revista portuguesa de ZOOTECNIA

Revista Portuguesa de Zootecnia
Associação Portuguesa dos Engenheiros Zootécnicos
fontain@utad.pt
ISSN (Versión impresa): 0872-7090
PORTUGAL

2005
B. E. P. Louro / A. L. S. Passos / D. M. Power
TRANSCRIPTOME ANALYSIS OF THE GILTHEAD SEA BREAM (SPARUS AURATUS) PITUITARY GLAND: TYPE I MARKERS FOR MOLECULAR GENETICS
Revista Portuguesa de Zootecnia, , año/vol. XII, número 002
Associação Portuguesa dos Engenheiros Zootécnicos
Vila Real, Portugal
pp. 91-105

Red de Revistas Científicas de América Latina y el Caribe, España y Portugal

Universidad Autónoma del Estado de México

http://redalyc.uaemex.mx

TRANSCRIPTOME ANALYSIS OF THE GILTHEAD SEA BREAM (*SPARUS AURATUS*) PITUITARY GLAND: TYPE I MARKERS FOR MOLECULAR GENETICS

B. E. P. Louro, A. L. S. Passos and D. M. Power[1]

Laboratory of Comaparative and Molecular Endocrinology, CCMAR, CIMAR Laboratório Associado, Universidadedo Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[1]towhom correspondence should be addressed

Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal-Tel: +351-289800100 ext 7335; Fax: +351-289818419

Email: dpower@alg.pt

(*Recepção: 8 de Setembro de 2005; Aprovado: 6 de Outubro de 2005)*

ABSTRACT

The pituitary gland of vertebrates produces a number of hormones, growth hormone (GH), prolactin (PRL), somatolactin (SL), gonadotrophin (GTH), thyroid stimulating hormone (TSH), and proopiomelanocorticotrophin (POMC), which regulate a range of important production traits. In the present study a gilthead sea bream (*Sparus auratus*) pituitary cDNA library was arrayed (948 clones) in 96 well plates and used to generate expressed sequence tags (ESTs) for pituitary hormones and novel pituitary transcripts. Using a combination of colony blotting and sequencing 750 clones were analysed, 61 ESTs were isolated for the first time in *Sparus auratus*, 12 ESTs were identified for the first time in teleosts and 70 ESTs which corresponded to 58 genes were unidentified. In addition to isolating ESTs for all the pituitary hormones, four novel transcripts with high levels of expression in the pituitary gland were also identified. The present data contribute to an ongoing European project (Bridgemap, www.bridgemap.tuc.gr) which aims to generate molecular resources for implementation of a selective breeding program for gilthead sea bream in Europe.
Key -words: BridgeMap, expressed sequence tags, novel pituitary transcripts, pituitary cDNA library, Sparus auratus, type I markers

INTRODUCTION

The diversification of aquaculture species is dependent on the establishment of sustainable production and depends on successful reproduction and completion of the life cycle in captivity (Donaldson, 1997). Molecular resources are currently available for a limited number of teleost fish used for aquaculture such as, winter

flounder, (*Pleuronectes americanus*, Douglas *et al.*, 1999), carp (*Cyprinus carpio*, Savan and Sakai, 2002), channel catfish (*Ictalurus punctatus,* Karsi *et al.* 1998; Liu *et al.*, 1999), salmon (www.salmongenome.no/cgi-bin/sgp.cgi) and trout (*Oncorhynchus mykiss*, Kono *et al.*, 2000). The principle resources developed for important aquaculture species are type I (genes) and type II (microsatellite) molecular markers for gene mapping, marker assisted selection (MAS) and also for genetic analysis of populations. However, so far relatively few selection programs exist for fish and their evolutionary diversity coupled with economic considerations represent a significant barrier to the implementation of molecular genetics in aquaculture.

The present study aimed to establish a technically simple, quick and relatively cheap method to characterise active genes in the transcriptome of the pituitary gland of *Sparus auratus*, an important Southern European aquaculture species and a member of the Sparidae, to which a number of other species of commercial interest belong eg. *Dentex dentex*, *Pagrus pagrus*, *Diplodus sargus*, *Diplodus puntazzo*, *Lithognathus mormyrus*, *Pagellus erythrinus*, *Pagellus bogaraveo*, *Pagrus pagrus*, *Pagrus aurita*, *Pagrus caeruleostictus*. The pituitary gland was chosen as in vertebrates it produces a range of hormones which regulate important production traits (Gelderman, 1975; Van Laere *et al.*, 2003). A rapid method based upon the generation of a pituitary macroarray, identification by specific hybridisation of clones corresponding to abundant pituitary hormones and then sequencing of non-hormone genes was developed.

MATERIALS AND METHODS

Preparation of pituitary Macroarray

A *Sparus auratus* pituitary cDNA library (1×10^6 primary recombinants with an average insert size of 1000bp) was constructed using the UNI-ZAP XR cloning kit (Stratagene, La Jolla, CA) and mRNA extracted from pituitary glands (n = 30) of adult Sparus auratus (600 g$\pm$ 25g) (full method given in Santos *et al.*, 1999).

In order to convert the I libraries into plasmid libraries a mass in vivo excision procedure was carried out. XL1-Blue MRF' bacteria were resuspended in 10 mM $MgSO_4$ to a final OD_{600} of 0.6 and the *Sparus auratus* pituitary library in lambda

bacteriophage (ratio 1:10 phage-to-bacteria) and helper phage (10:1 helper phage-to-bacteria) were added. Absorption of bacteriophage to bacteria was carried out at 37ºC for 15 minutes, heat inactivated (65-70ºC for 20 min), cell debris removed and used to transform SOLR cells. After overnight incubation individual colonies were picked into ten, 96 well plates containing 100ml of LB broth and ampicillin (50?g/ml) and grown as static cultures at 37ºC and 40ml of glycerol (50%w/v) added prior to storage at -20ºC. Fifty clones were selected at random from the arrayed library and DNA isolated for sequencing. The quality of the macroarray and the sequencing was assessed using these clones and hormone specific cDNA were also identified.

Plasmid DNA was prepared using the alkaline lysis procedure (Birnboim and Doly, 1979) and then extracted with phenol. The insert was sequenced with an automated sequencer (ABI 373) using the T3 sequencing primer in the polylinker of pBluescript II SK(+/-) which flanked the 5' region of the cDNA insert. cDNA inserts were sequenced once and had an average length of 600 bases and each constituted an expressed sequence tag (EST).

Macroarray hybridisation and EST generation

Isolated colonies were spotted in duplicate onto nylon membrane (Hybond-N, Amersham Biosciences, Lisbon, Portugal) using a 96 well replicating tool. Membranes were placed, colony side upwards, on LB agar and incubated overnight at 37ºC and used for colony blotting (Grunstein and Hogness, 1975). Membranes were washed with SDS (4%), denatured (1.5M NaCl/0.5MNaOH), neutralized (1.5M NaCl/0.5M Tris, pH 7.2/1mM EDTA) and washed briefly (2x SSC) before baking for 2hr at 80oC.

To identify clones containing cDNA of abundant genes encoding the pituitary hormones (target cDNA) a set of membranes was hybridized in series with [^{32}P]dCTP labeled probes specific for *Sparus auratus* prolactin (PRL), growth hormone (GH), somatolactin (SL) and proopiomelanocortin (POMC). Probes corresponded to full-length cDNA and were available in house (PRL, GH, Santos *et al*. 1999) or were identified among the initial 50 clones randomly selected and sequenced to assess macroarray quality. Membranes were prehybridised in Church-Gilbert solution (Church and Gilbert, 1984) at 58ºC for 2 hours and then hybridised overnight at the same temperature in Church-Gilbert to which labeled probe had been added. High stringency washes were carried out at 58ºC using 2 x SSC/0.1%SDS (2 x 10 minutes), 1 x SSC/0.1%SDS (2 x 15 minutes) and 0.1 x SSC/0.1%SDS (1 x 20minutes).

Positive signals were detected after probe hybridisation by exposure of membranes to autoradiography film (X-OMAT, Kodak) with an intensifying screen. Specificity of probe hybridization was established by sequencing 10-20 positive clones for SL and POMC and 5-10 clones of GH and PRL. Sixty percent of the macroarray clones were hormone-encoding cDNA. Almost half (48.3%) of the clones, which failed to hybridise with hormone probes, were randomly selected and sequenced.

The length of sequence obtained was approximately 600bp. Prior to sequence analysis quality was assessed (poor sequences were rejected) and the vector and polylinker sequence were clipped. Sequence identity was determined using the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) using the BlastX and Blastn algorithms (Altschul *et al.*, 1994) against the non-redundant nucleotide database (nr db). Matches were considered to be significant when approximately 300 bp of the sequenced EST had an E-value of < 1e-5. To establish EST homologues, sequences were also submitted to Blastn searches against the GenBank EST databases and homologues defined as those with an E-value of $< 1e^{-5}$ and a score of >40 (Martins *et al.*, 2001). The resulting sequences were compared to each other using "stand-alone" Blastn (Altschul *et al.* 1994) and identical sequences clustered and clusters classified following the recommendations of the Gene Ontology Consortium (http://www.geneontology.org/). Multiple sequence alignment using ClustalX (Thompson *et al.*, 1997) was carried out using the most abundant pituitary hormone ESTs for a higher cluster resolution.

Characterisation of tissue specificity of unidentified clones

In order to determine if unidentified clones (those in which sequence similarity searches failed to give a putative identity) were pituitary specific RT-PCR was carried out. The sequence obtained for each clone was introduced into primer3_www.cgi v0.2 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and specific primers were designed. It was not possible to generate functional primers for all 58 unidentified pituitary transcripts and so only 35 were analysed in this study. A panel of cDNAs from *Sparus auratus* liver, heart, kidney, brain, white muscle, skin, gonads, gill arches, spleen, duodenum, caudal fin bone and pituitary was prepared. In brief, cDNA was synthesised from 1mg of total RNA in a 40µl reaction containing 0.05M Tris-HCl, pH8.3, 0.075M KCl, 3mM MgCl2, 5mM DTT, 0.25mM dNTP, 0.1 mg/ml $pd(N)_6$, RNAase inhibitor (3.2U; Amersham Pharmacia) and M-MLV reverse

transcriptase (20U; Gibco BRL, Barcelona), for an hour and a half at 37ºC. PCR was carried out in a 10µl reaction containing 0.1mg of tissue cDNA, 10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% Triton X-100, 2mM MgCl2, 40?M dNTP, 0.3pmol of forward primer, 0.3pmol of reverse primer and Taq DNA polymerase (1.25U; Promega, Madison, WI), sterile water substituted cDNA in control reactions. In order to confirm a similar quantity of tissue cDNA was utilised in PCR reactions, a second amplification reaction of the house-keeping gene, elongation factor alpha (EF1a; Nowell *et al.*, 2000) was also carried out for each tissue cDNA. The PCR cycle utilized consisted of 2min at 94°C followed by 28 cycles of denaturing (94°C, 30 s), annealing (57°C, 30s), and extension (72°C, 30s). Control reactions in which reverse transcriptase was omitted from cDNA synthesis or in which cDNA was substituted with water in PCR reactions failed to give an amplified product. The presence or absence of reaction products was analysed on a 1.5% agarose gel containing ethidium bromide (0.5mg/ml). Gel images were collected using a Pharmacia Biotech ImageMaster® VDS system with Liscap® capture software and constant photographic parameters. Signal intensities for PCR products were quantified by densitometric analysis using ImageMaster 1D prime software v. 3.00, Pharmacia and the ratio of unidentified target mRNA/EF1-alpha in each sample was calculated and divided in three relative expression classes: low (0<=0,33), high (0.33<=0.66), and very high (0.66<=1).

RESULTS

Gene expression profile of Sparus auratus pituitary gland

Of the 948 clones analysed by colony blotting those encoding pituitary hormones were most abundant and accounted for 60 % (567 clones) of all clones in the macroarray. The hormones identified and their relative abundance in the macroarray were GH (10.3%), PRL (3.3%), SL (33.1%) and proopiomelanocorticotrophin (POMC; 13.1%). One hundred and eightly four, 5' ESTs were generated by randomly selecting and sequencing the non-hormone clones (Table 2), of these 114 corresponded to 65 known genes and were categorized according to cellular localisation of their products and the frequency of representation in the macroarray was noted (Table 2). Of all the known genes identified 61 were isolated for the first time in *Sparus auratus* and 12 were identified for the first time in teleosts while 70 ESTs which corresponded to 58 genes were unidentified (Table 2) with the present analysis. The ESTs generated from the pituitary macroarray have been deposited in GeneBank (accession numbers

TABLE I - SEQUENCE OF THE PCR PRIMERS UTILISED TO AMPLIFY PITUITARY HORMONE TRANSCRIPTS AND ALSO UNIDENTIFIED PITUITARY ESTS IN A RANGE OF DIFFERENT *SPARUS AURATUS* TISSUE. THE PRIMERS PAIRS ALBEIT DIFFERENT WERE ALL OPTIMISED FOR AN ANNEALING TEMPERATURE OF 57 ºC AND AMPLIFIED A PRODUCT BETWEEN OF 150-500BP.

Transcripts	Genebank Accession Nº	Forward primer 5'-3'	Reverse primer 5'-3'
GH	AY038038	GCCCCATCGACAAGCACG	CTACATTTTGCCACCGTCAG
SL	Y11144 L49205	GGAGTTCCTGACATGCTGCT	CGCTGTACGTTGTGGTCATC
PRL	AF060541	ACGGTGTTGTGCATGCTGGC	AGATGGCCTGAGCTGCTGGA
POMC	CB227624 CB227626	TGGAGTGCATCCAGCTCTGTC	CTGCTCCAGCGGAAGTG
TSHb	CB227560	ACCCACTGACTTCACCCTGT	GGCCGAGTATGTCCCTCA
GtHbII	CX244519	CCAAGGACCCAGTGATGAA	GACGTGTCCATAGCACAGA
GtHa	AF300425	CCCGAAGAACATCACCTC	TCTGGCAACACAAGAACAC
Unknown	DN048404	CAAAGTACGCCAACCTCACC	CCCTAGTTCCCGCATCATC
	CX244472	CCCATGAAGACCCAGGAG	AAGAGCAGCAGGGCAGAG
	CX244488	GACTAACTGTTGCAGCTCTCA	CCGCGGGTTTTAATACAGG
	CX244500	TCACTTCCTGTGACCAATCAG	CTGTCGGTTTCACCACTCAA
	CX244530	GATATGAGGAGCATTCACAGC	TTTCCACACGCATCAGCTC
	CB227588	CAGCGAGAGGAGAGTCAACC	GTTCTCAGACAGGGCACACA
	CB227601	AGGGTGTTTTGGGGACAC	ACCAGAGGGAAAGCAGGA
	DN048399	GACGGAGCATGTGACGAG	GGGAGGGTCTGTTAATCGAG
	CB227581	GCCAGATGGTTCTGGAAATG	TTCACCGGTCACAGTTCTACCT
	CB227579	CTGCATTGACTTGTGGGAGA	ATGACTCCGAAGCGAAACAC
	CB227593	GGTGCAACCCACCTAACAC	GTTTCCTCCCCACAGATAGC
	CB227603	GGGGCCTTAACAAGTGCAG	CTTTCGGCACGCTTCATC
	CB227606	GCTTGGTCCCCTTATTGC	CCACTGAGATGCGTCCAT
	CB227608	GACCACTTGAGGCTTGAAC	CTGCCTGCACAACAGTGAAT
	DN048390	AGGGCATGTTGGAGGAAC	GCACTCCATGTGGCTTCAG
	DN048398	GCCAGCATCACGAGTTTG	GAGCAACAGGGGACAAGG
	DN048405	GTCAGAAATGGGTCGCTAA	CGTCAGAAATGGGTCGCTAA
	CX244466	CGCCAGTTTCTCCGTTGT	CGCCAGTTTCTCCGTTGT
	CX244471	AAGCTGAAGCCAGCCAAT	AAGCTGAAGCCAGCCAAT
	CX244476	CAGATGCCAGCCGAGAAC	GTGGGGTGTGGGTTGTGT
	CX244480	GAAGAGGCGAGTGAGGAGAG	CCTGGAGTCAGCAAACAGG
	CB227611	GTCAGCGAGCGAATCTCAAC	GGGCCAACGACTGTTATGG
	CX244463	CCCATGCCTCCTCAGTTCT	GGAAACAAGTGCGCAACAC
	CX244468	CAGCCTCCAAATGGCAAC	ACAGTCGCCCCACAGGTA
	CX244510	TGCACTGTTCGGTTTGGA	GAGCTTTGGATCTGGGACTG
	CX244514	AGGTCAAAACTCCTCCCAGTC	GTCATTCCCGATACCAAACC
	CX244473	CAGCCGGTATGAAGAGAGGA	GTAACACAGCTGGTGGAGAGC
	CX244522	TCGGGGAATACTGTTGGTCT	GATTGTGGTGGGGCTGTT
	CB227550	GGTGTTTGCTGGGAGGAAC	ACGGATCTGACGGTGCTG
	CB227552	CTCCTCCAACAGGCACCTAC	GTCAGGCCACACATCCAAC
	CB227554	CGGTGTAAGTCCCACAAGG	GCAGCATCCCAGCAGTAGT
	CB227556	AAAGAGCACTCGGTCGGTAG	TGACAGAAGGGCCAGGTT
	CB227557	GTGGTGGTTGTCATCTGTGG	GGCTGACTGCAGGTGTGA
	CB227575	GCAAGAGGAGGCTGTGGAG	CCAGTGCAAGGGACAGACA
	CB227571	CAGGCTGTCAAGGCGATAA	TGCCTTAGGACGTGTAGCAA

indicated in table 2). Grouping of genes according to cellular localisation revealed that after hormones the second most abundant group corresponded to cDNA encoding mitochondrial proteins, the cytochrome-c-oxidases were particularly abundant. The third major group included translational machinery proteins such as large and small ribosomal proteins (Figure. 1).

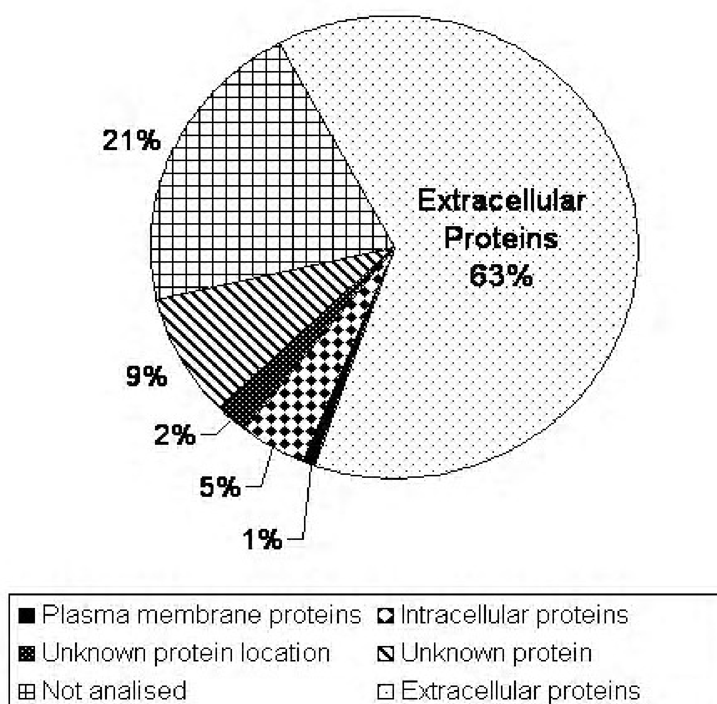

Figure 1. Scheme categorizing the transcripts identified in the *Sparus auratus* pituitary transcriptome based upon cellular localisation. ESTs were subject to BlastX and the protein products grouped according to cellular location, determined using GenBank+EMBL+ DDBJ+PDB "non-redundant" databases. The principal cellular localisations considered were; extracellular proteins, this group was principally composed of pituitary hormones (62%) and other extracellular proteins were a minor component (1%); plasma membrane proteins (1%) and intracellular proteins (5%). It was not possible to define the cellular location of some identified ESTs as they had an unknown protein location (2%). ESTs in which similarity searches failed to identify the protein product and unsequenced clones in the macroarray corresponded to 21%.

TABLE II - ESTS GENERATED FROM THE *SPARUS AURATUS* PITUITARY MACROARRAY (50 HORMONE ESTS GENERATED AS POSITIVE CONTROL FOR SPECIFICITY OF PROBE HYBRIDIZATION *). PUTATIVE GENE IDENTIFICATION WAS ESTABLISHED BY INTERROGATING THE NCBI NUCLEOTIDE DATABASE (http://www.ncbi.nlm.nih.gov/BLAST) USING BLASTX (OR BLASTN **). THE SIMILARITY SCORE (BITS) AND E VALUE AS IS THE ORGANISM POSSESSING THE SEQUENCE WITH THE GREATEST SIMILARITY IS GIVEN. CELLULAR LOCALISATION OF ESTS WAS EVALUATED USING THE RECOMMENDATIONS OF THE GENE ONTOLOGY CONSORTIUM (http://www.geneontology.org/).

Accession Nº	EST Identification clustered by cellular localisation	Frequency	Most blastX - Score (bits) / E Value (Closest species)
	1. Extracellular proteins	81	
	1.1. Secretory	80	
	1.1.1. Pituitary hormones	76	
CB227626	Proopiomelanocortin A*	4	272/ 7e^{-072} *Epinephelus coioides*
CX244494	Proopiomelanocortin B*	12	284/ 2e^{-075} *Acanthopagrus latus*
Y11144	Somatolactin (SAS)*	19	468/ e^{-131} *S. auratus*
L49205	Somatolactin (STS)*	15	465/ e^{-130} *S. auratus*
AF060541	Prolactin (PRL)*	9	425/ e^{-118} *S. auratus*
AF300425	Gonadotropin α subunit	6	385/ 1e^{-059} *S. auratus*
DN048396	Gonadotropin II beta subunit	2	179/ 3e^{-044} *Pagrus major*
CB227560	Thyrotropin β subunit	4	238/ 8e^{-062} *Oncorhynchus mykiss*
AY038038	Growth hormone (GH)*	5	375/ e^{-102} *S. auratus*
	1.1.2 Other secretory proteins	4	
CX244535	Neuronal activity-regulated pentraxin	1	149/ 8e^{-035} *Tetraodon nigroviridis*
CX244465	Apolipoprotein E	1	228/ 6e^{-059} *O. mykiss*
CX244485	Cystatin	2	112/ 1e^{-023} *T. nigroviridis*
	1.2. Matrix proteins	1	
CB227621	Acidic secreted protein in cartilage (ASPIC)	1	314/ 1e^{-084} *T. nigroviridis*
	2. Plasma membrane proteins	7	
CX244513	Type III iodothyronine deiodinase	1	149/ 4e^{-037} *Oreochromis niloticus*
CB227545	AMPA receptor subunit GluR3B	1	260/ 1e^{-072} *D. rerio*
AAO86517	Thyroid hormone receptor β	1	80/ 2e^{-014} *S. auratus*
CB227629	Growth hormone inducible transmembrane protein	1	172/ 1e^{-050} *Xenopus laevis*
CB227584	Phospholipase A2 group I	1	74 / 4e^{-010} *Chrysophrys major* **
CX244509	MHC class II invariant chain-like protein 1	1	222/ 2e^{-057} *Siniperca chuatsi*
CX244512	Solute carrier family 35, member B4	1	108/ 8e^{-025} *T. nigroviridis*

	3. Intracellular proteins	43	
	3.1.Nuclear proteins	8	
CB227544	Similar to Zinc finger protein 228	1	207/ 1e^{-052} *Canis familiaris*
CX244526	Zinc finger protein 238	1	129/ 3e^{-029} *T. nigroviridis*
CB227564	Id2 Protein	1	218/ 1e^{-055} *O. Mykiss*
CB227568	Similar to transcription elongation factor B	1	262/ 5e^{-069} *Rattus norvegicus*
CB227586	Similar to SWI/SNF related, matrix associated, actin dependent regulator of chromatin	1	206/ 3e^{-052} *T. nigroviridis*
X81646	GHF-1/PIT-1, pituitary transcription factor	2	165/ 1e^{-039} *S. auratus*
AF184170	Elongation factor 1-alpha (EF1-alpha)	1	437/ e^{-129} *S. auratus*
	3.2. Mitochondrial proteins	15	
-	Cytochrome b	2	347/ e^{-116} *S. auratus*
CB227570	Mitochondrial inner membrane translocating protein (timm23)	1	137/ 1e^{-031} *T. nigroviridis*
-	Ndufs1-prov protein	1	95/ 1e^{-018} *Xenopus laevis*
-	Cytochrome c oxidase subunit I	2	343/ 2e^{-093} *Galaxias maculatus*
-	Ubiquinol-cytochrome c reductase core I	1	326/ e^{-108} *T. nigroviridis*
-	Solute carrier family 25 alpha, member 5 (slc25a5)	1	442/ e^{-123} *T. nigroviridis*
-	Cytochrome-c-oxidase II	4	350/ 1e^{-095} *Cataetyx rubrirostris*
-	Cytochrome oxidase III	2	229/ e^{-106} *Pagrus auriga*
-	ATPase subunit 8 (ATPase8)	1	95/ 3e^{-020} *P. auriga*
	3.3. Cytoplasmic proteins	18	
	3.3.1. Ribosomal proteins	9	
CB227622	ribosomal protein L10	1	51/ 4e-014 *Ictalurus punctatus*
CB227558	Ribosomal protein S25	1	152/ 2e-036 *T. nigroviridis*
CB227569	Similar to 60S ribosomal protein L18a	1	356/ 3e-097 *T. nigroviridis*
CX244470	Ribosomal protein S7	2	365/ e-100 *Takifugu rubripes*
CB227591	Ribosomal protein P1	1	125/ 3e-028 *D. rerio*
CX244461	60S ribosomal protein L24	1	227/ 1e-058 *Pagrus major*
CX244496	Ubiquitin A-52 residue ribosomal protein fusion product 1 (Uba52)	1	249/ 1e-065 *Homo sapiens*
CX244523	Ribossomal protein L28	1	118/ 4e^{-026} *Hippocampus comes*

	3.3.2. Cytoskeletal proteins	7	
CX244462	Receptor for activated protein kinase C (RACK1)	2	299/ e^{-126} *P. major*
CX244493	Beta-actin protein	4	371/ e^{-114} *Epinephelus coioides*
CX244520	Similar to MAP1 light chain 3-like protein 2	1	238/ $2e^{-61}$ *T. nigroviridis*
	3.3.3. Others	2	
CB227573	Similar to Gdi-1 for complex RhoGDI-1	1	248/ $1e^{-066}$ *D. rerio*
CX244534	Alpha 4 subunit of 20S proteasome	1	421/ e^{-116} *T. nigroviridis*
	4. Unknown location	22	
CB227555	Similar to KIAA1076 protein [Homo sapiens]	1	295/ 3e-079 *T. nigroviridis*
CB227576	similar to calmodulin regulated spectrin-associated protein 1	1	60/ 2e-008 *Gallus gallus*
CB227594	Carbamoyl-phosphate synthetase III	1	137/ 5e-031 *Alcolapia grahami*
CB227596	cAMP-dependent protein kinase type I regulatory subunit	1	161/ 3e-074 *Gallus gallus*
CB227597	Like Gi2 alpha subunit	1	64/ 3e-007 *Oryzias latipes* **
CB227607	Similar to Glcci1; Glucocorticoid induced transcript 1[Mus musculus]	1	98/ 1e-019 *T. nigroviridis*
CB227610	ADP-ribosylation factor (ARF3)	1	234/ 2e-060 *Rattus norvegicus*
DN048391	NY-REN-58 antigen	1	130/ 3e-029 *Canis familiaris*
DN048393	Dachshund C protein (Dach1)	1	152/ $4e^{-036}$ *T. nigroviridis*
DN048394	KIAA1411 protein	1	291/ $6e^{-078}$ *T. nigroviridis*
CX244515	Enhancer of polycomb homolog 1, (Epc1), transcript variant 2 [Drosophila]	2	120/ $2e^{-026}$ *T. nigroviridis*
CX244490	protein phosphatase 1H (PP2C domain containing)	1	458/ e^{-128} *T. nigroviridis*
CX244498	5-aminolaevulinic acid dehyratase	1	422/ e^{-117} *T. nigroviridis*
CX244501	Hypothetical Protein TonB-dependent receptor protein containing protein	1	236/ $3e^{-059}$ *D. rerio* **
CX244502	similar to Osmotic stress protein 94 (Heat shock 70-related protein APG-1)	1	405/ e^{-120} *T. nigroviridis*
CX244521	Fatty acid binding protein H6-isoform (H6-FABP)	1	130/ $8e^{-031}$ *T. nigroviridis*
CX244544	Similar to RNA polymerase common subunit RPB6	1	48/ $7e^{-005}$ *Gallus gallus*
CX244489	Intestinal mucin 3	1	67/ $2e^{-010}$ *Mus musculus*

417_	Mucin 1 precursor	1	160/ $4e^{-038}$ *T. nigroviridis*
CX244514	similar to mastermind-like 2	1	116/ $2e^{-030}$ *T. nigroviridis*
CX244473	super cysteine rich protein; SCRP	1	22/ $7e^{-008}$ *Homo sapiens*
	5. Others	11	
CB227559	Reverse transcriptase pseudogene (ZfL3 LINE)	1	128/ $1e^{-027}$ *D. rerio* **
-	Mitochondrial 16S rRNA	1	839/ 0.0 *S. auratus* **
CX244472	Predicted gene Zgc:92146	1	72/ $9e^{-012}$ *D. rerio*
CX244474	clone DKEY-28J24 in linkage group 21	1	62/ $1e^{-006}$ *D. rerio* **
CX244469	Rex retrotransposon, D locus-related sequence	1	74/ $6e^{-010}$ *Xiphophorus maculates* **
CB227557	CAF89452.1 unnamed protein product	1	64/ $4e^{-010}$ *T. nigroviridis*
CX244522	CAF97170.1 unnamed protein product	1	187/ $3e^{-046}$ *T. nigroviridis*
CB227616	CNS0G9B4 full-length cDNA	1	88/ $3e^{-014}$ *T. nigroviridis* **
CB227603	CNS0FUWS full-length cDNA	1	66/ $1e^{-007}$ *T. nigroviridis* **
CB227579	CNS0GSZ4 full-length cDNA	1	72/ 1e-009 *T. nigroviridis* **
CB227556	CNS0FXGZ full-length cDNA	1	66/ $1e^{-007}$ *T. nigroviridis* **
	6. Unidentified	70	
	TOTAL	234	

Cluster analysis of the pituitary hormone ESTs generated from *Sparus auratus* pituitary and existing sequences deposited in genebank (http://www.ncbi.nlm.nih.gov) was carried out by multiple sequence alignment using ClustalX (Thompson *et al.*, 1997). This analysis revealed that a single cluster existed respectively for GH, PRL, GTH (*alpha* subunit) and GTH II (*beta* subunit) indicating the existence of a single cDNA transcript in the *Sparus auratus* for each of these hormones. In contrast, the ESTs of SL and POMC generated respectively 2 independent clusters indicating the existence of 2 different cDNAs for each of these hormones.

Tissue specificity of unidentified clones

The determination of the tissue distribution of unidentified Sparus auratus ESTs (n = 35) by RT-PCR demonstrated that the majority of transcripts had a ubiquitous tissue

TABLE III - RELATIVE EXPRESSION OF UNIDENTIFIED ESTS IN A *SPARUS AURATUS* CDNA TISSUE ARRAY. THE RELATIVE LEVEL OF EXPRESSION OF EACH TRANSCRIPT IN THE CDNA TISSUE ARRAY WAS CALCULATED USING THE RATIO BETWEEN THE EST BEING STUDIED AND EF1- A WHICH SERVED AS A CONTROL FOR THE QUANTITY OF CDNA USED IN RT-PCR REACTIONS. K - KIDNEY; L - LIVER; SP - SPLEEN; D - DUODENUM; SK - SKIN; H - HEART; WM - WHITE MUSCLE; B - BONE; BA - BRANCHIAL ARCHES; BR - BRAIN; G - GONADS; P - PITUITARY. THE RELATIVE EXPRESSION LEVELS WERE CLASSIFIED AS: - NULL; + LOW; ++ HIGH; +++ VERY HIGH AND NA - NOT ANALYSED.

Accession Nº	Tissues											
	K	L	Sp	D	Sk	H	WM	B	BA	Br	G	P
	Differential Expression											
DN048404	-	-	-	+	++	+	+	+	-	-	+	+++
CX244472	-	-	-	-	-	-	-	-	-	+	++	-
CX244488	-	-	-	-	+	-	-	-	-	+++	++	+++
CX244500	-	-	-	-	+	+	-	-	-	+++	++	++
CX244530	-	-	+	-	+++	-	-	-	-	+++	++	++
DN048390	-	-	+	-	-	-	-	-	-	+	-	-
CX244473	+++	-	+++	-	-	-	-	+	+	+++	+++	+
CB227601	++	-	+	++	++	-	++	-	+	++	-	+
	Ubiquitous High Expression											
CB227588	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CB227579	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++
CB227606	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CB227608	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
DN048398	+++	+	+++	+++	++	++	+++	+++	+++	+++	+++	+++
DN048405	++	++	++	++	++	+++	+++	++	++	++	++	++
CX244466	+++	++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++
CX244471	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CB227611	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CX244463	++	+	+++	+++	++	+++	+++	+++	++	+++	+++	+++
CX244468	+	+	++	++	+	++	++	++	++	+++	++	++
CX244510	++	+	++	+++	++	+++	+++	+++	+++	++	++	++
DN048399	-	-	++	++	+++	++	+++	++	++	+++	++	++
CB227581	-	-	+++	++	+++	+++	+++	+++	+++	+++	+++	++
	Ubiquitous Low Expression											
CB227593	+	+	++	+	+	+	+++	+	+	+	+	+
CX244514	+	+	+	+	+	+	+	+	+	++	++	+
CX244476	-	+	+	+	+	+	+	+	+	+	+	+
CX244480	+	+	+	+	+	++	+	+	+	++	+	+
CB227603	+	+	+	+	+	+	+	+	+	+	+	+
CX244522	+	-	+	+	++	+	+++	+	++	+	++	+
CB227550	+	+	na	na	na	+	+	na	na	+	+	+
CB227552	+	+	na	na	na	-	+	na	na	++	++	-
CB227554	-	-	na	na	na	-	+	na	na	+	+	+
CB227556	+	+	na	na	na	+++	-	na	na	+	+	++
CB227557	+	+	na	na	na	+	+	na	na	+++	+++	+
CB227575	+	+	na	na	na	-	+	na	na	+	++	+
CB227571	-	-	na	na	na	-	-	na	na	++	+	+

expression although a small group of transcripts (n = 4) were predominantly expressed in the pituitary gland. Assessment of the relative tissue abundance of different transcripts by normalising the data and using EF1-alpha as the internal control led to the identification of three principal groups of ESTs; i) those with a constant ubiquitous expression (Table 3); ii) those with a low ubiquitous expression (Table 3); and iii) those which were differentially expressed in tissue (Table 3). None of the unidentified ESTs were expressed exclusively in the pituitary gland, although several appeared to be highly expressed in the pituitary in relation to their expression in other tissue (Table 3). One EST was expressed predominantly in the Sparus auratus pituitary gland and skin (DN048404), a further EST was expressed predominantly in pituitary gland, brain, gonads and skin (CX244530) and two ESTs were expressed predominantly in the pituitary, brain and gonads (CX244488; CX244500). Currently further work is being carried out to characterise these clones in *Sparus auratus* and to identify homologues in fish in which the full genome has been sequenced (*Fugu rubripes, Tetraodon nigroviridis* and *Danio rerio*).

DISCUSSION

Hormone encoding genes were actively expressed in pituitaries from 3-year-old *Sparus auratus*, as illustrated by their frequent occurrence in the macroarray and the ease with which they were isolated. The abundance of cDNA clones for pituitary hormones is unsurprising and has previously been observed in expression profiles of the human pituitary gland (Hishiki *et al.*, 2000) and in channel catfish (*Ictalurus punctatus*) pituitary (Karsi *et al.*, 1998). In addition to cDNA for most of the pituitary hormones a range of other ESTs involved in pituitary hormone regulation and synthesis were isolated and included ESTs encoding factors involved in vesicular transport (ARFs, ADP-ribosylation factor); the pituitary-restricted POU domain transcription factor GHF-1/Pit-1, which is required for the expression of GH, PRL and TSH (Theill and Karin, 1993; Andersen and Rosenfeld, 1994) and several other transcription factors.

The tissue distribution of unidentified ESTs was established by RT-PCR using multi-tissue cDNA analysis and revealed that several were most abundant in the pituitary gland. The effectiveness of the approach utilised is clear, as it was possible to exclude 60% of macroarray clones by screening with specific probes and of the 184 none-hormone clones sequenced, five proved to be novel ESTs highly expressed in the

Sparus auratus pituitary gland relative to other tissue. Further work is underway to characterise the novel pituitary ESTs and establish their function.

CONCLUSION

The ESTs arising from the present study and deposited in Genebank represent a useful resource for future studies of *Sparus auratus* and other Sparidae important for southern European aquaculture. The data have been deposited in a database of molecular resources for *Sparus auratus* generated by the European project Bridge-Map (www.bridgemap.tuc.gr). Moreover, the approach presented is a relatively cheap and quick way to generate type I markers for prospective aquaculture species in which few resources are available.

ACKNOWLEDGEMENT

The authors thank Teresa Santos and Sara Almeida for technical assistance. This research was funded by the Commission of the European Union, Quality of Life and Management of Living Resources specific RTD programme (Bridge-Map, Q5RS-2001-01797) and Financiamento Plurianual from Fundação para a Ciência e Tecnologia (Portugal).

REFERENCES

Altschul, S.F., Boguski, M.S., Gish, W., Wootton, J.C., 1994. Issues in searching molecular sequence databases. Nature Genetics, 6: 119-129.

Andersen, B., Rosenfeld, M.G., 1994. Pit-1 determines cell-types during development of the anterior-pituitary gland - a model for transcriptional regulation of cell phenotypes in mammalian organogenesis. Journal of Biological Chemistry, 269: 29335-29338.

Birnboim, H.C., Doly, J., 1979. Rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research, 7: 1513-1523.

Church, G.M., Gilbert, W., 1984. Genomic sequencing. Proceedings of the National Academy of Sciences of the United States of America, 81: 1991-1995.

Donaldson, E., 1997. The role of Biotechnology in sustainable aquaculture. In: Bardach, J. (Ed.), Sustainable Aquaculture. John Wiley and Sons, Inc, New York, pp. 101-126.

Douglas, S.E., Gallant, J.W., Bullerwell, C.E., Wolff, C., Munholland, J., Reith, M.E., 1999. Winter flounder expressed sequence tags: establishment of an EST database and identification of novel fish genes. Marine Biotechnology, 1: 458-464.

Gelderman H, 1975. Investigation on inheritance of quantitative characters in animals by gene markers. I. Methods Theoretical and Applied Genetics 46, 300-319.

Grunstein, M., Hogness, D.S., 1975. Colony hybridization - method for isolation of cloned DNAs that contain a specific gene. Proceedings of the National Academy of Sciences of the United States of America, 72: 3961-3965.

Hishiki, T., Kawamoto, S., Morishita, S., Okubo, K., 2000. BodyMap: a human and mouse gene expression database. Nucleic Acids Research, 28: 136-138.

Karsi, A., Li, P., Dunham, R.A., Liu, Z.J., 1998. Transcriptional activities in the pituitaries of channel catfish before and after induced ovulation by injection of carp pituitary extract as revealed by expressed sequence tag analysis. Journal of Molecular Endocrinology, 21: 121-129.

Kono, T., Sakai, M., LaPatra, S.E., 2000. Expressed sequence tag analysis of kidney and gill tissues from rainbow trout (Oncorhynchus mykiss) infected with infectious hematopoietic necrosis virus. Marine Biotechnology, 2: 493-498.

Liu, Z.J., Karsi, A., Dunham, R.A., 1999. Development of polymorphic EST markers suitable for genetic linkage mapping of catfish. Marine Biotechnology, 1: 437-447.

Martins, R.P., Leach, R.E., Krawetz, S.A., 2001. Whole-body gene expression by data mining. Genomics, 72: 34-42.

Nowell, M.A., Power, D.M., Guerreiro, P.M., Llewellyn, L., Ramsurn, V., Wigham, T., Sweeney, G.E., 2000. Cloning and expression of an elongation factor-1 alpha in sea bream (Sparus aurata) larvae and adult tissue. Marine Biotechnology, 2: 173-179.

Santos, C.R.A., Brinca, L., Ingleton, P.M., Power, D.M., 1999. Cloning, expression, and tissue localisation of prolactin in adult sea bream (Sparus aurata). General and Comparative Endocrinology, 114: 57-66.

Savan, R., Sakai, M., 2002. Analysis of expressed sequence tags (EST) obtained from common carp, Cyprinus carpio L., head kidney cells after stimulation by two mitogens, lipopolysaccharide and concanavalin-A. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 131: 71-82.

Theill, L.E., Karin, M., 1993. Transcriptional control of GH expression and anterior-pituitary development. Endocrine Reviews, 14: 670-689.

Thompson, J.D., Gibson, T.J., Plewniak, F, Jeanmougin, F, Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876-4882

Van Laere AS, Nguyen M, Braunschweig M, Nezer C, Collette C, Moreau L, Archibald AL, Haley CS, Buys N, Tally M, Andersson G, Georges M, Andersson L., 2003. A regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. Nature, 425:832-836.