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Molecular cloning, tissue distribution and quantitative analysis of two proopiomelanocortin mRNAs in Japanese flounder (*Paralichthys olivaceus*)

Kyoung Sun Kim[1,#], Hyun-Woo Kim[1,#], Thomas T. Chen[2] & Young Tae Kim[3,]*

[1]Department of Marine Biology, Pukyong National University, Busan 608-737, Korea, [2]Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA, [3]Department of Microbiology, Pukyong National University, Busan 608-737, Korea

Proopiomelanocortin (POMC) plays an essential role in the stress response of the hypothalamic-pituitary-adrenal axis, and is the precursor of biologically active peptides such as adrenocorticotropin (ACTH), α-melanocyte-stimulating hormone (α-MSH), β-melanocyte-stimulation hormone (β-MSH) and β-endorphin. We have synthesized two different forms of POMC cDNA clones, POMC-I and POMC-II, from a pituitary cDNA library for Paralichthys olivaceus, or Japanese flounder. jfPOMC-I cDNA consists of 954bp and encodes a polypeptide of 216 amino acid residues, whereas jfPOMC-II consists of 971bp which encode a polypeptide of 194 amino acid residues. The high levels of jfPOMC-I and -II mRNAs detected in the pituitary tissue and moderate levels detected in the brain tissue plus our quantitative RT-PCR analysis, which showed there to be no significant difference between the levels of jfPOMC-I and -II mRNAs, indicate that there may be no functional separation between these two mRNAs in the flounder. [BMB reports 2009; 42(4): 206-211]

INTRODUCTION

Proopiomelanocortin (POMC)-derived peptides are thought to be involved a in wide range of physiological functions such as stress and the immune responses, skin pigmentation, sexual behavior, cardiovascular regulation and energy homeostasis (1, 2). This is so because the POMC gene is translated into a multifunctional precursor protein which generates several biologically active peptides, including adrenocorticotropin (ACTH), α-melanocyte stimulating hormone (α-MSH), β-melanocyte stimulating hormone (β-MSH), and β-endorphin by limited proteolysis (3). Tissue specific processing and posttranslational modification of the precursor protein are the result of cleavage by specific convertases which usually recognize single or paired basic amino acids (4).

The nucleotide and amino acid sequences were determined in various species, including mouse (5), guinea pig (6) sockeye salmon (7), rainbow trout (8), carp (9), and human (10). Those studies revealed that the structure of POMC has been highly conserved between species, especially in ACTH, α-MSH, and β-endorphin. Some amphibians and fish species were thought to have undergone chromosome duplication resulting in two POMC genes. Some teleosts including common carp, chum salmon, rainbow trout, and a chondrost-like white sturgeon have shown two POMC genes in fish species (11). Among them the rainbow trout has a unique 25 amino acid C-terminal extension in POMC-A and there has been convincing evidence suggesting that it may produce a novel neuropetide (12). Furthermore, evidence for functional differences between the two POMCs in rainbow trout have been postulated due to the fact that only POMC-A was expressed in sexually inactive fish (13). However, there has been no other report about functionally different POMC derived peptides in tetraploid fish species.

In the present study, we isolated and characterized two different POMC genes from the Japanese flounder (*Paralichthys olivaceus*). Since this species is one of the most important in aquaculture and marine resource management in Korea, how it responds to various environmental changes will aid in the proper containment of this fish, thus ensuring a high-quality end product. Therefore, understanding the endocrine system, the effect stress has on the metabolism of this fish and its food uptake systems are all a prerequisite. Additional studies regarding changes in POMC gene expression in response to various diseases would also prove beneficial. Although POMC is generally taken as major marker for stress and food uptake in mammals, its relevance in fish remains unclear due to insufficient evidence. We describe here the molecular cloning of two forms of POMC cDNAs and the sequence analysis of the cloned cDNAs with other species. We also measured the copy number of each transcript using real-time PCR to estimate their respective functional roles in each tissue, and any discrepancies between the two types of POMC gene.

*Corresponding author. Tel: 82-51-629-5616; Fax: 82-51-629-5619; E-mail: ytkim@pknu.ac.kr
[#]These authors contributed equally to this work.

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RESULTS AND DISCUSSION

Cloning of flounder POMC genes

Two PCR products 800 bp and 700 bp in length were obtained by PCR using POMCf-1 and T7 promoter primers. Both PCR products represented POMC genes, but there were significant differences in the nucleotide sequences of the two clones which suggested the presence of two different POMC genes. Consequently, using probes made with both PCR products, we obtained several positive clones by plaque hybridization screening method. We selected the longest clones which contained full ORF after analyzing their nucleotide sequences. Our results showed that there are two types of POMC genes, POMC-I and POMC-II in flounder. The longest clone with full ORF and poly A tail sequences was composed of 954 bp for POMC-I (AF184066) and 971 bp for POMC-II (AF191593) and their deduced amino acid sequences were 216 and 194 residues, respectively. PCR reaction using genomic DNA as a template showed that both flounder POMC-I & II have the same exon-intron boundary compared to other fish species (data not shown). Many previous studies have shown that the genomic structure of the vertebrate POMC gene is well conserved from human to zebrafish (14-17). The genomic structure of two flounder POMCs was also well conserved. Interestingly, POMC-II contains a very short second intron (114 bp) whereas POMC-I contains more than 2 kb of intron. There has been prior evidence for a bfPOMC-C gene in barfin flounder, the third type of POMC gene, but we failed to isolate a gene corresponding to the bfPOMC-C gene in the present study.

Structural analysis of POMC-I and -II

Multiple amino acid sequence alignment was performed to analyze protein structure and function of our POMCs from Japanese flounder (Fig. 1). Indeed, POMC-I and -II demonstrated a 57% homology. Interestingly, the two different POMC clones still shared such common active peptides such as ACTH, α-MSH, and β-endorphin, whereas both lacked γ-MSH, as is the case for other teleost POMCs (Fig. 1). In addition, two α-MSHs had conserved core amino acid residues of Met4, Phe7, Arg8, and Trp9 which suggested that both α-MSHs were biologically active in the endocrine system of the flounder.

Some fish and amphibians often have two genes encoding a certain hormone. So far, two POMCs had been cloned from sockeye salmon (7), common carp (9), rainbow trout, and zebrafish (17), whereas the channel catfish, *Ictalurus punctatus*, appeared to contain only one POMC gene (18).

There were two interesting differences in flounder POMCs when compared to the POMCs of other fish species. The first is that POMC-II has an extended 11 amino acid residue suggesting another it may be a hormonal peptide like POMC-A in rainbow trout. The second is that there are little joining sequences between biologically active peptides.

The gaps between the amino acid sequences were also considered and the highest and the lowest scores represented in

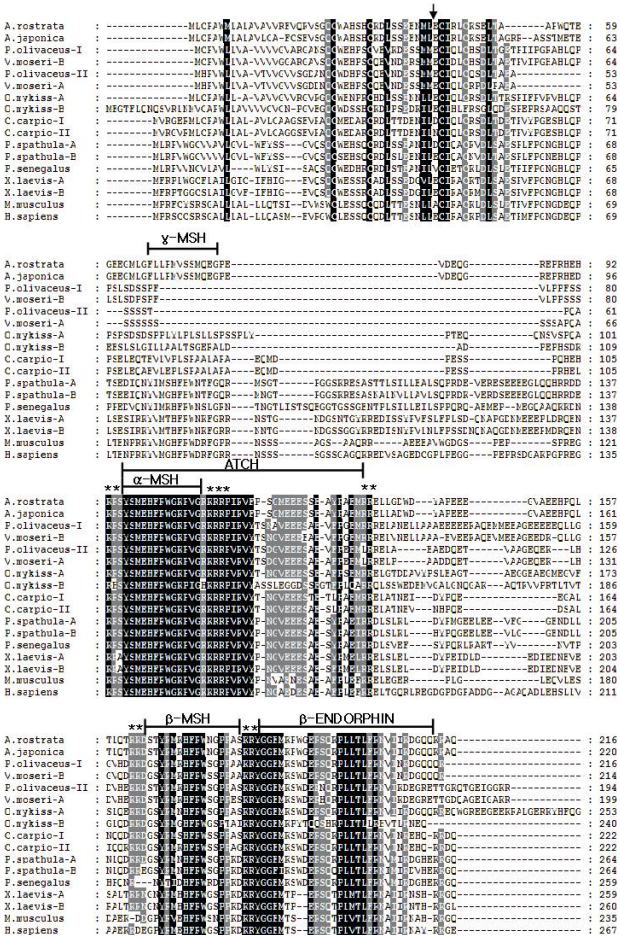

Fig. 1. Comparison of the deduced amino acid sequeces of POMCs from various species. A.rostrata (Anguilla rostrata, proopiomelanocortin I, NCBI# gi|6651501|gb|AAF22344.1|AF194969.1|); A.japonica (*Anguilla japonica*, proopiomelanocortin, NCBI# gi|30313582|gb|AAO17793.1|); P.olivaceus-I (*Paralichthys olivaceus*, POMC-I precursor protein, NCBI# gi|10441267|gb|AAG16978.1|); V.moseri-B (*Verasper moseri*, proopiomelanocortin B, NCBI# gi|11323308|dbj|BAB18468.1|); P. olivaceus-II (*Paralichthys olivaceus*, POMC-II precursor protein, NCBI# gi|11065889|gb|AAG28378.1|AF191593.1|); V.moseri-A (*Verasper moseri*, proopiomelanocortin A, NCBI# gi|11323306|dbj|BAB18467.1|); O.mykiss-A (*Oncorhynchus mykiss*, Corticotropin-lipotropin A precursor, NCBI# gi|416816|sp|Q04617|COLI1_ONCMY|); O.mykiss-B (*Oncorhynchus mykiss*, Corticotropin-lipotropin B precursor, NCBI# gi|416817|sp|Q04618|COLI2_ONCMY|); C.carpio-I (*Cyprinus carpio*, Corticotropin-lipotropin 1 precursor, NCBI# gi|14194542|sp|Q9YGK4|COLI1_CYPCA|); C.carpio-II (*Cyprinus carpio*, Corticotropin-lipotropin 2 precursor, NCBI# gi|14194543|sp|Q9YGK5|COLI2_CYPCA|); P.spathula-A (*Polyodon spathula*, proopiomelanocortin A, NCBI# gi|5257216|gb|AAD41263.1|AF117302_1|); P.spathula-B (*Polyodon spathula*, proopiomelanocortin B, NCBI# gi|5257219|gb|AAD41264.1|AF117303_1|); P.senegalus (*Polypterus senegalus*, proopiomelanocortin, NCBI# gi|18481671|gb|AAL73510.1|AF465781_1|); X.laevis-A (*Xenopus laevis*, proopiomelanocortin, NCBI# gi|148223411|ref|NP_001080838.1|); X.laevis-B (*Xenopus laevis*, Corticotropin-lipotropin B precursor, NCBI# gi|116894|sp|P06299|COLI2_XENLA|); M.musculus (*Mus musculus*, pro-opiomelanocortin-alpha, NCBI# gi|6679415|ref|NP_032921.1|); H.sapiens (*Homo sapiens*, proopiomelanocortin preproprotein, NCBI# gi|4505949|ref|NP_000930.1).

terms of raw and percentages scores. Tree view and Genedoc program were used to create a phylogenetic tree. The identity of the two flounder POMCs was as low as 57%. This was similar to that in rainbow trout POMCs (46%), whereas carp and sturgeon showed an 89% and 90% identity, respectively. In addition, there were few gaps between the two POMCs in carp and sturgeon, but frequent gaps within the amino acid sequences in flounder and rainbow trout. This long distance indicates a low relationship among species.

A phylogenetic diagram, based on amino acid sequence similarity, was constructed to better illustrate the shared evolution of our POMC genes (Fig. 2). As we expected, mammalian POMCs were grouped together. Amphibian genes appeared to have undergone duplication once they had diverged from mammalian genes. All other fish genes (which belong to the subclass Actinopterygii) were well grouped together also. In addition, the POMC genes from *Polyodon spathula* (Chondrostei) and *Polypterus senegalus* (Polypteriformes) had diverged from the other new ray-finned fish (Neopterygii). Both POMC-I and -II genes in Japanese flounder are closest, in an evolutionary sense, to those of the barfin flounder, *Verasper moseri*. The POMC-B gene in the rainbow trout *Oncorhynchus mykiss* did not group together with POMC-A. This may have been due to the presence of the extended amino acid tail at the C-terminus, or POMC-B gene. Thus, the presence of an ortholog in two flounder species would suggest that genome duplication occurred prior to their ancestral species evolving into the present day forms.

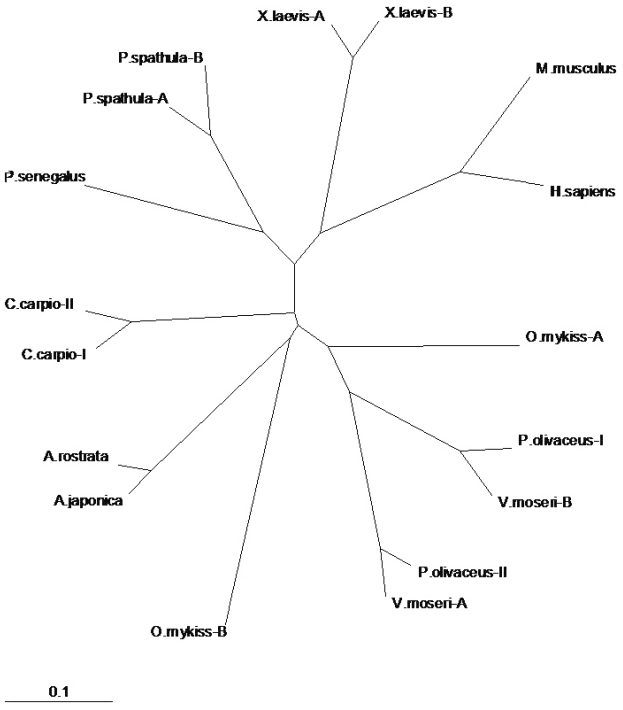

Fig. 2. Phylogenetic diagram of the POMCs based on the amino acid sequences.

Expression of POMC mRNAs

Regular RT-PCR was performed to investigate POMC expression in each tissue (Fig. 3). We identified the size of the single amplified band corresponding to each primer set after PCR (30 cycles using 50 ng of cDNA as template). Both POMC-I and -II were highly expressed in the pituitary gland and only moderately expressed in the brain tissue. Trace expression of POMC-II was also detected in the ovaries and testis. Thus, the pituitary tissue is the major source for both POMC genes, which is consistent with other previous fish studies. However, our findings are not entirely consistent with other studies. Takahashi *et al* (19) found that whilst bfPOMC-A (ortholog of fPOMC-II) was only expressed in the pituitary in barfin flounder, bfPOMC-B (ortholog of fPOMC-I) was expressed not only in the pituitary, but also in many other tissues such as the brain, gill, heart, spleen, liver, stomach, intestine, testis, muscle, blood, and skin.

Whilst there have been several reports measuring the precise tissue specific copy number of POMC mRNA (20), the transcriptional regulation of each POMC gene needs to be characterized. The POMC gene is expressed in both the pars distalis (PD) and pars intermedia (PI) of the pituitary gland. POMC-producing cells were identified in the pituitary of the barfin flounder and more recently three types of POMC mRNAs, and their derived peptides, were identified (21) and localized by various techniques, including in-situ hybridization and MOLDI-TOF (19, 22). To calculate the copy number of POMC mRNA, we performed a quantitative PCR. First we tested the efficiency of our primers for the exact quantification (Fig. 4). The melting temperature (Tm) for each amplicon was constant as 88.4°C for β-actin and 89.6°C for both POMC-I and POMC-II. These results indicated that each primer amplifies only a single PCR product and thus were good enough to quantify each mRNA transcript. As the slopes of the equation of copy number were between -0.2879 and -0.3099, each primer set had sufficient specificity for the target gene. This result also explained why POMC-II was amplified slightly

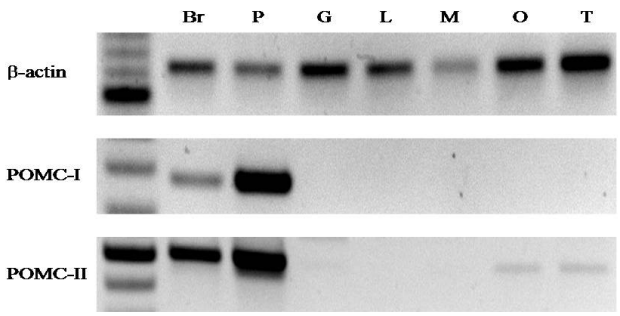

Fig. 3. Expression of the POMC gene. RT-PCR analyses of the expression of β-actin, POPOMC-I and II genes in various tissues of *P. olivaceus*. Br, brain; P, pituitary; G, gill; L, liver; M, muscle; O, ovary; T, testis.

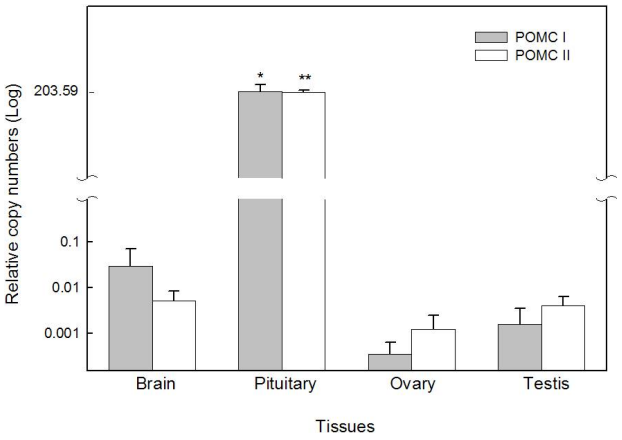

Fig. 4. Comparison of the relative copy numbers between POMC-I and II in brain, pituitary, ovary and testis of *P. olivaceus*. There were no significant differences between POMC-I and II in all tissues. $*P < 0.05$ and $**P < 0.01$.

better than POMC-I during RT-PCR (Fig. 3). Since any discrepancies between the two primer sets for POMC amplification were taken into consideration, a more precise mRNA copy number could be calculated. Quantitative PCR results were consistent with regular RT-PCR. The relative copy number of both POMC-I and -II genes was highest in the pituitary tissue. The number of POMC-I mRNA transcripts in the pituitary was 6.9×10^3 fold higher than in the brain, about 6.0×10^5 fold greater than in the ovaries, and 1.3×10^5 fold higher than in the testis. The copy number of POMC-II mRNA was 3.8×10^4 fold higher than in the brain, 1.6×10^5 higher than in the ovary, and 4.9×10^4 higher than in the testis. The statistical significance between the relative copy numbers of each tissue was also examined. The copy number of both POMC-I and -II in the pituitary was significant compared to all other tissues ($P < 0.05$) whereas there was no statistically significant difference among the other three tissues in both POMCs. In addition, we could not find any significant difference between the copy numbers of POMC-I and -II in all the tissues we examined. Our findings are contrary to a previous study in barfin flounder (19). In there study, the expression of bfPOMC-A, a homolog of jfPOMC-II, was detected only in the pituitary whereas bfPOMC-B gene, a homolog of jfPOMC-II, was expressed not only in the pituitary but also in brain, gill, heart, spleen, liver, stomach, intestine, testis, muscle, blood, and skin. This discrepancy may have arisen due to the different PCR strategies employed in the studies. In the aforementioned study, the transcript was measured using regular end-point PCR which did not take into consideration the efficiency of each primer. Since quantitative PCR is considered to be a more accurate and reliable technique it was necessary to examine multiple copies of POMC transcripts in other fish species as a comparative analysis. Our findings suggest that there may be no functional difference between POMC-I and -II in each tissue.

To our knowledge, little work has been carried out regarding expression levels of the two types of POMC genes. However, in the present study we have concluded that the expression levels of the two genes were not significantly different. Although its expression level was far lower in pituitary tissue, considerable expression in the brain tissue was also detected (Fig. 4). Expression of PCMC mRNA in the brain was predominately localized to the mediobasal hypothalamus and appeared to be well conserved between mammals and amphibians (23-25). In a fish study, POMC mRNA were found to be exclusively expressed in the mediobasal hypothalamus, a brain area associated with food intake and neuroendocrine regulation (1). Although we did not perform in-situ hybridization, we suspect that expression of POMCs in the brain was from the cell group of the hypothalamus.

In addition to hypothalamic-pituitary-adrenal axis, there is another axis called the hypothalamic-pituitary-gonadal axis. There have been many reports about the roles of opioid signaling in seasonal reproduction cycles and its correlation with the Luteinising Hormone Cells-Ovary Axis not only in the mammals (26, 27) but also in fish species (28). Besides the long axis, endogenous opioid peptides (EOP) are also implicated in local interactions. Both theca and granulose cells appear to be the major POMC mRNA producing cells of ovarian tissue in mammals (11, 29-31). Although its functional role is still unknown, the expression of POMC has also been detected in testis (19, 32, 33). However, the precise role of endogenous opioid peptides in the gonadal cells of fish remains to be elucidated. As with expression of POMCs in the hypothalamic-pituitary-adrenal axis, there was no significant difference between expressions of the two subtypes of POMC examined herein. This suggests that POMC-I and POMC-II may not be functionally different and may simply be the result of genome duplication.

In this study we isolated two subtypes of POMC mRNA, POMC-I and POMC-II from the Japanese flounder. Our subsequent structural and expressional analyses indicate that both flounder POMCs may have common functions both in hypothalamic-pituitary-adrenal axis and hypothalamic-pituitary-gonadal axis. In addition, we quantified POMC transcripts and pointed out the possible previous errors to measure. Such findings might prove exceedingly useful when quantifying POMC gene expression according to various physiological conditions, including food uptake, stress response, color deterioration, and disease states. Thus, POMC can be used as a reliable biomarker in the estimation of prime aquacultural farming conditions, so as to ensure a high quality product.

MATERIALS AND METHODS

The *E. coli* strain XL1-Blue was used for library titration, transformation, and color selection. The oligonucleotides used for PCR were POMCf-1 (5′-A(T/C)TCCATGGAGCA(T/C)TTCCGC-

3′; DEGENERACY FORWARD PRIMER), Po-POMCI F1 (5′-GG CGTGGCCAGAGGAGCTAACAGT-3′; specific forward primer for POMC-I), Po-POMCI R1 (5′-GGAGGTGTAGACTTTGATTG GACG-3′; specific reverse primer for POMC-I), Po-POMCII F1 (5′-GTGGGTGTGGTCAGCGGAGACGCCAAC-3′; specific forward primer for POMC-II) and Po-POMCII R1 (5′-CCGTTGTCT CCCGTCCTTCGTCT-3′; specific reverse primer for POMC-II). All of these were synthesized by Bioneer (Korea). Restriction enzymes, reverse transcriptase, and ribonuclease inhibitors were purchased from Promega (USA). Live adult Japanese flounders were purchased from a local seafood market and kept in a seawater tank where the water was continually circulated and aerated at 15°C until the fish were required.

RNA isolation and construction of cDNA library

The preparation of total RNA from flounder pituitary tissue was performed with total RNA isolation kit (Promega). cDNA libraries were constructed as described in the Manufacturer's instructions (Stratagene). Messenger RNA was isolated from flounder pituitary tissue using oligo (dT) cellulose. Flounder cDNA was synthesized with MMLV-reverse transcriptase and second-strand cDNA was synthesized with DNA polymerase and RNase H. Synthesized cDNA was packaged with the GigapackIII gold packaging extract kit (Stratagene). Its packing efficiency was calculated by counting plaques formed from the serially diluted primary packaging mixture.

Cloning of the two flounder POMC genes

Various POMC amino acid sequences were collected from the NCBI database (http://www.ncbi.nlm.nih.gov) and conserved sequences among fish species were determined by multiple protein alignment using the ClustalW program (http://www.ebi.ac.uk/clustalw/index.html). Degeneracy primers were synthesized based on the conserved amino acid sequences (Bioneer, Korea). PCR was carried out using these primers as described in (34). Identified partial POMC cDNAs were used to construct cDNA probes. Each probe was labeled with DIG (Digoxigenin)-oligonucleotide 3'-end labeling kit (Boehringer Mannheim). Positive plaques were screened and confirmed by the second screening. Positive plaques were recovered and the phagemid containing its insert was excised according to the manufacturer's instructions (Stratagene, USA). Inserted sequences were determined using an automated DNA sequencer (ABI Biosystem, USA). The DNA sequence similarity was examined by BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/). The analysis of multiple amino acid sequences was carried out as described above and a phylogenetic tree was constructed using the Treeview program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

POMC expression

The mRNA expression of each POMC gene was determined by RT-PCR. Tissue from the brain, pituitary, gill, liver, ovary, testis and muscle were dissected from adult flounders and stored at -70°C in the RNA later solution until they were used to isolated RNA. cDNA was synthesized as described above, but a random hexamer was used instead for the oligo dT primer. Synthesized cDNA was quantified to yield the same amount of template cDNA for each PCR. The IDTSciTools program was used to design primers (http://www.idtdna.com/SciTools/SciTools.aspx). DNA Engine Opticon® 2 Continuous Fluorescence Detection System (Bio-Rad Inc. USA) was used for quantitative PCR and raw data were analyzed by the MJ Opticon mornitor program (Ver. 3.1) which was provided by the manufacturer. Sybergreen PCR mixture (Takara Inc. Japan) was used with 50 ng of cDNA as a template for each quantitative PCR as per the manufacturer's instructions. PCR products were cloned and their identities reconfirmed from the nucleotide sequences. Real-time PCR was performed to quantify mRNA expression. Efficiency of each primer set was confirmed by constructing the standard curve from which the template's copy number was calculated as described in (35). Student's t test was used to evaluate the level of significance between the relative copy numbers of POMC-I and -II in the various tissue types used (SPSS version 11.5). The results were considered significant at $P < 0.05$.

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