COMPARATIVE EXPRESSION ANALYSIS IN MATURE GONADS, LIVER AND BRAIN OF TURBOT (*Scophthalmus maximus*) BY cDNA-AFLPS

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Abstract

Turbot is one of the most important farmed fish in Europe. This species exhibits a considerable sexual dimorphism in growth and sexual maturity that makes the all-female production recommended for turbot farming. Our knowledge about the genetic basis of sex determination and the molecular regulation of gonad differentiation in this species is still limited. Our goal was to identify and compare gene expression and functions between testes and ovaries in adults in order to ascertain the relationship between the genes that could be involved in the gonad differentiation or related to the sex determination system. The identification of differentially expressed sex related genes is an initial step towards understanding the molecular mechanisms of gonad differentiation. For this, we carried out a transcriptome analysis based on cDNA-AFLP technique which allowed us to obtain an initial frame on sex-specific gene expression that will facilitate further analysis especially along the critical gonad differentiating period. With the aim of widening the study on sex-biased gene expression we reproduced the same experiments in two somatic tissues: liver and brain. We have selected the liver because it is the most analyzed one regarding sexual dimorphic gene expression and due to its importance in steroid hormones metabolism and brain because the functional relationship between brain and gonad is documented. We found slight but important differences between sexes which deserve further investigation.

1. Introduction

Life stages knowledge is essential to reach a profitable animal domestication. Reproduction control is of special interest since it allows managing the number and timing of offspring for commercial purposes (Cnaani and Levari-Sivan, 2009). Nowadays the mechanisms controlling sex determination in fish are still poorly understood and the SD region has been identified in only eight fish including both model and aquaculture species (review in Penman and Piferrer, 2008)

A wide sprectrum of reproductive strategies exists in fish ranging from strict gonochorism to hermaphroditism (Devlin and Nagahama, 2002). Also, teleosts have the most diversified sex determination system and thus evolutionary proximity does not mean that species share the same sex determination mechanism (Yamazaki, 1983; Luckenbach et al., 2009). The phenotypic sex of an individual is the result of a combination of two processes: sex determination and sex differentiation. Sex determination, the processes by which the sex of an individual is established at the first stages of development, may be genetic (GSD), environmental (ESD) or a combination of both (Penman and Piferrer, 2008). The development toward an ovary or a testis from an undifferentiated gonad in response to a sex determination signal is referred to as sex differentiation (Siegfried, 2010). The mechanism that determines whether the bipotential gonad will differentiate as a testes or an ovary is one of the most plastic processes in the evolutionary developmental biology (Herpin and Schartl, 2008). Unlike sex determination, the process of gonad sex differentiation is conserved in vertebrates involving similar genes and pathways (Western and Sinclair, 2001). The cascades of genes controlling sex determination showed that the master genes at the top of the regulatory hierarchy are not conserved whereas downstream genes remain the same in a broad spectrum of species (Graham et al., 2003; Herpin and Schartl, 2008).

Transcriptomic and genomic studies related to gonad differentiation have been reported in model fish species, but few works have been addressed in species of aquaculture relevance. Most of these studies have been focused in a few genes, being *Dmrt1* the most analyzed because it is the only known non-mammalian vertebrate master sex-determining gene (Matsuda et al, 2002; Nanda et al,

2002). Large-scale gene expression studies have been made in a few fish species including zebrafish (Small et al., 2009), medaka (Lo et al., 2008), perch (Rossi et al., 2007), sea bass (Chini et al., 2006; Kulh et al., 2010), blue fin tuna (Chini et al., 2008) and three spine sticklebacks (Leder et al., 2010). These analyses are growing in farmed fish because mass EST sequencing from ovary and testes of several model fish has allowed improving the knowledge on their physiology.

Flatfish represent a very important group of commercial fish. The turbot is a marine flatfish species of great commercial value in Europe. Its production reached 9246 Tons in 2009 (APROMAR, 2010), and Spain with 8320 Tons (90, 0%), particularly the northwestern area, is the principal European producer. The turbot is a gonochoristic species which shows an important size sexual dimorphism. Females largely outgrow males and reach the commercial size between 3 and 6 months earlier (Purdom, 1972; Imsland et al., 1997). Besides, males achieve sexual maturity earlier than females, thus determining a delay in growth rate. Due to this sexual dimorphism, the knowledge of the sex determination and differentiation mechanisms is highly relevant for turbot farming.

Sex ratios are usually balanced in turbot culture (Imsland *et al.,* 1997), which indicates a major genetic factor in the sex determining system. However, variation between progenies has been noticed under certain culture conditions (Cal et al., 2006 a, b, Haffray et al., 2009). In this species the chromosome sex determination system is not fully understood, despite the fact that several karyotypic studies (Bouza et al., 1994, Pardo et al., 2001, Cuñado et al., 2001) and gynogenetic and triploid family analysis (Cal et al., 2006a) have been carried out. Recently, the application of a microsatellite genetic map has demonstrated the existence of a major genetic factor located on linkage group 5 and a chromosomic sex determining mechanism ZZ/ZW (Martínez et al., 2009). Haffray et al., (2009) also suggested a ZZ/ZW sex determination system based on sex ratios in families produced with sex-reversed parents. However, other genetic/environmental factors seem to be necessary to explain the observed differences between families (Martinez et al., 2009; Haffray et al., 2009).

Phenotypic sexual dimorphism is related to differences in gene expression between males and females at some particular genes. The availability of techniques that identify sex-specific expression

markers opens the possibility to study the initial expression of these genes in undifferentiated gonads and along the development until the mature gonad. As an initial step to explore the genes related to sex differentiation, here we report the analysis of adult turbot gonad transcriptome. It is possible that some of the sex-specific expressed genes play important roles in gonad development. Recently, expression studies on sexual dimorphism based on somatic tissues are gaining importance in mammals. In these organisms there are an important number of differentially expressed genes which exhibited sex-dependant expression (Rinn and Snyder, 2005, Clodfelter et al., 2006). Gene-by-gene studies are limited to fully understand the sex differentiation and sex determination processes, and thus we consider necessary to deal with sexual dimorphism expression in a more comprehensive approach that means analyzing sexual dimorphism expression in somatic tissues too. Because of this, we comparatively analyzed the expression profiling in other somatic tissues: brain and liver. The permanent communication between the brain and the gonad has been well established. In fishes reproductive activities are primarily controlled by the endocrine system consisting of the hypothalamus-pituitary-gonad axis (HPG axis). Furthermore we realized the expression analysis in liver not only because it is the most analyzed tissue regarding sexual dimorphism but also because sex differentiated hepatic metabolism of steroid hormones has been well studied (Isensee and Ruiz Noppinger, 2007). Besides all this, some proteins necessary for oocyte growth and development are synthetized in liver so HPG axis is referred in oviparous organism as HPGL (Hypothalamus-pituitary-gonadal-liver) axis in females.

DNA microarrays are the standard tools for genome-wide expression analysis; however their application is restricted to organisms where there are significant collections of known transcripts available (Vuylsteke et al., 2007). In turbot, despite large collections of immune-related genes being available (Pardo et al., 2008; Park et al., 2009) and an oligo-microarray having been designed and calibrated based on this database (Millán et al., 2010), no gonad-related expressed genes have been reported to date. In this work we used the cDNA-AFLP methodology to comparatively analyze gene expression in mature gonads, liver and brain of males and females. Our work addressed three major issues: i) a general screening of gene expression for identifying differentially expressed genes in adult

gonads of males and females ii) the characterization of those genes expressed only in males or females as a first step to know their implication in gonad differentiation; and iii) to compare the same primer combinations in liver and brain of both males and females.

2. Material and methods

2.1 Animals and sampling

Sexually mature females (n = 6) and males (n = 6) were sampled in November 2008 from the Instituto Español de Oceanografía (Vigo, Spain). Fish were killed by sedation and decapitation and gonads and livers were dissected and conserved on dry ice before RNA extraction.

2.*2 cDNA-AFLP analysis*

We applied the technique described by Vos et al. (1995) with the modifications by Papa et al. (2005) with additional slight modifications for our analysis with turbot cDNA. Total RNA was obtained from mature gonads and liver using TRIZOL (Gibco BRL) following manufacturer´s recommendations. RNA was treated with DNase I (Invitrogen) and its concentration and quality were assessed by spectrophotometry and by agarose gel electrophoresis. Double strand cDNA templates were synthetized with the "Superscript Double-Strand cDNA synthesis kit (Invitrogen).

We carried out an initial screening of dilutions and primer combinations for optimizing amplification (40 combinations were tested). The best results (band numbers, reproducibility) were obtained with the following conditions: 5-fold dilution of the restriction-ligation product; preselective amplification without extrabased primers; 2-fold dilution of the preamplification product; and selective amplification with an *Eco*RI primer having a three-base extension and a *Taq* primer with one extra-base, or vice versa. The technique was carried out on three individuals of each sex separately and in pools constituted by individuals of the same sex. cDNA (500 ng) was digested with *TaqI* (Promega) and *Eco RI* (Takara) followed by adapters ligation. The pre-amplification reaction was performed as follows: 2min at 34 ºC, 30 cycles of 30 s at 94 ºC, 1 min at 56 ºC, and 1 min at 72 ºC, and one final extension step of 5 min at 72 ºC. The resulting product was used for the selective

amplifications: 2 min at 94ºC, 30 s at 94ºC, 30 s at 65ºC, 60 s at 72ºC, 12 cycles of 30 s at 94ºC, 30 s at 65 ºC (decreasing 0.7 ºC each cycle), 60 s at 72ºC, 23 cycles of 30 s at 94ºC, 30 s at 34ºC, 60 s at 72ºC. Results were analyzed by capillary electrophoresis on an ABI3730 automatic sequencer (Applied Biosystems). The internal sizer used was GS500liz. The most interesting combination of primers (differences between sexes) were electrophoretically separated in 8% polyacrilamide gel in the vertical electrophoretic system Phor U2 (Ingeny) for 9 hours at 120 V. Gels were stained with SYBR Gold (Invitrogen) and their digitalization was performed with the Gel Doc 2000 (Biorad). Gel images were edited with Adobe Photoshop 7.0.

2.3 Cloning and sequencing of cDNA-AFLP products

cDNA-AFLPs were cloned following either the protocol developed by Tamborindeguy et al. (2004) or from differential sex-specific bands from gels. The first protocol consists of a mass cloning of specific cDNA-AFLP combinations. In this way, both differentially and non-differentially expressed transcripts between sexes are cloned. Mass cloning was performed at those combinations with highest different profiles between males and females (E-ACA/T-C; E-AAG/T-C, E-A/T-CAG, E-A/T-ACT, E-AAC/T-C, E-AAC/T-C, E-A/T-AAC, E-A/T-AAG, E-A/T-CAC). After selective amplification, the PCR reactions were purified with SpinClean kit (Mbiotech) according to the manufacturer´s protocol. These cleaned products were cloned in pGEM-T Vector (Promega) and then sequenced. In the second approach, differential bands between females and males were excised from acrylamide gel, resuspended in MilliQ water at 4ºC overnight and reamplified with primers and conditions used in selective amplification. PCR products were purified with SpinClean kit, cloned in pGEM-T Vector (Promega) and sequenced. A total of 104 clones obtained from cDNA-AFLP markers in male gonad, 104 clones in female gonad and the same number in liver of both genders were sequenced in the mass cloning analysis. cDNA sequencing was carried out using the ABI 3730 automatic sequencer (Applied Biosystems Inc. USA). After eliminating vector sequences, high quality sequences were then used to search GenBank databases using BlastN and/or BlastX

(http://blast.ncbi.nlm.nih.gov/Blast.cgi). The cutoff value used for determining the significance of the resulting alignments with the database sequences was < e-^{04}

2.4 Analysis of sexually dimorphic expression of selected genes by qRT-PCR

To verify the sexually dimorphic expression of some selected genes which might be differentially expressed we employed RT-qPCR with SYBR-green based detection. To carry out this study we have considered the recommendations of the MIQE (Minimum Information for publication of quantitative real-time PCR Experiments) guidelines (Bustin et al., 2009). Total RNA was extracted using TRIZOL (Gibco BRL) as we described previously. The Bioanalyzer Agilent 2100 (Agilent Technologies) system was used to calculate the RNA integrity number (RIN) which provides information about the RNA quality. The samples we worked with had RIN values above 7, 5 (in a scale 1 to 10). cDNA synthesis was carried out with the "Reverse Transcriptase Core kit" (Eurogenec) following manufacture ´s recommendations. Ribosomal protein S4 (RPS4), ubiquitin (UBQ), glyceraldehyde 3P dehydrogenase (GAPDH), ribosomal protein L17 (RPL17) and microglobulin b2 (B2M) were tested as reference genes for normalization of gene expression in gonads using BestKeeper (Pfaffl et al., 2004) and geNorm (Vandesompele et al., 2002). The primers for RPS4, UBQ and GAPDH were designed by Eurogentec taking sequences obtained in our laboratory as template while the primers for RPL17 and B2M were obtained from Dang and Sun (2011). We analyzed the five genes in 12 individuals. In the analyses realized with both BestKeeper and geNorm the UBQ is the most stable gene and therefore we used this gene as reference gene in the analysis done in gonad. We used RPL17 as reference gene for the liver, following the information published in Dang and Sun (2011). In order to check the primers efficiency we first tried different primer concentrations, choosing the one with the lowest Cq. Secondly, the amplification efficiency of each primer combination was tested with a calibration curve consisting of serial dilutions of our cDNA. All our primer pairs have efficiencies between 90 and 110% and a calibration curve R2 value above 95%. PCR reactions were made using qPCR Master Mix Plus for SYBR Green I No ROX (Eurogenetec) according to the supplier´s and RT-qPCR was performed on a MX3005P (Agilent Technologies) with the following conditions: 2 min at 50ºC, 10 min at 95ºC, 40 cycles of 15 s at 95ºC, 1 min at 60ºC, 1 min at 95ºC, 30 s at 65 ºC and 30 s at 95 ºC. The powerNest software (Kitchen et al., 2010) was used to decide the experimental optimization of the RT-qPCR assay. We developed a nested or hierarchical experiment consisting of 3 individuals, from which 3 samples (3 RNA extractions) were collected, then each sample was split into 3 RT

reactions and each RT was split into 3 qPCR amplifications. The nested design was 3x3x3x3, a total of 81 Cq values. After powerNest evaluation we decided a design based on 3 samples of RNA per individual analyzed, 1 reversotranscription of each sample and 1 qPCR reaction of each reversotranscription. We analyzed the expression levels of several genes in the gonad and/or liver of 6 individuals (3 males and 3 females) using the MxPro software. After running the experiment we confirmed that the observed differences between means of males and females are statistically significant by a T-test for independent samples using SPSS version 19.

3. Results

The cDNA-AFLP profiles obtained in liver and gonad tissues were very different (Figure 1). In both cases we obtained bands from 50 to 1000 bp, being 100 to 500 the most frequent transcript range. In gonad, it was possible to visualize around 50 bands per individual in contrast to the somatic tissues (only between 10 and 15 bands were identified per gel). In liver all primer combinations rendered a similar banding pattern, a few intense bands with no differences between sexes in the conditions tested. Only with the E-A/T-AAG combination a 700 bp male-specific band was identified (Figure 1). In gonad several sex-specific bands were distinguished with most primer combinations. These bands were excised from gels, cloned and sequenced. Banding profile obtained in brain is no shown because no differential bands were detected between males and females

In clones from male gonads, 54% of sequences didn´t give any not significant match to GenBank sequences. In Figure 2a it is represented the functional distribution of sequences which showed significant matching to specific GenBank entries. The most frequent classes of Gene Ontology database corresponded to microsatellite-like sequences (22%), unidentified category (16%), development *(*14%), cell division (11%), DNA synthesis, repair and replication (10%) and to gene regulation and protein synthesis (10%). In females the percentage of sequences that did not significantly match to GenBank was higher than in males (62%). Removing these sequences (Fig. 2b) an important fraction corresponded to unidentified sequences (22%), and to genes involved in gene expression, regulation and protein synthesis (36%) and to a minor extent to metabolism and inner/outer structures (9% in both cases). It is interesting to note that in our female sample no

sequences related to meiosis were identified, as it occurs in males (5%). The percentage of genes involved in neurology (3% in males and 4% in females) and mitochondrion (3% in males and 4% in females) was very similar. The genes related to development were underrepresented in female (4%) as compared to male gonad (14%)

In Tables 2 and 3 we present the results of the cDNA-AFLP mass cloning performed in male and female gonads of turbot, respectively, which showed significant similarity to the GeneBank database. Comparing the data in the mass cloning experiment between males and females, some transcripts were present in both sexes: S-phase-kinase-associated protein 1, an essential component of the SCF ubiquitin ligase complex which mediates the ubiquitinization of proteins involved in cell progression, signal transduction and transcription (Bai et al, 1996); *eif2b3*, a gene involved in regulation of protein synthesis (in contrast to the other common sequences, this was more represented in females (7) than in males (1)); *mesd1*, an essential gene for mesoderm differentiation and establishment of embryonic polarity; the transcript similar to *congentil dyserythroppoietic* anemia type I, a housekeeping gene with a not well understood function, but that may play an important role in the organization of heterochromatin; some sequences related to mitochondrial function and the microsatellite DNA locus ABGe596.

In the same experiment, some male-specific sequences were identified: *collagen type XII alpha1, B9 protein domain1*, *transmembrane protein 32*, *trub2*, and stabilizing protein of *WAF-1CIP1* or *p21* (WAF-1/CIP1), a cyclin-dependent kinase inhibitor and so an important regulator of cell cycle. This regulatory protein depends on *p53* and *Hsp90* for its function. Hsp90 is a chaperone which plays an essential role in many cellular processes. The majority of the known substrates of Hsp90 are signal transduction proteins such as steroid hormone receptors and kinases (Jascur et al., 2005). The NIMA related kinase 10 (*NEK1*0 gene)is similar to a serine/threonine protein kinase that was found in rainbow trout testis (Rolland et al., 2009); in humans the protein kinases of the NIMA family are important factors in the regulation of the eukaryotic cell cycle. The *meiosis-specific nuclear structural 1* gene (*MNS*1) may play a role in the control of meiotic division and germ cell differentiation through

regulation of pairing and recombination during meiosis. BET1-like is a Golgi associated membrane protein that participates in vesicular transport of proteins. In mass cloning females we could find specific transcrips, such as a sequence with high homology to the cold-inducible RNA-binding protein (*CIRP*) (E-value=$3e^{-14}$, size sequence 271 bp, (78%) identity). CIRP is likely a general stress response protein belonging to a highly conserved glycine-rich RNA-binding protein family and together with *RBM* they are the only two known cold-shock proteins. The role of these proteins is unknown but there is a general agreement that its main role is to ensure accurate and enhanced translation of specific mRNAs at low temperatures (Sahara et al., 2002). *Spna2* (spectrin alpha 2 gene), involved in actin cytoskeleton reorganization, is a family member of spectrins expressed in most metazoan cells. These proteins are known to organize and strengthen the plasma membranes of many cell types (Bennett and Healy, 2009). α spectrine is required for germ line cell division and differentiation in the *Drosophila* ovary. Notothenoids have an unusual abundance of spectrine suggesting that the various isoforms present may carry out different functions during oocyte maduration (Tammaro et al., 2007) *Pax5* encodes a member of the paired box family of transcription factors. This gene encodes the B-cell lineage specific activator protein and also its expression has been detected in developing central nervous system and testis and so may play a role in neural development and spermatogenesis (Adams et al., 1992).

Table 4 shows the clones obtained from differential bands extracted from gels in female or male gonads. Some clones were identical to those obtained in the mass cloning experiment and, as expected; most sequences were sex-specific. We sequenced a total of 23 clones corresponding to bands observed either in males or in females (Table 4). It is interesting to note that only three differential bands were found in females. These bands matched with a repeated sequence (microsatellite), with tubulin alpha-1 subunit and with mesoderm development candidate 1. Even though the last gene was found in both sexes in the mass cloning experiment, differences in expression (higher in females than in males) could account for these results. The most interesting male-specific bands corresponded to the synaptonemal complex central element, that was also recorded in the male mass cloning experiment, the *NEK-10*, the *suppressor of sable* gene which participates in several

processes related to the elimination of aberrant RNAs (Kuan et al., 2009) and a transcript similar to interferon-induced protein 44 (*IFIL44*). The sequences corresponding of this protein shown homology with the LG8 of stickleback (E-value = 2, 8 x 10^{-19}, size sequence 119, 87,39% Identity, Ensemble database) wich is syntenic with LG5 of turbot where the major sex-associated QTL was detected (Martínez et al., 2009)

The liver expression profile in the mass cloning experiment was very different to that found in gonads. Differences between sexes were very slight (Figure 2c, d). The most represented GO categories (biological function) in both sexes were cell communication, immune system-related and metabolism-related. The percentage of sequences with no significant similarity to GeneBank databases was lower than in gonads (15%, data not shown). However, there was an important proportion of unidentified sequences (16% in males and 19% in females). It is interesting to note that in male liver we found a transcript homologous to *GC1* mRNA. This gene encodes a nuclear protein that appears to be involved in spermatogenesis in human, mouse and *Drosophila*. Also, a transcript corresponding to VHSV-induced protein, an antiviral product only recorded in a few fish species, *Danio rerio, Onchorhynchus mykiies, Salmo salar*, was found. The only visible sex-different band found in liver (Figure 1) was extracted, purified, cloned and sequenced. The analysis of 11 clones obtained from this band showed an important homology in the database with C9 complement protein in other fish species.

Figure 2 clearly shows that there is a significant difference in genes expressed in the gonad when compared with the other somatic tissues. Figure 2 e, f shows categories of the sequences based on the GO database from male and female brain respectively removing those sequences that did not match with any sequences in the databases (21% in males and 27% in females). The graphics are more similar to those of the liver with the exception of the genes related to the immune system up-regulated in liver (32% in males, 23% in females, compared to 2% in brain). The proportion of the remaining functional categories is very similar in both tissues with the exception of the Gene expression, regulation and protein synthesis category that is a little higher in the brain in males and

females. In both sexes the most represented class corresponds to metabolism (27% in male, 31% in female) and cell communication (31% in male and 27% in female brain). In the analysis of the sequences obtained from brain (Tables 7 and 8) highlights *SRY-box 14 (Sox14)* gene. *Sox 14* belong to the *Sox* family, a group of genes that are involved in different development processes. High conservation of SOX14 protein has been demonstrated in several species of vertebrates which means that this protein has retained its functional properties (Popovic and Stevanovic, 2009). In male brain we detected *IF44L* (Interferon induced protein 44 like) which was present in gonad and in male liver; and the M subunit of the eukaryotic translation initiation factor 3 (*eif2b3*) (subunit gamma is present in gonad and in liver). In female and male brain was detected CIRP also present in female gonad.

To complete our study we analyzed the relative transcript abundance of some selected genes by RT-qPCR to investigate how broadly they might be expressed in male or female. In gonads*: mesd1* (it was found in female differential bands and in male gonad in the mass cloning experiment), *NEK10* (was detected in male differential bands and also in male gonad mass cloning experiment), I*FI44* (detected in male differential bands)*, eif2b3* and *CIRP* detected in female mass cloning, *MNS1* and *WAF1/CIP1* stabilizing protein detected in male mass cloning. In liver *C9*, because it is the only clearly differential band obtained in this tissue and is well represented in the male mass cloning. Finally, we analyzed *GC1* and *Skp1* genes in both tissues. Through RT-PCR using specific primers we detected significant differences between male and female gonad only in two of the eight genes analyzed: *MNS1* and *NEK10* being its expression higher in males than in females in both genes in gonad. RT-qPCR confirms data obtained in the mass cloning and for NEK-10 even with the data obtained from differential bands cloning. Some members of the NIMA-related kinases are differentially expressed during meiosis and some are also expressed in specific neurons (Arama et al., 1998). Nek 10 may take part in the regulation of the cell cycle in spermatogenesis in human (Zeng et al., 2011). The protein codified by *MNS1* (meiosis specific nuclear structural protein 1) was shown to be expressed at the pachytene stage during spermatogenesis and maintains appropriate the nuclear morphology during meiotic prophase in mouse (Furukawa et al., 1994). In the liver C9 is also up-

regulated in males (Figure3) which is in accordance with the mass cloning and with the gel profile where the only clear differential bands was located in males and correspond to C9 protein.

4. Discussion

Gene expression profiles were compared using the cDNA-AFLP technique. This method has been widely used as an attractive technology for gene discovery on the basis of fragment detection and for temporal quantitative gene expression analysis (Vuylsteke et al., 2007). Identification of differentially expressed genes can be accomplished by purifying cDNA-AFLP fragments from gels and subsequent sequencing. Several genes could be involved in sex determination and/or gonad sex differentiation since they are expressed differentially in male or female gonads.

The cDNA-AFLP method for global transcriptional analysis is an appropriate technology for gene expression studies in non model species such as turbot because it requires no primer knowledge of genomic sequence such us microarrays, is relatively inexpensive, quick and reproducible (Vuylstedke et al., 2007). This method increases the resolution of expression pattern detection using small amounts of mRNA (Reijans et al., 2003) and has demonstrated a good correlation with northern blot analyses (Durrant et al., 2000). The obtained fragments were cloned and classified into functional categories according to the Gene Ontology annotation. The expression level of each individual gene in a genome can vary considerably between cells, tissues or organs, and can even be very different in the same tissue or organ at different development stages or physiological conditions, however if the number of sequences analyzed is large the differences in gene expression will be reflected.

The expression pattern observed in turbot liver in our study shared several features with the same organ in other vertebrates and particularly in other fish: tuna (Chini et al., 2008), perch (Chini et al., 2006) and sea bass (Rossi et al., 2007). The C9 complement component, the G protein family and the fibrinogen were the most represented genes in both sexes. In turbot the percentage of expressed sequences related to immune response was higher than in tuna or sea bass and more similar to that found in *Perca fluviatilis* (Chini et al., 2006). In both species the most represented biological functions

according to the Gene Ontology classification were immune system, cell communication, metabolism and transport. Although we classified the biological process of transferrin as transport, a glycoprotein responsible for the transport and delivery of iron to cells, it is also related to the innate immune response of fish because of the importance of free iron for bacterial infections (Stafford and Belosevic, 2003; Pardo et al., 2008). The transferrin gene, together with other genes that we found in liver such as apolipoprotein and fibrinogen, has been shown to be regulated by estrogens in females (Pinto et al., 2006). In our analysis we found the transferrin transcript only in females whereas apolipoprotein and fibrinogen transcripts were detected in both sexes. The complement system plays an important role in innate immune response in fish. Complement proteins can lyse foreign cells or opsonise them for their destruction by phagocytes. Even though this transcript was present in both sexes, in males we found 21 transcripts (20%) that showed a significant match with C9 complement component and this was the only male specific transcript that we obtained from the unique clear differential band in liver (Figure 1). The sex-differential expression of C9 was confirmed by RT-qPCR (Figure 3). In a toxicological study made on killifish (*Fundulus heteroclitus)* this gene was highly expressed in males (Meyer et al., 2005). It is interesting to note that these authors claimed attention for including sex as an important factor in expression analyses. In a multiple somatic tissue study in mice differential complement protein expression between sexes was observed in several tissues, the authors refer a degree of sexual dimorphism close to 70% in liver (Yang et al., 2006). In females the most frequent transcript corresponded to β–family G protein (21 out of 104 transcripts). G proteins are a family of proteins involved in second messenger cascades. Additionally, it is interesting to note the presence of a *gc1* (Germ cell-less protein) homologous transcript in male liver, a gene related to spermatogenesis. This gene plays an important role in early differentiation of germ cell in *Drosophila* (Jongens et al., 1992). In medaka a strong expression in the ovary while it was detected and less in testis, brain, heart, liver and muscle tissues, (Scholz et al 2004) and other authors reported its importance for primordial germ cells formation in zebrafish (Li et al., 2006). The RT-qPCR analysis showed no significant expression differences of the *gc1* gene between the livers of males and females. For those genes we didn´t find any expression differences between sexes other periods of gonad development should be analyzed. In

the same way for the other genes that we have no found expression differences between sexes it should be analyzed in other period of gonad development.

In mammals, the liver is one of the highest sexually dimorphic organs in terms of gene expression (Yang et al., 2006). In fish, only a few studies have documented sexual dimorphism in this organ despite it taking part in a number of complex functions related to metabolism, immunity, detoxification and protein synthesis (Chini et al., 2006, 2008, Rossi et al., 2007, Robison et al., 2008, Leder et al., 2009, 2010). We do believe that sexual dimorphism in somatic tissues transcriptoma should deserve more scientific attention.

The identification of differentially expressed sex-related genes is an initial step towards understanding the molecular mechanisms of gonad development. Public databases are less enriched in gonad-related ESTs, as the large number of sequences with no significant match in our study suggests (54% in male and 62% in female), which indicates the poor molecular knowledge we have of these tissues. The sex-specific bands and the large number of ESTs, with unknown functions identified in males and females may have a role in gonad differentiation. In mass cloning we found both common as well as sex-specific sequences in males and females. Our analysis does not permit to soundly identify specific sequences of each sex because the number of sequences analyzed in the mass cloning of gonad transcriptome was not very large. Anyway, relevant insights on gonad differential expression emerged from our study. Thus, a male sequence similar to WAF-1/CIP1 stabilizing protein, an important regulator of cell cycle whose regulation depends on Hsp90, and the female sequence similar to cold-inducible RNA binding protein, were identified. However RT-qPCR analysis revealed no significant differences between sexes in these genes. As we mentioned before other periods of gonadal development should be done. A ZZ/ZW sex determination system is now accepted in turbot, although some environmental or even familiar factors could be involved in sex ratio biases (Martínez et al., 2009, Haffray et al., 2009). The interaction between family and temperature could explain some deviation of the expected sex ratio (Haffray et al., 2009) however the influence of temperature and sex in turbot is not fully confirmed. We believe that the expression of these two proteins should be

carefully analyzed. In this sense, the contribution of heat shock proteins to temperature-dependent sex determination in alligator is being explored because preliminary analysis showed that sexual dimorphism exist in mRNA expression of gonadal HSP27and HSP70 and adrenal HSP90 (Kohno et al., 2010). CIRBP may regulate expression or activity of other sex determining genes in the snapping turtle (Rhen and Schroeder, 2010). Some authors have hypothesized that in amphibian species, CIRP may function as a RNA "chaperone" facilitating expression of essential genes at low temperatures or having a role in the animal hibernation (Saito et al., 2000). Recently, the expression of this gene in the gonadal-adrenal-mesonephros complex was investigated in American alligator a species whose sex determination is temperature-dependent (Kohno et al., 2010). The presence of this gene in female brain (Table 8) remarks the importance of investigations its expression more profoundly.

It is remarkable the presence of sequences related to neurology (B9 protein domain, Ric8a). This result could reflect a connection among three physiologically constituents, the brain-pituitary-gonadal axis already demonstrated in fishes (Weltzien et al, 2004, Taranger et al., 2010).

Some microarray-based studies on sex-specific gene expression patterns showed an important masculinization of the transcriptome (Small, et al., 2009). In *Drosophila melanogaster* recent works have shown that sex-biased gene expression is significant in *Drosophila*, especially in gonads. Despite we handled lower sequence number, we could also identify much more male-specific bands, detecting only, 3 female-specific differential bands in contrast to the 21 ones detected in male gonads.

Results obtained in the mass cloning in brain (Tables 7 and 8) are in agreement with what has been found in other studies both in mammals (Galfalvy et al., 2003; Rinn et al., 2004) and fish (Guiry et al., 2010) which reported few gene expression differences between the adult brains of both sexes. Since the brain is a highly heterogeneous tissue, sex differences in gene expression within individual regions of the brain may be masked when the whole brain is studied (Iseense and Ruiz-Moppinger, 2007). However, it would be very interesting to investigate the function of *Sox14* because it belongs to a family which has several genes related to sex determination and sex differenciation in vertebrates (Kiefer, 2007)

Genomic and transcriptomic data are still scarce and mainly developed in model fish species and not in commercial ones. This fact is slowing down the development of technology in the culture of species such as turbot, with an important commercial interest Although our work represents a small sample of the male and female transcriptomes of gonad, liver and brain it will contribute to the existing data and will facilitate comparative interspecies studies. Transcript profiling is playing a substantial role in annotating and determining gene function which is fundamental to understand and to control one of the principal challenges for the aquaculture industries: the reproduction. In any case in order to gain new insights in key biological process in teleost, these genes have to be functionally characterized.

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FIGURE LEGENDS

Figure 1. cDNA-AFLP profiles in male and female liver (left) and gonad (right) of turbot. P indicates pool of individuals and numbers correspond to different individuals. In boxes are presented the most visible sex-different bands in both tissues.

Figure 2. Biological function classification, based on Gene Ontology, of transcripts with significant similarity to GeneBank database identified in this study a) male gonad b) female gonad c) male liver and d) female liver.

Figure 3. Mean expression level fold change of the males taking the mean females expression level as calibrator. Genes expression analyzed in gonads is represented in blue and liver gene expression in red.

Fig. 1.

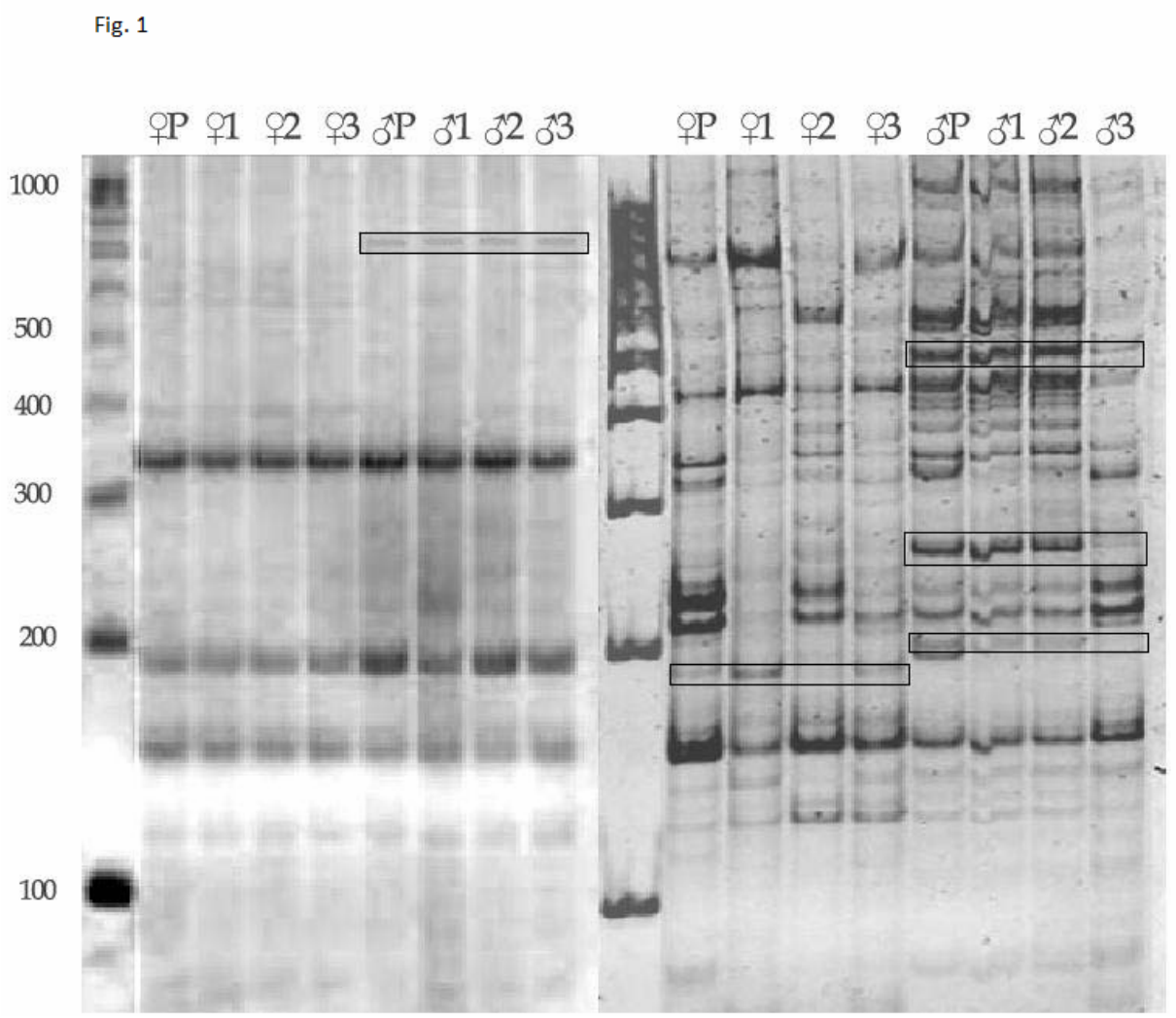

Fig. 2.

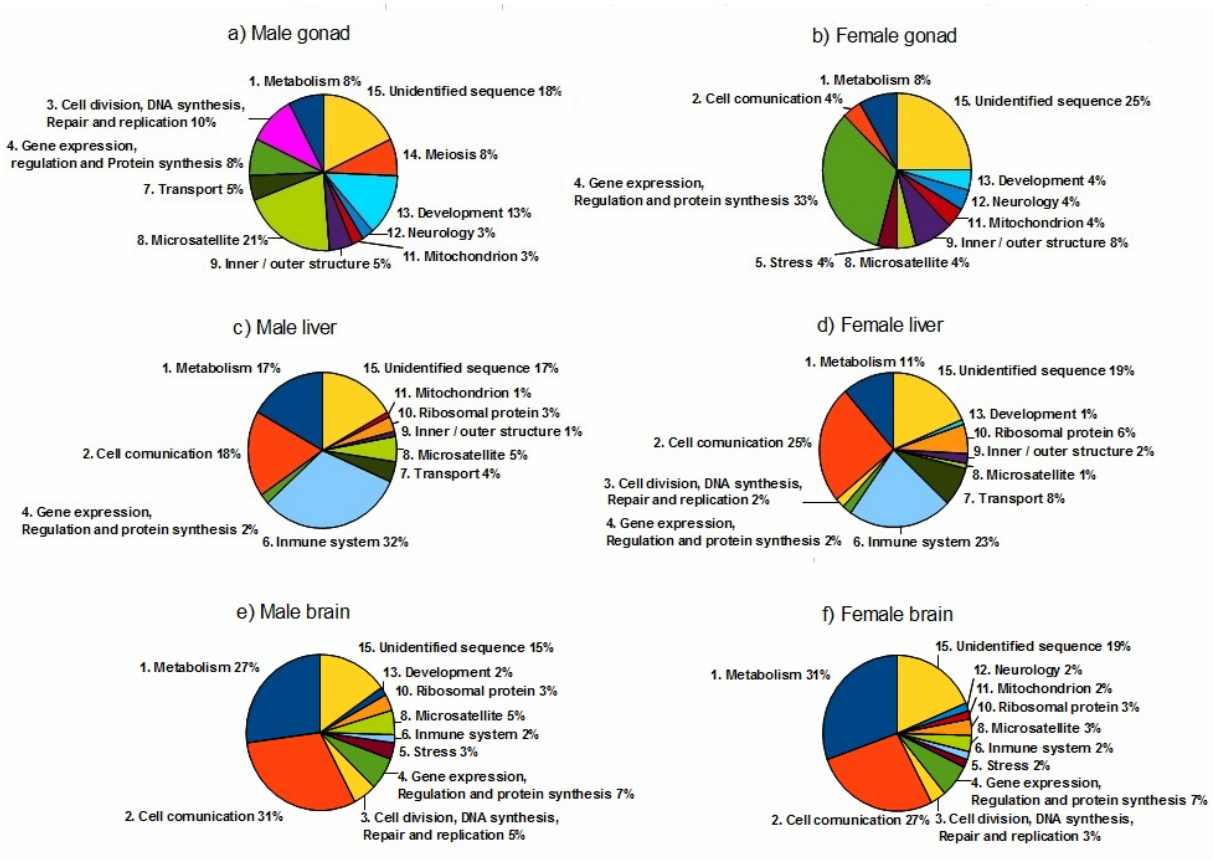
a) Male gonad
1. Metabolism 8%
15. Unidentified sequence 18%
3. Cell division, DNA synthesis, Repair and replication 10%
4. Gene expression, regulation and Protein synthesis 8%
14. Meiosis 8%
7. Transport 5%
13. Development 13%
8. Microsatellite 21%
12. Neurology 3%
9. Inner / outer structure 5%
11. Mitochondrion 3%
b) Female gonad
1. Metabolism 8%
2. Cell comunication 4%
15. Unidentified sequence 25%
4. Gene expression, Regulation and protein synthesis 33%
13. Development 4%
12. Neurology 4%
11. Mitochondrion 4%
9. Inner / outer structure 8%
5. Stress 4%
8. Microsatellite 4%
c) Male liver
1. Metabolism 17%
15. Unidentified sequence 17%
11. Mitochondrion 1%
10. Ribosomal protein 3%
9. Inner / outer structure 1%
2. Cell comunication 18%
8. Microsatellite 5%
7. Transport 4%
4. Gene expression, Regulation and protein synthesis 2%
6. Inmune system 32%
d) Female liver
1. Metabolism 11%
15. Unidentified sequence 19%
13. Development 1%
10. Ribosomal protein 6%
2. Cell comunication 25%
9. Inner / outer structure 2%
8. Microsatellite 1%
3. Cell division, DNA synthesis, Repair and replication 2%
7. Transport 8%
4. Gene expression, Regulation and protein synthesis 2%
6. Inmune system 23%
e) Male brain
15. Unidentified sequence 15%
1. Metabolism 27%
13. Development 2%
10. Ribosomal protein 3%
8. Microsatellite 5%
6. Inmune system 2%
5. Stress 3%
4. Gene expression, Regulation and protein synthesis 7%
2. Cell comunication 31%
3. Cell division, DNA synthesis, Repair and replication 5%
f) Female brain
15. Unidentified sequence 19%
1. Metabolism 31%
12. Neurology 2%
11. Mitochondrion 2%
10. Ribosomal protein 3%
8. Microsatellite 3%
6. Inmune system 2%
5. Stress 2%
4. Gene expression, Regulation and protein synthesis 7%
2. Cell comunication 27%
3. Cell division, DNA synthesis, Repair and replication 3%

Fig. 3.

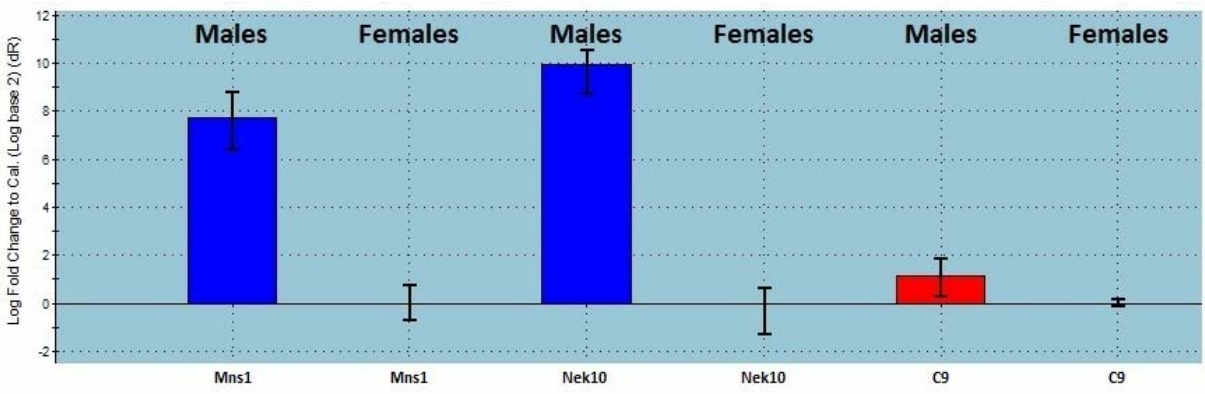
Males
Females
Males
Females
Males
Females
Log Fold Change to Cal. (Log base 2) (dR)
12
10
8
6
4
2
0
-2
Mns1
Mns1
Nek10
Nek10
C9
C9

Table 1. Gene specific primers used for qRT-PCR

Symbol	Name	Acces. Numb.	Primer sequence (5' → 3')
UBQ	Ubiquitin	FE946708.1	GCGTGGTGGCATCATTGAGC CTTCTTCTTGCGGCAGTTGACAG
RPS4	Ribosomal protein S4	FE943956.1	CAACATCTTCGTCATCGGCAAGG ATTGAACCAGCCTCAGTGTTTAGC
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	FE950888.1	CGCCCATAGCCCAGTCATAGC TGGCAGAGGGAGGTGGAGAG
RPL17	Ribosomal protein L17	DQ848879	AICAGTGCGTCCCCTTCA CTCATCTTCGGAGCCTTGTTC
B2M	β2-Microglobulin	DQ848854	CTCTGGCTGTTTTCGTCTGCT TCCTTTCCGTTCTCTCCCG
EIF2B3	Eukaryotic translation initiation factor 2B, subunit 3 gamma	HQ616895	ACAGCCCGTCCTCCAGATC GCAGAGAGACTTTGTTGGTGTG
MNS1	Meiosis-specific nuclear structural 1	HQ603850	GTGGTCACTGTAGCCTTAGC GACGACCTGGAGCACTTTG
NEK10	Similar to NIMA (never in mitosis gene a)-related kinase 10	HQ603844	CGACAGCACAGATGATGG CTTCGTAGTAACCGTTCTCC
FKBPL	Similar to WAF-1/CIP1 stabilizing protein	HQ603840	CCTGCCAGATGGTGAATG TCTACTTGCTATGTCCTTGAG
CIRBP	Cold-inducible RNA-binding protein	HQ616901	TCAGACTGGTGAACTACACATCC GTTGAGCACAGAAGATACCTTGG
IFRD1	Interferon-related developmental regulator 1	HQ603848	CGCTGAGAAGAAGAACATC CAAGTCACGATGGGTAAAG
MESDC1	Mesoderm development candidate 1	HQ603833	CCACAGAGCCTCAGTTCCTG GTTGACTGGCGGCAGAGT
C9	Complement component C9	HQ634399	TTTGGAGCCCATGAGAGC ACGAGAAGAAGTCTATGATTAAGG
GMCL1	Germ cell-less protein	HQ634428	TTCGCAGCATCGCAAACC CGTCCGTCTCCAGTACATTATC
SKP1	S-phase kinase-associated protein 1	HQ634394	TCACAGAGGAGGAGGAAGC CAACCAGTTAGCAGAGACAATC

Table 2: Transcripts with significant similarity to GenBank database obtained from male gonad in the mass cloning experiment. First column indicates the name of the clone and in parenthesis the number of sequence repeats is shown. The annotation from GenBank database, its significance (e-value), length of identity and the biological process based on gene ontology (GO) terms are included. Underlined, those male-specific fragments.

Seq. (repeats)	Database access	Alignment [Sp.]	E value	Length (Ident.)	Biological process
Gm01	HQ603829	S-phase kinase-associated protein 1 putative mRNA [*Anoploma fimbria]*	$3e^{-149}$	486 (91%)	Metabolism
Gm02	HQ603830	Collagen, type XII, alpha 1, transcript variant 1 mRNA [*Pan troglodytes]*	$3e^{-20}$	311 (76%)	Inner / outer structures
Gm03	HQ603831	Eukaryotic translation initiation factor 2B, subunit 3 gamma (eif2b3), mRNA [*Danio rerio*]	$3e^{-23}$	154 (80%)	Gene expression, regulation and protein synthesis
Gm04	HQ603832	B9 protein domain 1, mRNA (B9D1) [*Bos taurus*]	$2e^{-34}$	234 (81%)	Neurology
Gm05	HQ603833	Mesoderm development candidate 1 (mesd1), mRNA [*Salmo salar*]	$2e^{-8}$	220 (82%)	Development
Gm06	HQ603834	Transmembrane protein 32 precursor putative mRNA [*Anoploma fimbria*]	$2e^{-10}$	327 (69%)	Transport
Gm07	HQ603835	tRNA pseudouridine synthase 2 (trub2), mRNA [*Oncorhynchus mykiss*]	$2e^{-13}$	327 (70%)	Gene expression, regulation and protein synthesis
Gm08	HQ603836	DNA sequence from clone CH211-89M7 in linkage group 5 [*Danio rerio*]	$4e^{-9}$	512 (76%)	Unidentified sequence
Gm09	HQ603837	DNA sequence from clone CH211-264F5, complete sequence [*Danio rerio*]	$2e^{-4}$	261 (80%)	Unidentified sequence
Gm10	HQ603838	DNA sequence from clone CH211-133L11, complete sequence [*Danio rerio*]	e^{-4}	452 (83%)	Microsatélite
Gm11 (2)	HQ603839	Full-length cDNA *[Tetraodon*	$4e^{-6}$	273 (75%)	Unidentified sequence

		nigroviridis]			
Gm12	HQ603840	Similar to WAF-1/CIP1 stabilizing protein *[Danio rerio]*	$2e^{-18}$	267 (56%)	Gene expression, regulation and protein synthesis
Gm13	HQ603841	Similar to congenital dyserythropoietic anemia type I (human) [*Danio rerio*]	e^{-5}	344 (45%)	Inner / outer structures
Gm14	HQ603842	Mitochondrion [*Psetta maxima*]	0	412 (98%)	Mitochondrion
Gm15	HQ603843	Microsatellite DNA, locus ABGe10823 [*Equus caballus*]	e^{-4}	192 (79%)	Microsatellite
Gm16 (3)	HQ603844	Similar to NIMA (never in mitosis gene a)-related kinase 10 [*Bos taurus*]	$4e^{-6}$	238 (53%)	Cell division, DNA synthesis, repair and replication
Gm17	HQ603845	Similar to Synaptonemal complex central element protein 1, mRNA [*Danio rerio*]	$3e^{-16}$	307 (59%)	Meiosis
Gm18	HQ603846	Unnamed protein product [*Tetraodon nigroviridis*]	e^{-4}	203 (86%)	Unidentified sequence
Gm19	HQ603847	Clone AFE19M19.1 sex and growth traits AFLP marker sequence [*Penaus monodon*]	$3e^{-4}$	164 (93%)	Unidentified sequence
Gm20 (4)	HQ603848	Clone ssal-rgh-509-202 Interferon-related developmental regulator 1 putative mRNA [*Salmo salar*]	$4e^{-45}$	343 (85%)	Development
Gm22 (2)	HQ603850	Meiosis-specific nuclear structural 1 [*Danio rerio*]	$3e^{-15}$	340 (72%)	Meiosis
Gm23	HQ603851	Clone rpfsba0_002320 microsatellite sequence [*Scopthtalmus maximus*]	$3e^{-62}$	185 (89%)	Microsatellite
Gm24	HQ603852	BET1-like protein (bet1l), mRNA [*Oncorhynchus mykiss]*	$4e^{-15}$	484 (74%)	Transport
Gm25 (5)	HQ603853	Clone KVL2678 microsatellite sequence [*Sus scrofa]*	$2e^{-10}$	313 (85%)	Microsatellite

Gm26	HQ603854	Chromosome 8, clone CTD-2373N4 [*Homo sapiens*]	e^{-113}	258 (99%)	Unidentified sequence

Table 3: Transcripts with significant similarity to GenBank database from female gonad in the mass cloning experiment. The annotation from GenBank database, its significance (e-value), length of identity and the biological process based on GO terms are included. Underlined, those female-specific fragments.

Seq. (repeats)	Database access	Alignment [Sp.]	E value	Length (Ident.)	Biological process
Gf01 (7)	HQ616895	Eukaryotic translation initiation factor 2B, subunit 3 gamma (eif2b3), mRNA [*Danio rerio]*	$7e^{-25}$	158 (81%)	Gene expression, regulation and protein synthesis
Gf02	HQ616896	Beta-1,3-galactosyltransferase 2 putative mRNA [*Osmerus mordax*]	$5e^{-4}$	101 (93%)	Metabolism
Gf03	HQ616897	BAC clone RP23-16F4 from 12, complete sequence [*Mus musculus]*	$4e^{-4}$	612 (87%)	Unidentified sequence
Gf04	HQ616898	S-phase kinase-associated protein 1 putative mRNA [*Anoploma fimbria]*	$3e^{-23}$	468 (73%)	Metabolism
Gf05	HQ616899	Spectrin alpha 2 (spna2), mRNA [*Danio rerio*]	$3e^{-12}$	237 (86%)	Inner / outer structure
Gf06	HQ616900	Mesoderm development candidate 1 (mesd1), mRNA [*Salmo salar*]	$2e^{-7}$	219 (80%)	Development
Gf07	HQ616901	Cold-inducible RNA-binding protein putative mRNA [*Salmo salar*]	$3e^{-14}$	271 (78%)	Stress
Gf08	HQ616902	Clone AF-E53M34-3 sex and growth trait AFLP marker genomic sequence [*Penaus monodon*]	e^{-5}	271 (77%)	Unidentified sequence
Gf09	HQ616903	Similar to congenital dyserythropoietic anemia type I (human) [*Danio rerio*]	e^{-5}	344 (45%)	Inner / outer structures
Gf10	HQ616904	Mitochondrion [*Psetta maxima*]	0	414 (98%)	Mitochondrion
Gf11	HQ616905	Microsatellite DNA, locus ABGe5961 [*Equus caballus*]	$6e^{-11}$	119 (87%)	Microsatellite
Gf12	HQ616906	Chromosome 5 clone RPCI93-45E22 [*Trypanosoma brucei*]	$8e^{-4}$	130 (80%)	Unidentified sequence
Gf13 (2)	HQ616907	cDNA-AFLP fragment, clone Np466 [*Nicotinia plumbagifolia]*	$3e^{-4}$	188 (100%)	Unidentified sequence

Gf14	HQ616908	Chromosome 14 DNA sequence BAC R-689J19 of library RPCI-11 from chromosome 14 [*Homo sapiens*]	$5e^{-7}$	146 (94%)	Unidentified sequence
Gf16	HQ616910	Transcription factor (Pax5) gene, exons 1 through 4 [*Fugu rubripes*]	$7e^{-25}$	163 (85%)	Gene expression, regulation and protein synthesis
Gf17	HQ616911	Ric8a mRNA for resistance to inhibitors of cholinesterase 8 homolog A [*Danio rerio*]	$4e^{-5}$	279 (74%)	Neurology
Gf18	HQ616912	Putative beta family G-protein mRNA, partial cds [*Musa acuminata*]	e^{-5}	226 (84%)	Cellular comunication and signal transduction

Table 4: Differential transcripts obtained from female and male gonads from gels. Columns from left to right show the name of the clone (in parenthesis its frequency), the annotation from GenBank database, its significance (e-value), length of identity and biological process based on GO terms. Gm correspond to male-specific bands and Gf to female-specific bands

Differential band (repeats)	Database access	Alignment [Sp.]	E value	Length (Ident.)	Biological process
Gm27 (4)	HQ623653	Putative beta family G-protein mRNA, partial cds *[Musa<<< acuminata]*	$6e^{-4}$	301 (88%)	Cellular comunication and signal transduction
Gm28 (4)	HQ623654	Clone AFE50M24.1 sex and growth traits AFLP [*Penaus monodon*]	$6e^{-4}$	290 (83%)	Unidentified sequence
Gm29 (2)	HQ623655	PREDICTED: Danio rerio suppressor of sable-like (LOC557823), mRNA Similar to zinc finger CCCH-type containing 4 [*Danio rerio*]	$2e^{-39}$	202 (82%)	Gene expression, regulation and protein synthesis
Gf19	HQ623656	Clone FJAU011 SSR marker sequence [*Anas platyrhynchos*]	$5e^{-4}$	266 (86%)	Microsatellite
Gf20	HQ623657	ATCC 10500 tubulin alpha-1 subunit, mRNA [*Talaromyces stipatus*]	$9e^{-5}$	489 (94%)	Inner / outer structure
Gf21 (6)	HQ623658	Mesoderm development candidate 1 (mesd1),mRNA [*Salmo salar*]	$4e^{-8}$	442 (82%)	Development
Gm30	HQ623659	Clone FJAU009 SSR marker sequence [*Anas platyrhynchos*]	$2e^{-6}$	445 (91%)	Microsatellite
Gm31	HQ623660	18S ribosomal RNA gene, complete sequence [*Scopthtalmus maximus]*	$2e^{-69}$	476 (100%)	Ribosomal protein
Gm32 (6)	HQ623661	NIMA (never in mitosis gene a)-related kinase 10-like [*Bos taurus*]	$3e^{-7}$	257 (56%)	Cell division, DNA synthesis, repair and replication
Gm33	HQ623662	DNA sequence from clone DKEY-92I17 in linkage group 14 [*Danio rerio*]	$5e^{-4}$	243 (80%)	Unidentified sequence
Gm34 (9)	HQ623663	*Clone lmos2p07e08 mRNA sequence [Lithognathus mormyrus]*	$7e^{-26}$	192 (81%)	Unidentified sequence
Gm35 (4)	HQ623664	cDNA-AFLP fragment, clone Np517 (*Nicotinia plumbaginifolia*)	$3e^{-6}$	85 (91%)	Unidentified sequence
Gm36	HQ623665	Similar to CG18472 CG18472-PA (LOC100166175), mRNA [*Acyrthosiphon pisum*]	$2e^{-4}$	350 (83%)	Unidentified sequence

Gm37	HQ623666	Clone AFE19M19.1 sex and growth traits AFLP marker [*Penaus monodon*]	$5e^{-4}$	242 (96%)	Unidentified sequence
Gm38 (8)	HQ623667	Putative ATP synthase gamma chain mRNA, partial cds [*Pyrus communis]*	e^{-5}	236 (89%)	Metabolism
Gm39	HQ623668	Cyclin D1 (CCND1), mRNA [*Macaca mulatta*]	$3e^{-8}$	815 (81%)	Cell division, DNA synthesis, repair and replication
Gm40 (4)	HQ623669	PREDICTED: similar to Synaptonemal complex central element protein 1 [*Danio rerio*]	e^{-16}	358 (51%)	Cell division, DNA synthesis, repair and replication
Gm41	HQ623670	Clone AFE51M25 sex and growth traits AFLP marker [*Penaus monodon*]	$3e^{-5}$	569 (94%)	Unidentified sequence
Gm42 (2)	HQ623671	Clone AFE19M19 sex and growth traits AFLP marker [*Penaus monodon*]	$4e^{-14}$	846 (96%)	Unidentified sequence
Gm43(7)	HQ623672	Similar to interferon-induced protein 44-like, mRNA [*Danio rerio*]	$2e^{-4}$	846 (81%)	Inmune system
Gm44	HQ623673	Clone AF-E53M34-3 sex and growth traits AFLP marker [*Penaus monodon*]	$7e^{-5}$	416 (80%)	Unidentified sequence
Gm45	HQ623674	cDNA-AFLP fragment, clone Np067 (*Nicotinia plumbaginifolia*)	$3e^{-4}$	149 (78%)	Unidentified sequence
Gm46	HQ623675	Clone AFE50M24.1 sex and growth traits AFLP marker [*Penaus monodon*]	$4e^{-5}$	270 (79%)	Unidentified sequence

Table 5: Transcripts obtained from male liver in the mass cloning experiment. First column indicates the name of the clone and in parenthesis the number of sequence repeats is shown. The annotation from GenBank database, its significance (e-value), length of identity and the biological process based on GO terms are included. Underlined, those male-specific fragments.

Seq. (repeats)	Database access	Alignment [Sp.]	E value	Length (Ident.)	Biological process
Lm01	HQ634394	S-phase kinase-associated protein 1 putative mRNA [*Anoplopoma fimbria*]	$4e^{-149}$	486 (87%)	Metabolism
Lm02	HQ634395	Clone rpfsba0_002320 microsatellite sequence [*Scopththalmus maximus*]	$4e^{-62}$	229 (89%)	Microsatellite
Lm03	HQ634396	1334.C1 mRNA sequence [*Hippoglossus hippoglossus*]	$2e^{-42}$	275 (83%)	Unidentified sequence
Lm04	HQ634397	Chromosome 15, clone RP23-189K4, complete sequence [*Mus musculus*]	e^{-4}	79 (94%)	Unidentified sequence
Lm05	HQ634398	Microsatellite DNA, CA-repeat (AC)11.5 [*Salmo salar*]	$9e^{-6}$	187 (90%)	Microsatellite
Lm06 (21)	HQ634399	mRNA for complement component C9 [Paralichthys olivaceus]	$3e^{-36}$	441 (81%)	Inmune system
Lm07	HQ634400	Clone lithmor328 mRNA sequence [*Lithognathus mormyrus*]	$2e^{-5}$	441 (78%)	Unidentified sequence
Lm10 (16)	HQ634401	Putative beta family G-protein mRNA [*Musa acuminata*]	$4e^{-6}$	286 (94%)	Cellular comunication and signal transduction
Lm11	HQ634402	Ferredoxin-dependent glutamate synthase-like mRNA [*Pyrus communis*]	$3e^{-4}$	497 (82%)	Metabolism
Lm12 (2)	HQ634403	Hepatic lipase mRNA [*Siniperca chuatsi*]	$6e^{-55}$	887 (89%)	Metabolism
Lm13	HQ634404	Transcript-derived fragment TDF57 mRNA [*Phytophthora infestans*]	$7e^{-6}$	461 (88%)	Unidentified sequence
Lm14	HQ634405	mRNA for antimicrobial peptide precursor (TAPP gene) [*Scophtalmus maximus*]	$2e^{-114}$	515 (97%)	Inmune system
Lm15 (3)	HQ634406	cDNA-AFLP fragment, clone NP466	$6e^{-7}$	467 (94%)	Unidentified sequence

		[*Nicotinia plumbaginifolia*]			
Lm16 (7)	HQ634407	Fibrinogen alpha chain mRNA [*Epinephelus coioides*]	$5e^{-110}$	551 (84%)	Inmune system
Lm17 (2)	HQ634408	Clone FJAU011 SSR marker sequence [*Anas Platyrhynchos*]	$3e^{-6}$	198 (75%)	Microsatellite
Lm18 (2)	HQ634409	mRNA for apoliprotein B [*Salmo salar*]	$7e^{-19}$	510 (78%)	Transport
Lm19	HQ634410	80673.Onmycontig mRNA sequence [*Oncorhynchus mykiss*]	$4e^{-39}$	356 (79%)	Unidentified sequence
Lm20 (2)	HQ634411	Clone AFE50M24.1 sex and growth traits AFLP marker sequence [*Penaus monodon*]	$4e^{-4}$	189 (90%)	Unidentified sequence
Lm21	HQ634412	DNA sequence from clone DKEY-92I17 [*Danio rerio*]	$3e^{-4}$	171 (81%)	Unidentified sequence
Lm22 (2)	HQ634413	Phosphatidyl glycerol specific phospholipase C-like mRNA [*Pyrus communis*]	$7e^{-5}$	393 (100%)	Metabolism
Lm23 (10)	HQ634414	Putative ATP synthase gamma chain mRNA [Pyrus communis]	$9e^{-5}$	165 (100%)	Metabolism
Lm24 (4)	HQ634415	Clone lmos2p07e08 mRNA sequence [*Lithognatus mormyrus*]	$5e^{-24}$	279 (82%)	Unidentified sequence
Lm25 (2)	HQ634416	18s Ribosomal RNA gene [*Scophthalmus maximus*]	$3e^{-69}$	551 (100%)	Ribosomal protein
Lm26 (2)	HQ634417	Eukaryotic translation initiation factor 2B, subunit 3 gamma (eif2b3), mRNA [*Danio rerio*]	$6e^{-22}$	221 (80%)	Gene expression, regulation and protein synthesis.
Lm27	HQ634418	Alpha globin gene cluster [*Sphoeroides nephelus*]	e^{-63}	808 (77%)	Transport
Lm28	HQ634419	IGFALS [*Sphoeroides nephelus*]	$6e^{-38}$	808 (79%)	Cellular comunication and signal transduction (Cell adhesion)
Lm29	HQ634420	Clone Rsa456 microsatellite	e^{-10}	200 (94%)	Microsatellite

		sequence [*Salmo salar*]			
Lm30	HQ634421	16S rRNA gene [Uncultured *Crenarchaeota*]	e^{-7}	127 (94%)	Ribosomal protein
Lm31	HQ634422	Mitochondrion [Psetta maxima]	$5e^{-53}$	183 (100%)	Mitochondrion
Lm33	HQ634424	Mitochondrial ornithine transporter 1 putative mRNA [*Anoplopoma fimbria*]	$2e^{-140}$	463 (86%)	Transport
Lm34	HQ634425	cDNA-AFLP fragment, clone Np010 [*Nicotiana plumbaginifolia*]	$2e^{-4}$	295 (78%)	Unidentified sequence
Lm35	HQ634426	Clone lmos9p07g08 mRNA sequence [*Lithognatus mormyrus*]	e^{-7}	337 (79%)	Unidentified sequence
Lm36	HQ634427	Tetraspanin-9 (tsn9), mRNA [*Oncorhynchus mykiss*]	$2e^{-10}$	118 (92%)	Inner / Outer structures
Lm37	HQ634428	Germ cell-less protein (gc1), mRNA [*Oryzias latipes*]	e^{-123}	423 (86%)	Highest levels in pachytene and diplotene stage spermatocytes and primordial germ cells of the male and the female.
Lm38	HQ634429	VHSV-induced protein-10, mRNA [*Oncorhynchus mykiss*]	e^{-66}	575 (74%)	Inmune system

Table 6: Transcripts obtained from female liver in the mass cloning experiment. First column indicates the name of the clone and in parenthesis the number of sequence repeats is shown. The annotation from GenBank database, its significance (e-value), length of identity and the biological process based on GO terms are included. Underlined, those female-specific fragments.

Seq. (repeats)	Database access	Alignment [Sp.]	E value	Length (Ident.)	Biological process
Lf01	HQ634430	28s ribosomal RNA gene [Uncultured eukaryote] (2)	$2e^{-08}$	254 (100%)	Ribosomal protein
Lf02	HQ634431	Low density lipoprotein receptor-like [*Danio rerio*]	$3e^{-28}$	328 (75%)	Transport
Lf03 (3)	HQ634432	S-phase kinase-associated protein 1 puttive RNA [*Anoplopoma fimbria*]	$7e^{-146}$	488 (86%)	Metabolism
Lf04	HQ634433	312.C1 mRNA sequence [*Hippoglossus hippoglossus*]	$4e^{-60}$	492 (88%)	Unidentified sequence
Lf05	HQ634434	Nucleotide-binding oligomerization domain containing 2-like, mRNA [*Danio rerio*]	$6e^{-20}$	492 (72%)	Inmune system
Lf06	HQ634435	Kelch-like 22 (Drosophila)-like, mRNA [*Danio rerio*]	$3e^{-24}$	517 (72%)	Cell division, DNA synthesis, repair and replication
Lf07	HQ634436	Actin-related protein 2/3 complex subunit 4 putative mRNA [*Anoplopoma fimbria*]	$8e^{-12}$	517 (90%)	Inner / outer structures
Lf08	HQ634437	151081.Onmycontig mRNA sequence [*Oncorhynchus mykiss*]	e^{-4}	517 (77%)	Unidentified sequence
Lf09	HQ634438	Poly polymerase 14 putative mRNA, pseudogen cds [*Salmo salar*],	$2e^{-59}$	582 (73%)	Cell division, DNA synthesis, repair and replication
Lf10	HQ634439	Clone rpfsba0_002320 Microsatellite sequence [*Scophtalmus maximus*]	e^{-59}	396 (89%)	Microsatellite
Lf11	HQ634440	Clone FJAU014 SSR marker sequence [*Anas platyrhynchos*]	$7e^{-5}$	396 (79%)	Unidentified sequence
Lf12	HQ634441	Transferrin mRNA [*Pagrus major*]	$5e^{-13}$	361 (72%)	Transport
Lf13 (21)	HQ634442	Putative beta family G-protein mRNA [*Musa acuminata*]	$4e^{-7}$	361 (87%)	Cellular comunication and signal

					transduction
Lf14	HQ634443	Similar to rho/rac guanine nucleotide exchange factor 18, mRNA [*Danio rerio*]	$6e^{-15}$	637 (80%)	Cellular comunication and signal transduction
Lf15 (4)	HQ634444	mRNA for apoliprotein B [*Salmo salar*]	$2e^{-20}$	548 (73%)	Transport
Lf16 (4)	HQ634445	Putative ATP synthase gamma chain mRNA [*Pyrus communis*]	$8e^{-4}$	367 (98%)	Metabolism
Lf17 (9)	HQ634446	Complement component C9 mRNA [*Pseudopleuronectes americanus*]	$4e^{-111}$	564 (84%)	Inmune system
Lf18	HQ634447	mRNA for antimicrobial peptide precursor (TAPP gene) [*Scophtalmus maximus*]	$4e^{-118}$	596 (99%)	Inmune system
Lf19	HQ634448	220.C1 mRNA sequence [*Hippoglossus hippoglossus*]	$9e^{-159}$	671 (85%)	Unidentified sequence
Lf20	HQ634449	Matrix metallopeptidase 30 (mmp30), mRNA [*Danio rerio*]	$2e^{-39}$	671 (72%)	Metabolism
Lf21 (2)	HQ634450	Synaptobrevin homolog ykt6 putative mRNA [*Oncorhynchus mykiss*]	$3e^{-74}$	433 (82%)	Transport
Lf22 (2)	HQ634451	28s ribosomal RNA gene [*Scarus ghobban*]	$2e^{-9}$	302 (96%)	Ribosomal protein
Lf23	HQ634452	755.C1 mRNA sequence [*Hippoglossus hipoglossus*]	$5e^{-63}$	351 (91%)	Unidentified sequence
Lf24	HQ634453	Inter alpha (globulin) inhibitor H3 [*Danio rerio*]	$3e^{-5}$	351 (63%)	Metabolism
Lf25	HQ634454	Ribosomal protein L12 mRNA [*Scophthalmus maximus*]	$5e^{-134}$	398 (99%)	Ribosomal protein
Lf26 (3)	HQ634455	Clone lmos2p07e08 mRNA sequence [*Lithognatus mormyrus*]	e^{-22}	551 (81%)	Unidentified sequence
Lf27 (8)	HQ634456	Fibrinogen alpha chain mRNA [*Epinephelus coioides*]	$5e^{-116}$	507 (85%)	Inmune system
Lf29	HQ634458	Full-length cDNA [*Tetraodon nigroviridis*]	$6e^{-57}$	445 (73%)	Unidentified sequence
Lf30	HQ634459	Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform [*Mus musculus*]	$3e^{-7}$	267 (88%)	Metabolism
Lf31	HQ634460	cDNA-AFLP fragment, clone Np015 [*Nicotiana plumbaginifolia*]	$2e^{-5}$	119 (90%)	Unidentified sequence

Lf32	HQ634461	DNA sequence from clone DKEY-92I17 [*Danio rerio*]	$5e^{-4}$	235 (81%)	Unidentified sequence
Lf33 (3)	HQ634462	Clone AFE51M31 sex and growth traits AFLP marker sequence [*Penaus monodon*]	e^{-4}	219 (84%)	Unidentified sequence
Lf34	HQ634463	Alpha globin gene cluster [*Sphoeroides nephelus*]	$6e^{-54}$	786 (78%)	Transport
Lf35	HQ634464	IGFALS [*Sphoeroides nephelus*]	e^{-17}	786 (68%)	Cellular comunication and signal transduction (Cell adhesion)
Lf36	HQ634465	Dihydrolipoyl dehydrogenase, mitochondrial precursor putative mRNA [*Salmo salar*]	$2e^{-25}$	446 (83%)	Metabolism
Lf37	HQ634466	Interferon-related developmental regulator 1 putative mRNA [*Salmo salar*]	e^{-46}	425 (87%)	Development
Lf38	HQ634467	Clone 050 AFLP marker mRNA sequence [*Citrus reticulata*]	$2e^{-7}$	160 (87%)	Unidentified sequence
Lf39 (2)	HQ634468	18s Ribosomal RNA gene [*Scophthalmus maximus*]	$2e^{-46}$	793 (93%)	Ribosomal protein
Lf40	HQ634469	Tetraspanin-9 (tsn9), mRNA [*Oncorhynchus mykiss*]	$8e^{-26}$	517 (93%)	Inner / Outer structures
Lf41	HQ634470	DNA sequence from clone DKEY-211E20 [*Danio rerio*]	$3e^{-11}$	517 (80%)	Unidentified sequence
Lf42	HQ634471	Disulfide-isomerase A3 precursor putative mRNA [*Salmo salar*]	$2e^{-78}$	639 (75%)	Different functions
Lf43	HQ634472	Clone lmos9p07g08 mRNA sequence [*Lithognatus mormyrus*]	$^{e-7}$	288 (79%)	Unidentified sequence
Lf44 (3)	HQ634473	Beta-2-glycoprotein 1 precursor putative mRNA [*Anoplopoma fimbria*]	$7e^{-127}$	551 (80%)	Inmune system
Lf45 (2)	HQ634474	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 putative mRNA [*Anoplopoma fimbria*]	e^{-9}	77 (91%)	Gene expression, regulation and protein synthesis.
Lf46	HQ634475	Full-length cDNA [*Tetraodon nigroviridis*]	$3e^{-55}$	424 (85%)	Unidentified sequence
Lf47	HQ634476	Transcript-derived fragment TDF57 mRNA sequence [Phytophthora infestans]	$3e^{-4}$	424 (85%)	Unidentified sequence
Lf48	HQ634477	Clone lmos2p09a05 mRNA sequence [*Lithognatus mormyrus*]	e^{-6}	113 (84%)	Unidentified sequence

Table 7: Transcripts with significant similarity to GenBank database obtained from male brain in the mass cloning experiment. First column indicates the name of the clone and in parenthesis the number of sequence repeats is shown. The annotation from GenBank database, its significance (e-value), length of identity and the biological process based on gene ontology (GO) terms are included. * Indicates sequences less than 200 bp.

Seq. (repeats)	Database access	Alignment [Sp.]	E value	Length (Ident.)	Biological process
Bm1 (16)	JN650438	Putative beta family G-protein mRNA [*Musa acuminata*]	$2e^{-6}$	342 (95%)	Cellular comunication and signal transduction
Bm2	JN650439	SRY-box containing gene 14 [*Danio rerio*]	e^{-12}	869 (76%)	Gene expression, regulation and protein synthesis
Bm3	JN650440	Transcript-derived fragment TDF57 mRNA [*Phytophthora infestans*]	$3e^{-6}$	221 (88%)	Unidentified sequence
Bm4	JN650441	Clone FJAU002 SSR marker sequence [*Anas platyrhynchos*]	$2e^{-5}$	340 (81%)	Microsatellite
Bm5	JN650442	Basic leucine zipper and W2 domains 1 transcriptional factor mRNA [*Sparus aurata*]	$2e^{-35}$	903 (91%)	Gene expression, regulation and protein synthesis
Bm6	JN650443	mRNA for corticotropin releasing hormone (crh gene) [*Solea senegalensis*]	$6e^{-55}$	917 (74%)	Stress
Bm7 (15)	JN650444	Putative ATP synthase gamma chain mRNA [*Pyrus communis*]	$6e^{-9}$	643 (79%)	Metabolism
Bm8	JN650445	FAM3B, Mx2, Mx1, TMPRSS2 genes [*Sus scrofa*]	$3e^{-9}$	362 (92%)	Unidentified sequence
Bm9	JN650446	clone ssal-rgf-501-107 Cold-inducible RNA-binding protein putative mRNA [*Salmo salar*]	e^{-12}	738 (78%)	Stress
Bm10 (3)	JN650447	Female-specific AFLP marker VevaF160 genomic sequence [*Verasper variegatus*]	$3e^{-6}$	219 (95%)	Unidentified sequence
Bm11	JN650448	Isolate WH1o 18S ribosomal RNA gene [*Kareius bicoloratus*]	$4e^{-20}$	959(78%)	Ribosomal protein
Bm12	JN650449	Clone eluc-evq-503-302 Ubiquitin-conjugating enzyme E2 variant 2 putative mRNA [*Esox lucius*]	$2e^{-41}$	638 (93%)	Development
Bm13	JN650450	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2 [*Salmo salar*]	$7e^{-13}$	471 (80%)	Cellular comunication and signal transduction
Bm14 (2)	*	Clone FJAU011 SSR marker sequence [*Anas platyrhynchos*]	$3e^{-6}$	85 (91%)	Microsatellite

Bm15	JN650450	18S ribosomal RNA gene [*Scophthalmus maximus*]	$4e^{-69}$	820 (100%)	Ribosomal protein
Bm16	JN650451	Plasminogen activator inhibitor 1 RNA-binding protein-like [*Danio rerio*]	$2e^{-6}$	503 (75%)	Metabolism
Bm17	JN650452	Clone C372 nuclear NF-kappaB activating protein mRNA [*Siniperca chuatsi*]	$6e^{-11}$	884 (83%)	Gene expression, regulation and protein synthesis
Bm18	JN650453	Ubiquitin mRNA [*Pachycara brachycephalum*]	e^{-5}	627 (94%)	Cellular comunication and signal transduction
Bm19	JN650454	Full-length cDNA [*Tetraodon nigroviridis*]	$2e^{-9}$	248 (72%)	Unidentified sequence
Bm20	JN650455	Clone AF-E53M34-3 sex and growth trait AFLP marker genomic sequence [*Penaeus monodon*]	$4e^{-6}$	251 (71%)	Unidentified sequence
Bm21 (3)	JN650456	Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform [*Mus musculus*]	$6e^{-7}$	438 (87%)	Cell division, DNA synthesis, repair and replication
Bm22 (2)	*	Chromosome sequence corresponding to linkage group 18 [*Dicentrarchus labrax*]	$4e^{-35}$	155 (86%)	Unidentified sequence
Bm23	*	Clone FJAU014 SSR marker sequence [*Anas platyrhynchos*]	$5e^{-5}$	95 (79%)	Microsatellite
Bm24	JN650457	Interferon-induced protein 44-like [*Danio rerio*]	$3e^{-18}$	516 (79%)	Inmune system
Bm24	JN650458	Clone afim-evh-006-067 Eukaryotic translation initiation factor 3 subunit M putative mRNA [*Anoplopoma fimbria*]	$5e^{-9}$	516 (94%)	Gene expression, regulation and protein synthesis

Table 8: Transcripts with significant similarity to GenBank database obtained from female brain in the mass cloning experiment. First column indicates the name of the clone and in parenthesis the number of sequence repeats is shown. The annotation from GenBank database, its significance (e-value), length of identity and the biological process based on gene ontology (GO) terms are included. * Indicates sequences less than 200 bp.

Seq. (repeats)	Database access	Alignment [Sp.]	E value	Length (Ident.)	Biological process
Bf1 (16)	JN650459	Putative ATP synthase gamma chain mRNA [*Pyrus comunis*]	$2e^{-11}$	874 (80%)	Metabolism
Bf2	JN650460	SRY-box containing gene 14 (sox 14) [*Danio rerio*]	$2e^{-11}$	889 (83%)	Gene expression, regulation and protein synthesis
Bf3	JN650461	cDNA-AFLP fragment, clone Np067 [*Nicotiana plumbaginifolia*]	$2e^{-5}$	295 (75%)	Unidentified sequence
Bf4	JN650462	Clone ssal-rgf-521-309 Plasminogen activator inhibitor 1 RNA-binding protein putative mRNA [*Salmo salar*]	$5e^{-19}$	338 (77%)	Gene expression, regulation and protein synthesis
Bf5	JN650463	FYN-binding protein [*Salmo salar*]	$7e^{-38}$	472 (71%)	Inmune system
Bf6	JN650464	Clone AFE19M19 sex and growth traits AFLP marker sequence [*Penaeus monodon*]	$3e^{-19}$	634 (79%)	Unidentified sequence
Bf6	JN650465	Protein phosphatase 2A regulatory subunit B55 delta isoform mRNA [*Carassius auratus*]	$3e^{-6}$	634 (89%)	Cell division, DNA synthesis, repair and replication
Bf7	JN650466	Clone FJAU011 SSR marker sequence [*Anas platyrhynchos*]	e^{-7}	900 (80%)	Microsatellite
Bf8 (15)	JN650467	Putative beta family G-protein mRNA [*Musa acuminata*]	$4e^{-6}$	275 (91%)	Cellular comunication and signal transduction
Bf9 (2)	JN650468	Voucher S.gho-V-NBFGR-LKO 28S ribosomal RNA gene [*Scarus ghobban*]	$8e^{-9}$	834 (95%)	Ribosomal protein
Bf10	JN650469	Chromosome sequence corresponding to linkage group 1 [*Dicentrarchus labrax*]	$7e^{-7}$	478 (91%)	Unidentified sequence
Bf11 (6)	*	cDNA-AFLP fragment, clone Np015 [*Nicotiana plumbaginifolia*]	e^{-5}	198 (91%)	Unidentified sequence
Bf12	JN650470	Mitochondrion [Psetta maxima]	$3e^{-103}$	932 (99%)	Mitochondrion
Bf13	JN650471	Phosphatidyl glycerol specific phospholipase C-like mRNA [*Pyrus communis*]	$4e^{-5}$	232 (100%)	Metabolism

Bf14	JN650472	G alpha s/olf-1 protein [*Oncorhynchus mykiss*]	$3e^{-12}$	649 (88%)	Cellular communication and signal transduction
Bf15	JN650473	Clone FJAU014 SSR marker sequence [*Anas platyrhynchos*]	$7e^{-5}$	354 (79%)	Microsatellite
Bf16	JN650474	Ectonucleoside triphosphate diphosphohydrolase 4 [*Meleagris gallopavo*]	$9e^{-9}$	280 (84%)	Metabolism
Bf17	JN650475	DNA sequence from clone DKEY-34K9 in linkage group 19 [Danio rerio]	$4e^{-6}$	236 (84%)	Unidentified sequence
Bf18	JN650476	DNA sequence from clone CH73-367P20 in linkage group 1 [Danio rerio]	e^{-6}	241 (85%)	Unidentified sequence
Bf19	JN650477	Clone FJAU011 SSR marker sequence [*Anas platyrhynchos*]	$5e^{-5}$	781 (77%)	Microsatellite
Bf20	JN650478	Clone ssal-rgf-501-107 Cold-inducible RNA-binding protein putative mRNA [*Salmo salar*]	$6e^{-13}$	422 (78%)	Stress
Bf21	JN650479	Protein phosphatase 2, regulatory subunit B, beta [*Rattus norvegicus*]	$2e^{-5}$	391 (87%)	Cell division, DNA synthesis, repair and replication
Bf22	*	Neurofilament medium polypeptide-like [*Equus caballus*]	$6e^{-8}$	168 (91%)	Neurology
Bf23 (2)	JN650480	Sine oculis homeobox homolog 4.3 (six4.3) [*Danio rerio*]	$5e^{-6}$	720 (86%)	Gene expression, regulation and protein synthesis