Generation of a large scale repertoire of Expressed Sequence Tags (ESTs) from normalised rainbow trout cDNA libraries
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Abstract

Background: Within the framework of a genomics project on livestock species (AGENAE), we initiated a high-throughput DNA sequencing program of Expressed Sequence Tags (ESTs) in rainbow trout, *Oncorhynchus mykiss*.

Results: We constructed three cDNA libraries including one highly complex pooled-tissue library. These libraries were normalized and subtracted to reduce clone redundancy. ESTs sequences were produced, and 96,472 ESTs corresponding to high quality sequence reads were released on the international database, currently representing 42.5% of the overall sequence knowledge in this species. All these EST sequences and other publicly available ESTs in rainbow trout have been included on a publicly available Website (SIGENAE) and have been clustered into a total of 52,930 clusters of putative transcripts groups, including 24,616 singletons. 57.1% of these 52,930 clusters are represented by at least one Agenae EST and 14,343 clusters (27.1%) are only composed by Agenae ESTs. Sequence analysis also reveals that normalization and especially subtraction were effective in decreasing redundancy, and that the pooled-tissue library was representative of the initial tissue complexity.

Conclusion: Due to present work on the construction of rainbow trout normalized cDNA libraries and their extensive sequencing, along with other large scale sequencing programs, rainbow trout is now one of the major fish models in term of EST sequences available in a public database, just after Zebrafish, *Danio rerio*. This information is now used for the selection of a non redundant set of clones for producing DNA micro-arrays in order to examine global gene expression.

Background

Rainbow trout, *Oncorhynchus mykiss*, is an important fish species for aquaculture and has been introduced throughout the world. It is also probably one of the most widely studied fish species with a long history of research carried out in physiology, nutrition, ecology, genetics, pathology, carcinogenesis and toxicology (reviewed in [1]). Its relatively large size compared to model fish like zebrafish or medaka, makes rainbow trout a particularly suited alternative model to carry out biochemical and molecular studies on specific tissues or cells that are impossible to decipher in small fish models. The genomic resources in...
rainbow trout are now being extensively developed and a few high-throughput DNA sequencing programs of ESTs have been recently initiated [2, 3]. AGENAE (Analyse du GENome des Animaux d’Elevage) [4] is a project led by the French National Institute for Agricultural Research (INRA), that focuses on genomics of several livestock species (cattle, pigs, chickens and rainbow trout). The objectives of this program are the identification and characterization of the expressed part of genomes, the mapping of entire genomes, and the study of genetic diversity in animal populations. As a first step for the characterization of the expressed part of the genome of rainbow trout, we initiated a high-throughput EST sequencing program. Among other interests, this resource will allow large scale expression profiling experiments using microarrays based on a well characterized cDNA clone collection.

Results and discussion
cDNA libraries construction and characterization
We constructed three directionally cloned rainbow trout cDNA libraries: two from reproductive tissues i.e., ovarian (previtellogenesis) and testicular (gonial proliferations) tissues, and one highly complex pooled tissue cDNA library. The pooled tissue library was made in order to be as representative as possible of the entire expressed genome of rainbow trout. For this purpose, mRNA from 14 different tissues (liver, kidney, adipose tissue, gills, intestine, pituitary, brain, ovary, testes, differentiating male and female gonads, muscle, interrenal and blood cells), sampled at different developmental stages or in different physiological conditions, and mRNA from entire eyed-stage embryos and hatching larvae, were used for this pooled-tissue library construction. The three resulting libraries displayed a high initial clone complexity (>1 × 10^6 colony-forming units). Approximately 98% of the cDNA inserts were larger than 450 bp and the average insert size ranged between 1.3 and 1.5 kb depending on the library. Each of the 3 libraries was normalized according to previously described protocols [5, 6], in order to decrease the representation of abundant mRNA. All normalized libraries were subsequently submitted to one (testis library) or two (pooled-tissue library) runs of subtraction with the already sequenced clones in order to decrease redundancy.

ESTs sequencing
High-throughput EST sequencing was carried out on these initial, normalized and subtracted libraries (Table 1). The pooled-tissue library was the most extensively sequenced with 82% of the total sequencing effort (88 704 reads) as this library was not focused on a particular biological function, and thus of broad interest for a vast community of physiologists. The testis library was also quite extensively sequenced (13 825 reads) as this library was found to be very informative in terms of production of new sequences, while the sequencing of the ovary library was interrupted after a first round of sequencing (5 376 reads) as this library was found to be very redundant (see below in "Redundancy and quality of the libraries"). Until now, a total of 107 904 reads sequences have been performed on 84 864 clones; among those, 88.9% were found valid (96 472 sequences corresponding to high quality sequence reads of at least 100 bp with a Phred score over 20). All the valid sequences have been released in international databanks (EMBL, GenBank). The proportion of empty vectors was found to be very low.

In order to provide an important set of well annotated clones, the 5’ end sequencing strategy was favoured. However, due to the use of an excess of oligo(dT) during the first reverse transcription of the library construction, the polyA sequence remained short enough to allow sequencing of the cDNA 3’ ends. A 3’ end sequencing strategy was therefore carried out on 23 040 (27.1%) of the sequenced clones with a good success rate (83.1% of good quality released sequences). This 3’ strategy is a useful way to distinguish genes in a closely related family using the more divergent 3’ end non coding region.

Sequence analysis
Influence of normalization/subtraction on the pooled-tissue library
Following EST sequence assembly into putative transcript groups (EST clusters), we calculated the percentage of novel EST clusters as a function of the number of clones sequenced (Fig. 1). Before normalization (1 000 sequenced clones, see insert in Fig. 1), the percentage of new clusters decreased very rapidly. Normalization considerably slowed down this decrease and induced a 10% increase of novel clusters. Before the first subtraction, a few large clusters were however still observed, including a
high percentage of the sequenced clones encoding putative orthologs of trypsin (for instance more than 300 ESTs for a trypsin 1 precursor homolog). The fact that these sequences were overrepresented in the library, even after normalization, was surprising, and we do not have any rational explanation. However, during the first subtraction we specifically removed these abundant clones by increasing their representation as driver DNA in the subtraction protocol [5]. After this subtraction, the EST analysis in the pooled-tissue library revealed that only 2 clones out of 48 800 (0.004%) belonged to these trypsin clusters. This demonstrated the efficiency of the specific subtraction strategy in decreasing redundancy. As a matter of fact, the gain of novelty was then rather high following the first subtraction (55%), probably due to the removal of these largest clusters. Following the second subtraction the gain in novelty was around 15–20%.

Redundancy and quality of the libraries

With regards to clone redundancy, we listed the 20 biggest clusters considering all publicly available ESTs (Table 2), and the 20 biggest clusters containing only Agenae ESTs (Table 3). Among these clusters, the 2 biggest ones correspond to ESTs which are highly represented in (BX072800.1.p.om.3) or specific to (CR361581.1.p.om.3) the Agenae ovary library. The best Swissprot hit for cluster BX072800.1.p.om.3 corresponds to a zona pellucida sperm-binding protein precursor, a protein that is known to be highly expressed in the ovary [7] and whose cDNA has already been described as overrepresented in a fish ovary library [8]. Cluster CR361581.1.p.om.3 does not exhibit any significant homology and a careful examination of the ESTs belonging to this cluster show that 98.5% of these ESTs actually start at exactly the same nucleotide position, which probably reflects an amplification artefact that occurred during the process of library construction. Similarly, 10 out of the 20 biggest clusters of ESTs specific to the Agenae libraries are also either specific to, or overrepresented in this ovary library (Table 3). Due to this rather poor quality in terms of novel sequence discovery, we stopped sequencing this library. Apart from these highly redundant ESTs from the ovary library, redundancy was found to be relatively low in other Agenae libraries with the vast majority of the EST sequences within cluster classes containing less than 33 ESTs (Fig. 2). As shown in table 2, the pooled-tissue library produced 2 of the biggest trout clusters. One cluster (CA369471.1.p.om.3; 389 ESTs) corresponds to a homolog of the zebrafish protein ES1 that is specifically expressed in the adult retina [9]. The other (CA368365.1.p.om.3; 342 ESTs) is one of the large clusters of putative orthologs of trypsin overrepresented in the non subtracted pooled-tissue library mentioned above. No highly redundant clusters of ESTs were identified in the testis library with the largest clusters represented by only 4 ESTs (CR362356.1.p.om.3, CR370007.1.p.om.3 and CR365721.1.p.om.3). This demonstrated the high quality of this testis library in terms of diversity and probably reflects the high complexity of the repertoire of genes expressed in testicular tissue as previously shown in large scale ESTs sequencing programs in other fish species [8,10]

Contribution of the Agenae EST collections

The overall released data, including sequences from the testis and ovary libraries, represented 42.5 % of the total rainbow trout sequences (96 472 over 226 825) in international databases in February 2006. Based on the SIGENAE trout assembly version 3 [11], our EST sequences added 14 343 unique clusters (27.1%) to the total of 52 930 clusters of putative transcript groups characterized after clustering of all ESTs available in rainbow trout (Fig. 3). Among these 52 930 clusters (including 24 616 Singletons), 30 221 (57.1 %) were represented by at least one Agenae EST. These figures were obtained by running CAP assemblies under default parameters (our values were: at least 75 bp with 96 % similarity), and are close to those found in the TIGR clustering. However, we do realise that the number of contigs (52 930) can vary when different stringencies are used, and that when using the default parameters there may be cases where paralogous genes and certainly alleles are clustered, although we have not yet gone deeply into that subject. The UNIGENE cluster number is smaller (32 400), may be in part because it contains more paralogous gene clustering, but more likely because it does not use a large part of the singletons available.

Figure 1

Percentage of novel EST clusters found as a function of the number of clones sequenced in the rainbow trout pooled-tissue cDNA library. N = Normalization. S1 = First subtraction. S2 = Second subtraction.
Sequence annotation

For 12 out of the 14 single organs initially gathered to construct the pooled-tissue library, a rapid search identified at least one EST matching for a gene considered as "specifically" expressed in this tissue (Table 4). This shows that this library was potentially representative of the various tissues used, although we do realize that some of these cited genes are probably not strictly tissue specific but could be better described as genes which are highly expressed in a particular tissue. Low abundance cDNA may be difficult to detect through such EST sequencing projects. However, the fact that most genes are expressed in many tissues, combined with the normalization procedure probably increased the chances of picking them up in such a pooled-tissue library.

Although cDNA library construction and EST sequencing is a time and money consuming task, the most common strategy still consists in sequencing numerous tissue specific libraries in order to provide a large number of clusters. For instance, in the medaka, Oryzias latipes, 26 689 clusters were generated from 147 802 EST obtained from 29 different tissue specific cDNA libraries (TIGR gene Index, Release 5.0, May 17, 2004). In trout, with slightly more ESTs (157 116) coming mainly from 2 pooled-tissue libraries (AGENAE and 1RT-NCCCWA USDA), the last TIGR clustering (Release 5.0, January 31, 2005) detected twice as many clusters (50 773). The pooled-tissue libraries strategy combined with normalization/subtraction methods may thus be a better approach for enrichment of different transcripts. Actually, some recent EST projects rely on pooled-tissue libraries [2,12,13]. However the pooled-tissue strategy suffers from a lack of information concerning mRNA tissue origin and it is then not possible to carry out in silico analysis of tissue differential expression [14]. A strategy based on pooled-tissue library with a tissue specific DNA identification tag, has recently been proposed [13]. This would combine the advantages of the pooled-tissue library with keeping the information on the tissue origin of each EST.

Conclusion

In conclusion our rainbow trout cDNA libraries provided a large set of well characterized clones for future studies. The Agenae sequencing project together with ongoing collaborative efforts of the ARS-USDA program [2] and the Genome BC project [3] now places rainbow trout in the position of being one of the major fish models, in terms of ESTs present, in public databases just after the zebrafish, Danio rerio (for instance 24 466 clusters in Unigene Build #17 09 Feb 2006 for rainbow trout and 32 400 clusters for zebrafish in UniGene Build #89, 05 Dec

Table 2: Top 20 most redundant EST clusters.

| Cluster Name | Cluster depth | Best swissprot hit | Hit description | Over-expressed in Agenae libraries |
|--------------|---------------|--------------------|-----------------|-----------------------------------|
| BX072800.p.3 | 1463          | P48831             | Zona pellucida sperm-binding protein 3 precursor | Yes (Ovary) |
| CR361581.p.3 | 1460          | NULL               |                 |                                   |
| CA352033.p.3 | 1434          | P10995             | Actin, alpha sarcomeric/cardiac (Actin alpha 2) | No |
| BX073087.p.3 | 431           | P21993             | Prolactin precursor (PRL) | No |
| CA342041.p.3 | 402           | P02141             | Hemoglobin beta-4 subunit | No |
| CA369471.p.3 | 389           | P30042             | EST protein homolog, mitochondrial precursor (Protein KNP-I) | Yes (Pooled-Tissue) |
| CA368365.p.3 | 342           | P35031             | Trypsin I precursor | Yes (Pooled-Tissue) |
| BX074651.p.3 | 314           | P20322             | Somatotropin 2 precursor (Growth hormone 2) | No |
| CA341574.p.3 | 264           | P35979             | 60S ribosomal protein L12 | No |
| CA343108.p.3 | 255           | P02609             | Myosin regulatory light chain 2, skeletal muscle isoform (G2) | No |
| BX073970.p.3 | 224           | P07064             | Somatotropin precursor (Growth hormone) | No |
| CA341678.p.3 | 197           | P70083             | Sarcoplasmic/endoplasmic reticulum calcium ATPase I | No |
| CA341906.p.3 | 178           | Q90Y7              | 60S ribosomal protein L11 | No |
| CA341950.p.3 | 173           | Q90Z10             | 60S ribosomal protein L13 | No |
| CA341578.p.3 | 170           | P0AFB7             | Nitrogen regulation protein NR(II) | No |
| CA345253.p.3 | 161           | P47954             | Glutathione S-transferase P | No |
| CA341772.p.3 | 159           | O57561             | 60S ribosomal protein L18a | No |
| CA342754.p.3 | 157           | Q91408             | 60S ribosomal protein L13a (Transplantation antigen | No |
| CA342547.p.3 | 155           | P02457             | Collagen alpha 1(I) chain precursor | No |
| CA378499.p.3 | 153           | NULL               |                 | No |

The 20 most redundant EST clusters in all rainbow trout cDNA libraries are listed with their Sigenae cluster name and the number of ESTs within each cluster (cluster depth). When a homology search using blastx was carried out against the Swissprot database returned a significant homology (blast score > 100), the accession number of this best putative homolog is given along with its associated description. When a cluster contains an over-representation of ESTs found only in one Agenae library, the name of this library is given in the last column.
We are now using this important sequence information and our corresponding clone collections for producing DNA arrays in order to examine global gene expression in rainbow trout [15,16]. A micro-array, containing 9,000 well annotated and unique cDNAs chosen for their informative annotation and their low redundancy, is now produced in large numbers in our resource centre (CRB-GADIE) [17], and used for gene expression profiling by several research teams.

Methods

Tissues samples and RNA preparation
Research involving animal experimentation has been approved by the authors' institution (authorization number 35-14) and conforms to the principles for the use and care of laboratory animals in compliance with French and European regulations on animal welfare. Rainbow trout were obtained from the Drennec experimental farm (Drennec, France). For the pooled-tissue cDNA library, more than 30 different individual fish of both sexes, issued from 3 different strains (autumnal, spring and winter spawning strains) were used; these strains themselves

Table 3: Top 20 most redundant Agenae specific EST clusters.

| Cluster Name           | Cluster depth | Best swissprot hit | Hit_description                                                                 | Over-expressed in a specific Agenae library |
|------------------------|---------------|--------------------|----------------------------------------------------------------------------------|---------------------------------------------|
| CR361581.1.p.om.3      | 1460          | NULL               | Yes (Ovary)                                                                      |                                             |
| CR361588.1.p.om.3      | 139           | NULL               | Yes (Ovary)                                                                      |                                             |
| BX304386.1.p.om.3      | 63            | Q9PP2              | Zinc finger protein 318 (Testicular zinc finger protein)                         | Yes (Ovary)                                 |
| BX871489.1.p.om.3      | 59            | NULL               | Yes (Ovary)                                                                      |                                             |
| BX310674.1.p.om.3      | 49            | NULL               | No                                                                               |                                             |
| CR361690.1.p.om.3      | 48            | Q9IY70             | NADH-ubiquinone oxidoreductase 51 kDa subunit                                    | Yes (Ovary)                                 |
| BX300811.1.p.om.3      | 48            | Q94480             | VEG136 protein (Fragment)                                                         | Yes (Pooled-Tissue)                         |
| BX304242.1.p.om.3      | 40            | P98165             | Very low-density lipoprotein receptor precursor                                  | No                                           |
| BX310459.1.p.om.3      | 37            | Q9ESN4             | Complement C1q-like protein 3 precursor (Gliacolin)                              | No                                           |
| CR361738.1.p.om.3      | 36            | NULL               | Yes (Ovary)                                                                      |                                             |
| BX300472.1.p.om.3      | 36            | NULL               | No                                                                               |                                             |
| CR361612.1.p.om.3      | 33            | Q8N6G5             | Chondroitin beta-1, 4-N-acetylgalactosaminyltransferase 2                         | Yes (Ovary)                                 |
| CR361716.1.p.om.3      | 33            | P28570             | Sodium- and chloride-dependent creatine transporter I                            | Yes (Ovary)                                 |
| BX860653.1.p.om.3      | 33            | NULL               | Yes (Ovary)                                                                      |                                             |
| BX321717.1.p.om.3      | 32            | P50416             | Carnitine O-palmitoyltransferase I, mitochondrial liver isoform                   |                                             |
| BX304069.1.p.om.3      | 31            | Q9NR09             | Baculoviral IAP repeat-containing protein 6                                       | No                                           |
| BX321042.1.p.om.3      | 30            | Q9R190             | Metastasis-associated protein MTA2                                                | Yes (Pooled)                                |
| BX299242.1.p.om.3      | 28            | O08849             | Regulator of G-protein signaling 2 (RGS2)                                         | Yes (Pooled)                                |
| BX317551.1.p.om.3      | 28            | Q9HYK0             | Endonuclease III (DNA-(apurinic or apyrimidinic site) lyase)                      | Yes (Ovary)                                 |
| BX298697.1.p.om.3      | 28            | NULL               | Yes (Pooled-Tissue)                                                              |                                             |

The 20 most redundant EST clusters composed of only ESTs originated from Agenae rainbow trout cDNA libraries are listed with their Sigenae cluster name and the number of ESTs within each cluster (cluster depth). When homology search using blastx against the Swissprot database returned a significant homology (blast score > 100) the accession number of this best putative homolog is given along with its associated description. When a cluster contains an over-representation of ESTs found in only one Agenae library, the name of this library is given in the last column.

Figure 2

Histogram of cluster sizes. Repartition in the different cluster size classes of the complete collection of trout clusters (black squares) and of Agenae specific clusters (open squares), both expressed in proportion of all clusters, based on Sigenae rainbow trout EST clustering version 3. The table represented the number of clusters in each cluster size class.

Histogram of cluster sizes

Figure 2

Classes (EST numbers)

| Classes | All | Agenae |
|---------|-----|--------|
| 1       | 24616| 9009   |
| 2       | 8610 | 2845   |
| 3-4     | 7580 | 1650   |
| 5-8     | 6026 | 630    |
| 9-16    | 3816 | 160    |
| 17-32   | 1660 | 35     |
| 33-64   | 477  | 12     |
| 65-128  | 110  | 0      |
| 129-256 | 26   | 1      |
| 257-512 | 6    | 0      |
| 513-1024| 0    | 0      |
| >1025   | 3    | 1      |

2005). We are now using this important sequence information and our corresponding clone collections for producing DNA arrays in order to examine global gene expression in rainbow trout [15,16]. A micro-array, containing 9,000 well annotated and unique cDNAs chosen for their informative annotation and their low redundancy, is now produced in large numbers in our resource centre (CRB-GADIE) [17], and used for gene expression profiling by several research teams.
originated from at least 3 different French or Belgium regions. The following tissues, obtained at different stages of their development for several of them, were sampled and stored at -80°C before RNA purification: liver, kidney, adipose tissue, gills, intestine, pituitary, brain, ovary, testes, early differentiating male and female gonads, muscle, interrenal, leucocytes, blastula embryos, eyed-stage and hatching larvae, skin and blood cells. For the testis and ovary libraries, testes contained only spermatogonia (Stage I and II according to Billard’s classification [18]), and the ovary was at the previtellogenesis stage. Total RNA was extracted from each frozen tissue using TRIzol™ reagent (Gibco BRL, Gaithersburg, MD). The quality of total RNA was first checked by electrophoresis on a 1% agarose gel, then by a reverse transcription test using trace amounts of [α-32P] dCTP [19]. The radioactive cDNA obtained was analyzed by autoradiography after electrophoresis on a denaturing alkaline agarose gel. Some total RNA samples (originating from blastula embryos, leucocytes, and skin) were found to be unsuitable for oligo(dT) primed reverse transcription and were not incorporated into the pool of total RNA used for the final construction of the pooled-tissue cDNA library. Total RNAs from the 14 tissues (liver, kidney, adipose tissue, gills, pituitary, brain, ovary, testes, differentiating gonads, muscles, intestine, interrenal and blood cells) plus entire eyed stage embryo, and hatching larvae RNAs were pooled in equal proportions. Poly-A-selected mRNA was prepared by purification of pooled total RNA on a oligo(dT) – cellulose column as previously described [19]. Quality of mRNA purification was checked by electrophoresis of a small aliquot on 1% agarose and by a reverse transcription test using trace amounts of [α-32P] dCTP.

Table 4: Tissue representation in the pooled-tissue cDNA library.

| Tissues       | Protein                  | Species       | References | TBLASTN | BLASTX | Sequence ID                  |
|---------------|--------------------------|---------------|------------|---------|--------|------------------------------|
| Testis        | Testis Creatine kinase   | O.mykiss      | [23]       | 0       | /      | tccao001c.e.17 (BX073510)    |
| Ovary         | Factor in germ line alpha (Figa) | M. musculus | [24]       | 89, 7e-19 | 89, 9e-17 | tccad001a.a.19 (BX074064–BX074066) |
| Adipose tissue| Perilipin (PLIN)         | H. sapiens    | [25]       | 197, 7e-51 | 197, 2e-49 | tccbc0010c.i.14 (BX877787)    |
| Kidney        | Collectrin               | H. sapiens    | [26]       | 141, 1e-34 | 141, 1e-32 | tccy0014b.i.05 (BX304379–BX304380) |
| Fetal gonads  | DMRT1                    | O.mykiss      | [27]       | 0       | /      | tccad0009a.n.11 (BX081098–BX081099) |
| Liver         | Liver-basic fatty acid binding protein | D. rerio     | [28]       | 222, 1e-59 | 222, 3e-57 | tccay0037b.e.14 (BX319797–BX319797) |
| Gill          | FHL5                     | A. japonica   | [29]       | 399, 1e-12 | 404, 1e-117 | tccad002a.a.17 (BX074365)    |
| Pituitary     | Growth Hormone factor 1 (Pit-1) | O. keta      | [30]       | 156, 4e-40 | 156, 2e-38 | tccay007b.j.13 (BX301445–BX301446) |
| Blood         | ERMAP                    | H. sapiens    | [31]       | 163, 1e-40 | 163, 9e-43 | tccbc0052c.i.01 (BX885983) |
| Brain         | Brain cell membrane protein 1 (BCMP1) | C. familiaris | [32]       | 369, 3e-73 | 247, 2e-64 | tccbc0029c.j.07 (BX885584) |
| Intestine     | Intestinal mucin-like peptide | R. Norvegicus | [33]       | 217, 2e-56 | 194, 1e-48 | tccac0004c.g.02 (BX083186–BX083187) |
| Muscle        | Muscle LIM protein       | R. Norvegicus | [34]       | 315, 4e-97 | 270, 1e-71 | tccba0018c.g.07 (BX861190) |

Representative “tissue specific” protein homologs in the pooled-tissue cDNA library. Clone identity (clone ID) is given in the sequence ID column with the GenBank accession numbers in brackets. When more than one sequence was carried out on one clone (5’ and 3’ EST sequences), the second accession number is noted -X. For rainbow trout EST matching a rainbow trout gene only a blastN strategy was used.
Library construction

cDNA libraries were constructed in the pT7T3-Pac vector as initially described by B. Soares, M. Bonaldo and collaborators [5,6,19]. Briefly, starting from the mRNA, cDNA synthesis was carried out with a NotI-dT18 primer to allow directional cloning. After size selection chromatography (≥ 500 bp), the double-stranded cDNA were ligated to EcoRI adapters, digested with NotI, and directionally cloned into the NotI and EcoRI digested pT7T3-Pac vector. The library was electroporated and then amplified in DH10B competent cells (Invitrogen). Normalization and subtraction was carried out according to [19]. Briefly, single strand DNA circles were produced from the directional cDNA libraries (tester DNA). These single strand DNA circles were also used to produce doubled strands DNA (driver DNA) corresponding to the inserts, by PCR using vector primers T7 and T3, flanking the insertion sites. Tester DNA was then melted and reannealed with an excess of driver DNA and the remaining single strand driver DNA (normalized or subtracted library) was then purified by hydroxyapatite chromatography. These single strands DNA molecules were then converted to partial duplex by random priming and electroporated into bacteria to produce the final normalized or subtracted library (see [19] for additional details).

The cDNA mean insert sizes of the libraries have been estimated on 50 individual clones by PCR using T3 and T7 as primers flanking the inserts.

Sequencing

The libraries were plated onto 2XY medium and arrayed robotically into 96 well plates at the INRA National Biological Resources Centre for Animal Genomics (CRB GADIE) [17]. Plates were then sent to a sequencing company [20], and bacterial clones were sequenced following plasmid DNA purification with T7 primer for 5' end sequencing and T3 primer for 3' end sequencing.

Sequence analysis and EST clustering

EST sequences were cleaned from vector and adaptor sequences and sequences containing contaminants such as E. Coli, Yeast, Mitochondria, Ribosome or Univec were removed from the analysis. Only sequences with a PHRED score over 20 on at least 100 bp were released in the EST division of the EMBL-EBI (European Molecular Biology Laboratory – European Bioinformatic Institute) Nucleotide Sequence Database. The calculation of the redundancy and proportion of clusters generated by the different EST sequencing projects was carried out using the SIGENAE trout clustering version V3 [11]. The percentage of novelty in the pooled-tissue cDNA library was calculated as follows: knowing that some clones have been sequenced at both ends (5' and 3') one representative sequence was selected for each clone (the selected sequence was the 3' end if it existed). Then the clones were ordered by name for each block of clones [using an incremental step of 400 clones] and the number of clusters was counted. The figures shown in the graph are therefore the number of new clusters generated by the 400 next sequenced clones. This work was done using an R [21] home made routine extracting data from a PostgreSQL database. Sequences corresponding to putative "tissue specific" proteins in the pooled-tissue cDNA library were found using a best blast hit strategy for the approximation of orthologs rainbow trout ESTs. Tissue specific proteins were chosen according to their description as "tissue specific" in the literature and their amino-acid sequence was used to search at NCBI [22] for a putative orthologs in rainbow trout using a TblastN algorithm on Database "EST-others" with a query limited by the term "Oncorhynchus" and other parameters set to default. The best hit sequence was then double checked by a blastx query on a non-redundant Database. For already known tissue specific genes in rainbow trout a blastn query was carried out and EST sequences showing 100% identity were selected.

Authors’ contributions

MG carried out most of the work needed for construction and normalization of the cDNA libraries with substantial help from FLG and YG. FLG and YG conceived the study, participated in its design and coordination. YG also drafted the manuscript. All authors read and approved the final manuscript.

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References

1. Thorgaard GH, Bailey GS, Williams D, Buhler DR, Kaattari SL, Ristow SS, Hansen JD, Winton JR, Bartholomew JL, Nagler J, Walsh P, Vijayan MM, Devlin RH, Hardy RW, Overturf KE, Young WP, Robison BD, Rexroad C, Pati Y. Status and opportunities for genomics research with rainbow trout. Comp Biochem Physiol B Biochem Mol Biol 2002, 133(4):609-46.
2. Rexroad CE 3rd, Lee Y, Keele JW, Karamycheva S, Brown G, Koop B, Gahr SA, Pati Y, Quackenbush J. Sequence analysis of a rainbow trout cDNA library and creation of a gene index. Cytogenet Genome Res 2003, 102(1-4):347-54.
3. Rise ML, von Schallburg KR, Brown GD, Mawer MA, Devlin RH, Kuipers N, Busby P, Beets-Sargent M, Alberto R, Gibbins AR, Hunt P, Shukin R, Zeznik JA, Nelson C, Jones SR, Smallus DE, Jones SJ, Schein JE, Marra MA, Butterfield YS, Scott JM, Ng SH, Davidson WS, Koop
BF: Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. Genome Res 2004, 14(3):478-90.

4. AGENAE [http://www.inra.fr/agenae/]

5. Soares MB, Bonaldo MF, Jelene P, Su L, Lawson L, Efratiadis A: Construction and characterization of a normalized cDNA library. Proc Natl Acad Sci USA 1994, 91(20):9228-32.

6. Bonaldo MF, Lennon G, Soares MB: Normalization and subtraction: two approaches to facilitate gene discovery. Genome Res 1996, 6(9):791-806.

7. Conner SJ, Hughes DC: Analysis of fish ZP1/ZPB homologous genes – evidence for both genome duplication and species-specific amplification models of evolution. Reproduction 2003, 126:347-352.

8. Zeng S, Gong Z: Expressed sequence tag analysis of early gametogenesis in rainbow trout.genome Res 2002, 12(1–2):45-53.

9. Zhang H, Gilbert W: A Novel Zebrafish Gene Expressed Specifically in the Photoreceptor Cells of the Retina. Biochem Biophys Res Commun 1997, 237:84-89.

10. Davey GC, Caplice NC, Martin SA, Powell R: A survey of genes in the Atlantic salmon (Salmo salar) as identified by ExQuest, a specific amplification models of evolution. Reproduction 2003, 126:121-130.

11. SIGENAE [http://www.sigenae.org/]

12. Smith TP, Grosse WM, Freking BA, Roberts AJ, Stone RT, Casas E, Wray JE, White J, Cho J, Fahrenkrug SC, Bennett GL, Heaton MP, Lagneid WW, Rohrer GA, Chikto-Mickown CG, Pertaie G, Holt J, Kavaymchega S, Lianf F, Quackenbush J, Kaelj J. Sequence evaluation of four pooled-tissue normalized bovine cDNA libraries and construction of a gene index for cattle. Genome Res 2001, 11(4):626-30.

13. Gavin AJ, Schleitz TE, Roberts CA, O'Leary B, Braun TA, Sheffield VC, Soares MB, Robinson JP, Casavant TL: Pooled library tissue tags for EST-based gene discovery. Bioinformatics 2002, 18(9):1162-6.

14. Brown AK, Kai K, May ME, Brown DC, Roopenian DC: ExQuest, a novel method for displaying quantitative gene expression from ESTs. Genomics 2004, 83(3):528-39.

15. Baron D, Houlgatte R, Fostier A, Guiguen Y: Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 2005, 73:959-966.

16. Zhang H, Montfort J, Delalande C, Le Gac F: Transcriptional analysis of testsis maturation using trout cDNA macroarrays. Gen Comp Endocrinol 2005, 142:143-154.

17. GADIE Biological Resources Centre [http://w3.jouy.inra.fr/unites/lreg/CNB/BRC/index.html]

18. Ballard R: Spermatogenesis and spermatology of some teleost fish species. Reproduction Nutrition Development 1986, 26:877-920.

19. Soares M, Bonaldo M: Constructing and screening normalized cDNA libraries. In Genome analysis: a laboratory manual: detecting genes Edited by: Birren B, Green E, Klapholz S, Myers R, Roskams A. Cold Spring Harbor. Laboratory Press; 2000:49-157.

20. Millegen [http://www.millegen.com/]

21. The Comprehensive R Archive Network [http://cran.r-project.org/]

22. The National Center for Biotechnology Information Basic Local Alignment Search Tool [http://www.ncbi.nlm.nih.gov/BLAST/]

23. Garber AT, Winkfein RJ, Dixon GH: A novel creatine kinase cDNA whose transcript shows enhanced testicular expression. Biochem Biophys Acta 1990, 1087(2):256-8.

24. Billard L, Soylay SM, Dean J: FIGAlph, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. Development 1997, 124(24):4939-4947.

25. Nishii J, Tanaka T, Nakamura Y: Isolation and chromosomal mapping of the human homolog of perilipin (PLIN), a rat adipose tissue-specific gene, by differential display method. Genomics 1998, 48(2):254-7.

26. Zhang H, Wada J, Hida K, Tsuchiya Y, Hiragushi K, Shikata K, Wang H, Lin S, Kanwar YS, Makino H: Collectrin, a collecting duct-specific transmembrane glycoprotein, is a novel homolog of ACE2 and is developmentally regulated in embryonic kidneys. J Biol Chem 2001, 276(20):17132-9.

27. Marchand O, Govoroun M, D'Costa H, McMeel O, Lareyre J, Bernot A, Laudet V, Guiguen Y: DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, Oncorhynchus mykiss. Biochim Biophys Acta 2000, 1493(1–2):180-7.

28. Denovan-Wright EM, Pierce M, Sharma MK, Wright JM: cDNA sequence and tissue-specific expression of a basic liver-type fatty acid binding protein in adult zebrafish (Danio rerio). Biochim Biophys Acta 2000, 1492(1):227-32.

29. Mistry AC, Kato A, Tran TH, Honda S, Tsukada T, Takei Y, Hirose S: FHL5, a novel actin fiber-binding protein, is highly expressed in gill pillar cells and responds to wall tension in eels. Am J Physiol Regul Integr Comp Physiol 2004, 287(5):R1141-54.

30. Ono M, Takayama Y: Structures of cDNAs encoding chum salmon pituitary-specific transcription factor, Pit-1/GHF-1. Gene 1992, 116(3):275-279.

31. Xu H, Foltz L, Sha Y, Madlandsacy MR, Cain C, Lindemann G, Vargas J, Nagy D, Harriman B, Mahoney W, Schueler PA: Cloning and characterization of human erythroid membrane-associated protein, human ERMAP. Genomics 2001, 76(1–3):2-4.

32. Christophe-Hobertus C, Szpirer C, Guyon R, Christophe D: Identification of the gene encoding brain cell membrane protein 1 (BCMP1), a putative four-transmembrane protein distantly related to the peripheral myelin in protein 22/epithelial membrane proteins and the claudins. BMC Genomics 2001, 2(3):1471-2164.

33. Chen X, Hu W, Tanaka Y, Nakamura Y: Development and application of a salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. Genome Res 2004, 14(3):478-90.

34. Arber S, Halder G, Caroni P: Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. Cell 1994, 79(2):221-231.