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Genes expressed in Blue Fin Tuna (*Thunnus thynnus*) liver and gonads

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Abstract

Blue Fin Tuna (BFT), *Thunnus thynnus*, has been seriously endangered by global massive overfishing and by the pollution of marine environment. Feeding and fattening of caught tuna in marine cages is a recent resource, but the development of a self-sustained aquaculture activity, being independent from the supply of wild fish, is required from both industrial and conservation perspectives. At this scope, several technical problems have to be solved and the control of reproduction is the cardinal one. Beside the technological developments of farming facilities and protocols, a molecular approach seems promising for the studies of appropriate nutritional strategies, reproduction physiology and animal welfare, as well as lifestyle and response to endocrine disruptor pollutants. In this context, we have started an EST project on this species sequencing 2743, 2907, and 3014 clones from expression libraries of ovary, testis and liver, respectively, and 1499 clones from an ovary normalized library. Thanks to this project, we have identified several sequences with known function in other organisms, but not previously described in this species. Among the new genes, 712 were found only in the expression library of the ovary, 613 in that of the testis and 318 in that of the liver, while 324 additional genes were shared by two or more expression libraries; other 127 genes not found in the expression libraries were obtained from the ovary normalized library. This represents a contribution to the knowledge of the molecular basis of BFT and a necessary step for facilitating further molecular studies on this species.

Keywords: Marine biotechnology; Aquaculture; Animal biotechnology; Transcriptome; EST

1. Introduction

When compared with the studies on genomics and proteomics of other vertebrates, the data set concerning the fishes remains highly incomplete despite the complexity of phylogenetic differentiation of this group of animals. In fact, only teleosts have been more widely studied, and, among them, only two species (*Danio rerio*, and *Takifugu rubripes*) had their genome annotated and mapped (Project ID 13922 at the NCBI) (Aparicio et al., 2002). The more recent addition of a huge number of sequences from *Tetraodon nigroviridis*, a member of the same family of *T. rubripes*, extensively lacks ontogenetic annotations (Jaillon et al., 2004). Both these two members of the Tetraonidae Family are characterized by an exceptionally compressed genome, feature that, although convenient for genome sequencing projects, renders this family, as far as it concerns genome, poorly representative of fishes. Genomic and transcriptomic data, however, have been produced mainly on "model

Abbreviations: bp, Base pairs; BFT, Blue Fin Tuna; BLAST, Basic Local Alignment Search Tool; cDNA, Complementary to RNA; db, Database; dNTP, Deoxynucleotide triphosphate; DOE, US Department of Energy; ds cDNA, Double strand cDNA; DSN, Duplex specific nuclease; EBI, European Bioinformatics Institute; EST, Expressed sequence tag; *E*-value, Expectation value; EU, European Union; FAO, Food and Agriculture Organization; HPRD, Human Protein Reference Database; IPTG, Isopropyl β-D-thiogalactopyranoside; mRNA, Messenger RNA; NCBI, National Center for Biotechnology Information; PCR, Polymerase chain reaction; TEF, *Thunnus thynnus* Expression library from liver; TEO, *Thunnus thynnus* Expression library from Ovary; TET, *Thunnus thynnus* Expression library from Testis; TtN, *Thunnus thynnus* Normalized library from Ovary; T_m, Melting temperature; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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organisms" and much less on species valuable for fishing and aquaculture. This represent an obstacle for studies of functional genomics, transcriptomics and proteomics on species important for human nutrition, therefore of great commercial interest. Vice versa, an effort to reduce the gap that separates farming species from "model organisms" would offer a powerful tool for studies on global quality, control of reproduction, and assessment of life style on fish species where the fishing and farming efforts are concentrated. In this regard, we have recently produced EST collections of a well domesticated species, the sea bass *Dicentrarchus labrax*, (Chini et al., 2006) and of a species potentially adapted to farming conditions, the European perch *Perca fluviatilis* (Rossi et al., 2007). With this paper, we have taken into consideration Blue Fin Tuna (BFT), *Thunnus thynnus*, a species included in the Family of Scombridae with a great commercial value and higher nutritional characteristics. This pelagic oceanodromous species, whose habitat spreads from Artic to the subtropical regions of the northern hemisphere, has been seriously endangered by global massive overfishing and by the pollution of marine environment (Srebocan et al., 2007). Feeding and fattening of caught tuna in marine cages is a recent resource, being started only some ten years ago and which seems able to process no more than 10 thousand fish per year at the present day (FAO Fishery Statistic, www.fao.org; Ottolenghi et al., 2004; Fromentin and Powers, 2005). Of course, there is a great interest in developing the aquaculture technology also for this species. At this scope, several technical problems have to be solved and the control of reproduction is the cardinal one. In fact, the development of a self-sustained aquaculture activity, being independent from the supply of wild fish, is required from both an industrial and conservation perspective.

2. Materials and methods

2.1. Construction of cDNA libraries

Tissues were sampled from two adult individuals of *T. thynnus* obtained from New Eurofish srl tuna farm at Castellamare del Golfo, Sicily, Italy, with the kind collaboration of Biotecno soc. Coop, Marsala, Sicily, Italy. Total RNA was extracted using Trizol reagent (Invitrogen) and mRNA was enriched by oligo-(dT) using Gene Elute mRNA miniprep kit (Sigma) according to the manufacturer's instructions.

The ovary, testis and liver cDNA expression libraries and the ovary normalized library were prepared by EVROGEN JSC (www.evrogen.com) as follows. For the expression libraries, 0.15 μg of mRNA samples were used for ds cDNA synthesis using SMART approach (Zhu et al., 2001). First strand cDNA synthesis was initiated by mixing oligo-(dT) primers containing Sfi1A restriction site, CDS-Sfi1B primer and Power Script Reverse Transcriptase (BD Biosciences Clontech). The first strand cDNA was used for amplification by Long Distance PCR. Amplified cDNAs were purified using QIAquick PCR Purification kit (Qiagen, CA), then cDNA and pAL17.3 vector were digested with restriction enzymes Sfi1A and Sfi1B. cDNAs were ligated into the vector and used for *E. coli* transformation with the BioRad Micropulser (Bio-rad).

For the normalized library, total RNA was isolated from 1 g of ovary of *T. thynnus* using TRIzol (Invitrogen). The library was prepared by EVROGEN JCS (www.evrogen.com). RNA was used for ds cDNA synthesis using SMART approach (Zhu et al., 2001). SMART prepared amplified cDNA was then normalized according to Zhulidov et al. (2004). Normalization included cDNA denaturation and reassociation, treatment by duplex specific nuclease (DSN) as described by Shagin et al. (2002), and amplification of normalized fraction by PCR. The cDNAs were cloned using pGEMT-easy T/A cloning kit (Promega), transformed into XL1blue competent cells and plated into LB with ampicillin, X-Gal and IPTG.

2.2. PCR and DNA sequencing

Random clones were picked and then resuspended in 20 μL of sterile water. PCR amplification of cDNA inserts was performed using the DyNAzyme II DNA Polymerase kit (Finnzyme) according to the manufacturer's instructions; 10 μL of samples were mixed with 0.2 mM dNTPs mix, 2 μM solution of primers (T7: 5′-T A A T A C G A C T C A C T A T A G G G-3′, T_m=53.2 °C; SP6: 5′-C A T T T A G G T G A C A C T A T A G-3′, T_m=50.2 °C), 2.5 μL 10× optimized DyNAzyme reaction buffer, 0.25 μL of DyNAzyme (2 U/μL) and 10.75 μL water. The PCR mix was subjected to the following amplification cycle conditions: 94 °C for 4 min and 30 cycles at 94 °C for 30 s, annealing at 53 °C for 30 s, elongation at 72 °C for 2 min. The clones with an apparent size superior to 500 bp were subjected to sequencing reaction using T7 primer and the MEGABace DYEnamic ET DYE terminator kit (GE Healthcare). The sequencing reactions (in a total volume of 10 μL) were run using 5 pmol of T7 primer, 2 μL of sequencing reagent Premix, 100 ng of DNA at following condition: 25 cycles at 95 °C for 20 s, 50 °C for 15 s, 60 °C for 1 min. The sequencing of randomly selected *T. thynnus* cDNA clones was performed using the MEGABace 500 (GE Healthcare).

2.3. Sequence analysis

All sequences were analysed with Vector NTI 10.0 software, freely available at Invitrogen. Sequences were first subjected to 5′-trimming to eliminate the vector and, when necessary, to 3′-trimming to eliminate fragments having a higher uncertainty degree (rich in undetermined bases). Trimmed sequences were searched in the NCBI database (www.ncbi.nlm.nih.gov/BLAST) by nucleotide blast (BlastN, against non-human, non-mouse organism's nucleotide and EST collections) and by protein blast (BlastX for non-redundant protein sequences). Matches were considered to be significant only when *E*-value was less than e^{-10}. Comparisons between libraries or groups were performed with χ^2 or Student *t* test.

3. Results

Of the three expression libraries of *T. thynnus*, we have sequenced 8664 clones: 2743 clones from the ovary expression library (TEO) deposited in the NCBI Expressed Sequence Tags