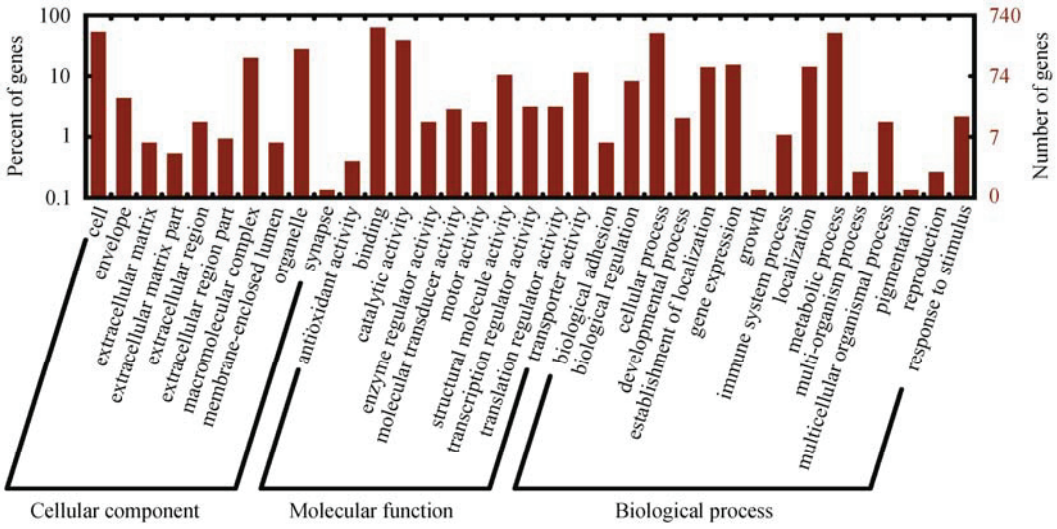

Figure 1 Functional classification of muscle tissue ESTs from *S. chuatsi*. Of 1,625 unique sequences, 740 were assigned with one or more GO terms.

Table 2 Summary statistics of EST clusters

No. of ESTs in a cluster	>100	41-100	11-40	10	9	8	7	6	5	4	3	2	1
No. of clusters	10	5	13	2	9	9	4	15	13	26	39	132	558

Table 3 The 15 most abundant genes detected from the *S. chuatsi* EST library

Gene	No. of ESTs	% of total EST	Pathway
Nuclease diphosphate kinase B	449	8.86%	Nucleotide metabolism
Parvalbumin	432	8.53%	-
Actin, alpha skeletal muscle A (Alpha-actin-1 A)	350	6.91%	Muscle structure
Myosin light chain 3	237	4.68%	-
Muscle-type creatine kinase CKM	192	3.79%	Amino acid metabolism
Glyceraldehyde-3-phosphate dehydrogenase	161	3.18%	Carbohydrate metabolism, Neurodegenerative diseases
Skeletal muscle myosin heavy chain	146	2.88%	Muscle structure
Tropomyosin-1 alpha chain (Alpha-tropomyosin)	130	2.57%	-
Aldolase A	126	2.49%	Carbohydrate metabolism, Energy metabolism
Troponin I, fast skeletal	105	2.07%	-
Troponin C, fast skeletal	90	1.78%	Signal transduction
Hypothetical protein	70	1.38%	-
Troponin T, fast skeletal	63	1.24%	Cellular processes and signaling, Cytoskeleton proteins
Myosin light chain 2	45	0.89%	Cell motility and cell communication
ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1 like	41	0.81%	Energy metabolism, signal transduction

were represented by 2 clones; 15 genes with more than 40 ESTs each are considered as the most abundant genes in the cDNA library of *S. chuatsi* muscle, consisting of 2,792 ESTs (55.1% of the total 5,063 ESTs). The majority of the 15 genes are related to muscle structure and metabolism (**Table 3**). These genes could be classified into three categories, including structural, metabolic and regulating genes. Actin, myosin light chain, and myosin heavy chain are the major structural genes, which determine the

differentiation and formation of muscle fiber (*14-16*). The nuclease diphosphate kinase B, parvalbumin, muscle-type creatine kinase CKM, glyceraldehyde-3-phosphate dehydrogenase, aldolase A, hypothetical protein and ATPase-Ca^{2+} transporting are all related to muscle metabolism and they are highly abundant accounting for 8.86%, 8.53%, 3.79%, 3.18%, 2.49%, 1.38% and 0.81% of the total ESTs, respectively. Trypomyosin-1, troponin I, troponin C and troponin T are also rich in *S. chuatsi* muscle at a ratio of 2.57%, 2.07%, 1.78% and 1.24% of the total ESTs and these genes regulate muscle differentiation (*17*).

Metabolic profiling

To understand the systemic behavior of *S. chuatsi*, the 1,625 unique sequences were assigned to Kyoto Encyclopedia of Genes and Genomes (KEGG) reference pathways (**Table 4**) (*12, 18*). A set of 12 genes with 487 ESTs were identified to participate in the process of nucleotide metabolism. The high abundance of transcripts in nucleotide metabolism is consistent to the high concentrations of nuclease diphosphate kinase B, which is the most abundant gene represented by 449 ESTs. The other high abundant metabolic pathway is the amino acid metabolism, in which 14 amino acid pathways exist. The highest abundance of transcripts involved in argnine and praline metabolism is the creatine kinase gene represented by 192 ESTs (3.79% of the total ESTs) in *S. chuatsi*.

Table 4 EST abundance in functionally grouped EST clusters

Functional group	No. of genes	No. of ESTs
Nucleotide Metabolism		
Purine metabolism	10	484
Pyrimidine metabolism	3	452
Amino Acid Metabolism		
Arginine and proline metabolism	5	198
Glutamate metabolism	3	5
Methionine metabolism	3	3
Selenoamino acid metabolism	2	2
Cysteine metabolism	3	5
Tyrosine metabolism	3	4
Alanine and aspartate metabolism	2	3
Glycine, serine and threonine metabolism	2	3
Valine, leucine and isoleucine degradation	1	1
Lysine degradation	2	3
Histidine metabolism	1	1
Phenylalanine metabolism	2	2
Aminophosphonate metabolism	1	1
Glutathione metabolism	3	7

Discussion

The scanty sequence information on muscle-related proteins seriously impedes the understanding of the muscle structure. Recently available EST data of *S. chuatsi* provide us an opportunity to research the flesh quality and nutritional components of this commercially important fish (*11*). Our work presents a transcript expression profile in the white muscle of the mandarin fish.

Fish skeletal muscle consists of two spatially separated fibers. The white, fast-twitch muscle makes up the bulk of the fish body, whereas the red, slow-twitch muscle founds a narrow midlateral band immediately under the skin (*19*). Many proteins are specifically expressed in muscle tissue, mainly including structural and contractile proteins (myosin, actin and troponin) as well as muscle-specific regulating factors (*20-22*). In this report, a total of 1,625 unique genes were identified in the *S. chuatsi* muscle tissue and 15 of them are highly expressed. These genes are functionally related to muscle structure, metabolism and muscle differentiation regulation. Myosin heavy chain, myosin light chain and actin are major structural genes that control muscle fiber structural formation (*16, 17*). They have several isoforms, which may reflect their adaptation to different environments (*23*). Skeletal alpha-actin is a primary component of skeletal muscle thin filament (*24*). Tropomyosin/troponin complex play a central role in the calcium-dependent regulation of vertebrate-striated muscle contraction (*17*). In striated muscle cells, tropomyosin wraps itself around actin filaments and interacts with troponin through troponin T subunit. Troponin consists of three components: troponin I, which inhibits ATPase activity of actomyosin; troponin T, which contains the binding site for tropomyosin; and troponin C, which binds calcium to abolish the inhibitory action of troponin on actin filaments (*25*). Different isoforms of

each component in tropomyosin/troponin complex can modulate sarcomeric performance by either increasing or decreasing Ca^{2+} sensitivity that regulates muscle contraction and relaxation (*26, 27*). All of these genes identified in this work indicate that the cDNA-EST technology is an efficient tool for gene discovery and expression profile establishment, and the results further strength our earlier reports (*6*).

We observed that the most abundant gene in the library is nuclease diphosphate kinase B (NM23-H2) with 449 ESTs (8.86%). Nuclease diphosphate kinase (NM23) is a protein that catalyzes phosphoryl transfer from a nucleoside triphosphate to a nucleoside diphosphate (*28, 29*). In human, eight NM23 isoforms (NM23-H1 to NM23-H8) have been detected. NM23-H2 was reported to play an important role in tumor metastasis (*30*). In the EST database of *S. chuatsi*, gene NM23-H2 encodes a full-length cDNA consisting of 149 amino acids. As we know, two other fish species (*Danio rerio* and *Gillichthys mirabilis*) also contain the NM23-H2 gene. Comparison of the NM23-H2 sequences among these three species shows that the NM23-H2 gene in *S. chuatsi* has high similarity (85%) to that in *G. mirabilis* (**Figure 2**). However, how this gene functions in fish remains to be investigated. Furthermore, fructose-bisphosphate aldolase A is also highly expressed in *S. chuatsi*. It is related to a high rate of glycolysis by catalyzing the cleavage of fructose-1,6-bisphosphate in the glycolytic pathway, giving rise to glyceraldehyde-3-phosphate and dihydroacetate phosphate. It was reported that resistance to hypoxia in fish is associated with increasing in binding of selected glycolytic enzymes to subcellular fractions (*31*). High abundant expression of the gene in *S. chuatsi* may explain why the fish is extremely tolerant of stress aquaculture environment including lower oxygen condition.

A variety of amino acid metabolism processes were detected in *S. chuatsi* and enzymes related to the metabolisms of glutamate, methionine, selenoamino acid, cysteine, tyrosine, arginine and other amino acids were identified in this study. Particularly, transcripts related to arginine and proline metabolism are extremely abundant. The creatine kinase gene is highly abundant in *S. chuatsi,* which produces a muscle-specific soluble enzyme and functions in the catalysis of ADP to form high energy ATP during muscle contraction (*32*). The existence of a variety of amino acid metabolism processes could indicate that fish is a good source of essential amino acids, as we reported earlier (*4*). Besides, many cofactor and vitamin metabolism processes were also detected, including thiamine metabolism, retinol metabolism, nicotinamide metabolism, biotin metabolism, folate biosynthesis as well as porphyrin and chlorophyll metabolism. The results suggest that there exist highly active amino acid metabolism in *S. chuatsi*, thus giving the fish unique nutritional and flesh flavor traits.

```
Homo sapiens            MAN-LERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVAMKFLRASEEHLK
Danio rerio             ..GKT......V.........M.....................Q...DL..
Gillichthys mirabilis   ----M......V.........C.D..H...KR..Q...A..IQ.T.DFM.
Siniperca chuatsi       ----M......V.........C........HR......A..MQ...D.M.

Homo sapiens            QHYIDLKDRPFFPGLVKYMNSGPVVAMVWEGLNVVKTGRVMLGETNPADS
Danio rerio             ...........Y.......S....L.......................
Gillichthys mirabilis   ...L....M..YG..C...S....F......E.I..L..M.........
Siniperca chuatsi       K..L....M..YA..C...S....L......Q.I..LA.M.........

Homo sapiens            KPGTIRGDFCIQVGRNIIHGSDSVKSAEKEISLWFKPEELVDYKSCAHDW
Danio rerio             ...........E............D..NT............SF....QQ.
Gillichthys mirabilis   ...S....L..NI.........TLEN.KR.VA.......F.T.T..FKPF
Siniperca chuatsi       ...S....L..NI.........T.EN.KM.VG....D..F.A.TP..QA.

Homo sapiens            VYE
Danio rerio             I..
Gillichthys mirabilis   L..
Siniperca chuatsi       L..
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Figure 2 Amino acid sequence alignment of NM23-H2 gene of *S. chuatsi* compared with homologs of *D. rerio*, *G. mirabilis and Homo sapiens*.

Our work provides a valuable resource for further study on those ecologically significant fish species. Application of this knowledge will reveal many candidate genes involved in metabolism and flesh quality of fish.

Materials and Methods

Tissue preparation

The mandarin fish (*S. chuatsi*) individuals were reared at Hunan Aquatic Research Institute, Changsha, China. The white muscle was dissected from adult fishes aged about 2 years with an average body weight of 500 g. The isolated muscle tissues were immediately frozen in liquid nitrogen and stored at −80°C until use.

EST assembly, annotation and analysis

A total of 5,191 EST sequences were obtained. Accession numbers of all the ESTs are GR473858 to GR479048. Vector sequences were trimmed, and sequences with length of less than 100 bp or low quality were removed. As a result 5,063 (97.5%) of the sequences were retained for further analysis. The 5,063 high-quality ESTs were assembled into contigs using the Phrap program (http://www.phrap.org/phredphrap/phrap.html), and 443 contigs and 1,182 singletons were obtained. A total of 1,625 unique sequences were searched for their similarity to other known proteins from the nr database in NCBI with BLASTX (*33*). For annotation of the unique sequences, the top hit of the BLASTX output was used. E-value of 1e-20 and 1e-05 were for significant similarity and the threshold of weak similarity, respectively. Based on the GO classification, unique sequences were assigned to "molecular function", "biological process" and "cellular component" from the GO database (*13*). E-value of 1e-05 was used as the threshold for the functional assignment. These unique sequences were submitted to the KEGG online server to acquire concrete metabolism information (http://www.genome.jp/kegg/).

The most abundant ESTs

To analyze the most abundant ESTs from the muscle cDNA library of *S. chuatsi*, we grouped the unique sequences based on a BLASTX search against the nr database. The sequences were clustered when two or more query sequences were annotated to the same gene.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 30972263 and 30771644), and the Natural Science Foundation of Hunan Province, China (Grant No. 09JJ6037 and 08jj3064).

Authors' contributions

MT, RZ and FZ performed the experiment. FD and PC analyzed the data and designed the tables and figures. FD and WC wrote the manuscript. SH and JZ designed and supervised the research. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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