Thesis for the Degree of Master of Science

Characterization of the melanin-concentrating hormone receptor genes from olive flounder (*Paralichthys olivaceus*)

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양식 넙치의 멜라닌 농축 호르몬 수용체 유전자 특성

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ABSTRACT

The melanin-concentrating hormone (MCH), a 17-aa cyclic hypothalamic peptide, was identified initially in teleost fish as a regulator of pigmentary changes in background adaptation. The MCH was later also found, a 19-aa cyclic hypothalamic peptide in mammals, to be a regulator of feeding, and energy homeostasis. Recently, several groups have reported the identification of an orphan G protein-coupled receptor as a receptor for MCH. I hereby report the characterization of a flounder (*Paralichthys olivaceus*) melanin-concentrating

hormone receptor genes termed fMCHR1 and fMCHR2.

Using specific primer sets on the partial sequence of MCHR1 and rapid amplification of cDNA ends (RACE) system, full-length of MCHR1 and MCHR2 cDNA were generated from flounder brain cDNA. MCHR1 has an open reading frame (ORF) of 1077 base pair, encoding to 359 amino acids from flounder brain cDNA. MCHR2 full-length cDNA were generated with an open reading frame (ORF) of 1032 base pair, 344 amino acids in flounder brain cDNA.

The flounder MCHR1 shared about 58% amino acid identity with human MCH-1R and flounder MCHR2 share about 38% with human MCH-2R. The flounder MCHR2 shared about 29.2% amino acid identity with flounder MCHR1. These receptors belong to class I (rhodopsin-like) of the G protein-coupled receptors.

MCHR1 gene was amplified from all total RNAs extracted from the liver, spleen, kidney, small intestine, muscle, and brain tissues, indicating that MCHR1 gene was expressed in all tissues.

However, MCHR2 gene was amplified from all total RNAs extracted from the liver, spleen, kidney, small intestine, muscle, and brain tissues, indicating that MCHR2 gene was only expressed in brain.

Key words : MCH(melanin-concentrating hormone), MCH receptor, MCHR1, MCHR2, RACE(rapid amplification cDNA ends)

INTRODUCTION

Melanin concentrating hormone (MCH) was initially isolated from the pituitary of chum salmon (*Oncorhynchus keta*) as a hormone of cyclic heptadecapeptide mediating skin color change (Kawauchi *et al.,* 1983), was characterized from rat hypothalamus (Thompson and Watson 1990; Vaughan *et al.,* 1989) and finally identified as cyclic nonadecapeptide in human (Presse *et al.,* 1990). The human and rat MCH precursor are similar to one another but differ extensively from salmon counterpart. The novel putative neuropeptides named neuropeptide G-E (NGE) and neuropeptide E-I (NEI) were found in the regions of mammalian prohormone (Presse, Nahon, Fischer, and Vale 1990). Although fish MCH was originally discovered in pituitary of teleost fish for regulating skin melanocyte aggregation, MCH function in regulating skin color in higher mammals has not been confirmed (Shi 2004). MCH is expressed in neurons of mammalian hypothalamus (lateral hypothalamic area and zona incerta) and has a role in control of energy homeostasis and food intake in mammals (Qu *et al.,* 1996; Shimada *et al.,* 1998) as well as behavior and emotion (Shi 2004).

MCH of olive flounder has 25 amino acid residues in mature form and shows highly conserved in core region within single disulfide bond except 2 or 3 amino acids residues extending to N-terminus and C-terminus region as comparing with human and other fish MCH (Jeon and Song 2003).

MCH signals via two specific G protein-coupled receptors (GPCR) in human as MCHR1 and MCHR2. MCHR1 was initially identified as SLC-1 through its homology to somatostatin receptors (Kolakowski, Jr. *et al.,* 1996). The SLC-1 GPCR was discovered as an expressed sequence tags (ESTs) exhibiting about 40% homology in it hydrophobic domains to the five somatostatin receptors and cloned by PCR amplification of human genomic DNA. The rat orthologue sharing 91% overall sequence identity to human SLC-1 receptor was identified, but is shorter 49 amino acid residues in its N-terminal region (Lakaye *et al.,* 1998). These SLC-1 receptors, GPR24, have been remained as an orphan GPCR (oGPCR) until MCH was identified simultaneously by several groups a specific ligand for SLC-1 receptor using different biochemical and reverse pharmacology techniques (Bachner *et al.,* 1999; Chambers *et al.,* 1999; Lembo *et al.,* 1999; Saito *et al.,* 1999; Saito *et al.,* 2000). MCHR2 was also identified by its sequence homology with MCHR1 searching human genome database (An *et al.,* 2001; Hill *et al.,* 2001; Mori *et al.,* 2001; Rodriguez *et al.,* 2001; Sailer *et al.,* 2001; Wang *et al.,* 2001). Two receptors exhibit significant sequence homology in their MCH-binding pocket (Hintermann *et al.,* 2001). While MCHR1 is conserved among all mammals, MCHR2 (GPR145) gene only exists in carnivores and primates, not in rodents. Several non-human species (rat, mouse, hamster, guinea pig and rabbit) do not have functional MCHR2 gene or exists as nonfunctional pseudogene (Tan *et al.,* 2002). Although human MCHR2 gene was isolated since 2001, its functions are not

characterized at all because of lacking proper experimental model organisms like mouse or rat.

Several MCH receptor genes have been partially identified from Zebrafish (MCHR1A, MCHR1B and MCHR2) and Fugu (MCHR1 and MCHR2) by searching whole genome database (Logan *et al.,* 2003). Expression of zebrafish MCHR1A is embryo specific and can not be detected in adult tissue. MCHR1B is expressed in young embryo and adults. MCHR2 transcripts were detected in young embryo and the period of larval melanophore function. It suggests that either MCHR1B or MCHR2 is likely to regulate pigmentation.

The pigmentary system is more sophisticated in most fish, reptiles and amphibian than human. At least six distinct pigment cell types identified in fish, although zebrafish have only three types: iridophores, xanthophores and melanophore. αMSH exerts an influence on each cell types, but its role on melanophore function has been characterized. When administrated in vivo to mice, αMSH produce a change in melanin biosynthesis while MCH shows no effect, because mouse lacking MCH or its receptor are normally pigmented (Marsh *et al.,* 2002; Shimada *et al.,* 1998)

Recently MCH1-R antagonists were characterized as showing antidepressants and anxiolytic effects, suggesting that MCHR1 might be involved in other physiological process (Borowsky *et al.,* 2002). The hypothalamus, where MCH and MCH receptor are expressed mainly in and which has important roles in the regulation of food intake and fat mass, may be involved in the regulation of bone mass (Ducy *et al.,*

2000). *MCHR1*$^{-/-}$ mice have osteoporosis, caused by a reduction in the cortical bone mass, while the amount of trabecular bone is unaffected, indicating that MCHR1 signaling is involved in a tonic stimulation of bone mass (Bohlooly *et al.,* 2004).

The function of MCH receptor is not fully identified until yet because of the limited structural information of MCH receptor genes from diverse species, lack of MCHR2 gene in model organism and the complicated signaling mechanisms. In this thesis, two subtypes of MCH receptor genes were cloned and characterized from total brain cDNA library of olive flounder. This studies is first case to be identified characterize MCHR2 gene except human and will be a good example to reveal the function of MCHR2.

MATERIALS AND METHODS

MATERIALS

Fish tissues

The brain and liver tissues were obtained from mature olive flounder (*Paralichthys olivaceus*; n = 8; size; 40 ± 10 ㎝, body weight; 1000 ± 200 g; 3 years old) and stored at -70 ℃ until used.

Bacterial strain

The *E. coli* strain XLI-Blue [F'::Tn10 *pro*A^+B^+ *lac*I^q △(lacZ) M15/*recA1 endA1 gyrA96*(Nal^r) *thi hsdR17*(r_k^- m_k^+) *supE44 relA1 lac*] was used for transformation and color selection.

Enzymes

Restriction enzymes were purchased from Promega (USA), Amersham Biosciences (UK) and TAKARA (Japan). Taq polymerase and *pfu* polymerase were obtained from Bioneer (Korea) and Clontech (USA). Reverse transcriptase were obtained from Invitrogen (USA).

Oligonucleotide primers

The primer for MCHR1 and MCHR2 cloning were designed on the basis of the conserved sequences from known MCHR1 and MCHR2 of different species (human, rat, fugu, zebrafish, *et al.*) and synthesized from Bioneer (Korea) and GenoTech (Korea).

Other materials

The primer walking system (DNA walking *SpeedUp*™ Premix Kit) was purchased from Seegene (Korea). The RACE system (BD SMART™ RACE cDNA Amplification Kit) was purchased from Clontech (USA). The Plasmid isolation, Gel and PCR purification system were purchased from Bioneer (Korea) and Promega (USA). The mRNA isolation Kit (Micro-FastTrack 2.0™) was purchased from Invitrogen (USA). The TRIzol reagent for total RNA isolations was purchased from Difco (USA). The *AccuPrep*® PT/PCR PreMix was purchased from Bioneer (Korea). The DIG labeling and detection kit were purchased from Roche (Germany).

Table 1. Primers used in this study

Name	Sequence	Remark
DGMR1-F	5'-GVTAYBTGGCBACNGTCC-3'	fMCHR1 degenerated,Foward
DGMR1-R	5'-TTRGCRTARCCCAWGCTDATGGC-3'	fMCHR1 degenerated,Reverse
DGMR2-F	5'-GAGAACMAKSYAGTGGACCATCA-3'	fMCHR2 degenerated,Foward
DGMR2-R	5'-CAGATGCTGATGTTGTADGCGTA-3'	fMCHR2 degenerated,Reverse
PWMR1-Tsp1	5'-CCGAGGAATGATGTAGGAAGTC-3'	fMCHR1 primer walking TSP 1
PWMR1-Tsp2	5'-TGCCTCAGACTCCCACGCTTG-3'	fMCHR1 primer walking TSP 2
PWMR1-Tsp3	5'-CTACCTGACCAACGAACACGC-3'	fMCHR1 primer walking TSP 3
PWMR2-Tsp1	5'-CCTTTTACTCGCTCACCCTCTAC-3'	fMCHR2 primer walking TSP 1
PWMR2-Tsp2	5'-TCTTGATCTGCTGGTCACCCTAC-3'	fMCHR2 primer walking TSP 2
PWMR2-Tsp3	5'-ACCTTCGTCTACGCCTACAACATCC-3'	fMCHR2 primer walking TSP 3
RCMR1-G1-5	5'-GCCTCTTGAAAGTGTCACTGAGGATG-3'	fMCHR1 5' RACE GSP 1
RCMR1-G1-5	5'-GCTATGTTGTACGCATAGGAGAACGC-3'	fMCHR1 5' RACE GSP 2
RCMR1-G1-5	5'-TGTAGTAAGGAGCCCAGCAGATGAAG-3'	fMCHR1 5' RACE GSP 3
RCMR1-G1-3	5'-CTGCTATGACCCTTGACCGTTACTTG-3'	fMCHR1 3' RACE GSP 1
RCMR1-G1-3	5'-GTCTGTCTTTCCTCACCATTATCC-3'	fMCHR1 3' RACE GSP 2
RCMR2-G1-5	5'-GATAGTTCTGGGCGAAGATGAGCAG-3'	fMCHR2 5' RACE GSP 1
RCMR2-G1-5	5'-ATGCAGCTGTGAGAGTAGCTGAGACA-3'	fMCHR2 5' RACE GSP 2
RCMR2-G1-5	5'-CTGATGTGGTAGGCATAGACGAAGGT-3'	fMCHR2 5' RACE GSP 3
RCMR2-G1-3	5'-AGGTACATAGCCATAGTCCACCCCAC-3'	fMCHR2 3' RACE GSP 1
RCMR2-G1-3	5'-GTCATGATGTACGCCAAGGTTGAG-3'	fMCHR2 3' RACE GSP 2
SP1-F	5'-TT**AAGCTT**GGCACTTCTTAACTTGGATACACC-3'	forward primer for fMCHR1 cloning to pcDNA3.1(+), containging **Hind Ⅲ site**
SP1-R	5'-AA**GAATTC**GTGCAGATAAGATGGTGATGGAGT-3'	reverse primer for fMCHR1 cloning to pcDNA3.1(+), containging **EcoR I site**
SP2-F	5'-TT**AAGCTT**CTGAGAAGAACTCCAGGAAGAAAG-3'	forward primer for fMCHR2 cloning to pcDNA3.1(+), containging **Hind Ⅲ site**
SP2-R	5'-AA**GAATTC**CCCAGGTTCAATTGTACAGTATCC-3'	reverse primer for fMCHR2 cloning to pcDNA3.1(+), containging **EcoR I site**

METHODS

Isolation of genomic DNA

The olive flounder (*Paralichthys olivaceus*) was purchased from Hae-byun fish market. The genomic DNA of the olive flounder was prepared by *AccuPrep*® Genomic DNA Extraction Kit (Bioneer). 100 ㎎ of the fresh liver was placed in liquid nitrogen and grinded to a fine powder with mortar and pestle under liquid nitrogen, placed them within 200 ㎕ of Tissue Lysis Buffer (TL) in a new microcentrifuge tube. After that RNase (final concentration : 20 ㎍/㎖) was added and incubated at 37 ℃ for 1 hr. Proteinase K (final concentration : 10 ㎍/㎖) was added to the sample tube, mixed by vortex mixer and incubated at 60 ℃ for 2 hr. The tube was briefly spined down and added 200 ㎕ of Binding buffer (GC), and immediately mixed by vortex mixer. The tube was incubated at 60 ℃ for 10 min. 100 ㎕ of isopropanol was added and the sample was mixed by pippetting and the lysate was carefully transferred into the upper reservoir of the binding column tube and centrifuged at 8,000 rpm for 1 min. The Binding column tube was transferred to a new 2 ㎖ tube for filtration. 500 ㎕ of Washing buffer 1 (W1) was added to the Binding column tube without wetting the rim, and centrifuged at 8,000 rpm for 1 min. The solution was poured into a disposal bottle and 500 ㎕ of Washing buffer 2 (W2) was added to the Binding column tube without wetting the rim, and centrifuged at 8,000 rpm for 1 min. The Binding column tube was

centrifuged once more at 12,000 rpm for 1 min to completely remove ethanol, and transferred to a new 1.5 ㎖ tube for elution, and 200 ㎕ of Elution buffer (EL) was added onto Binding column tube.

The sample tube was waited for 1 min at room temperature, and centrifuged at 8,000 rpm for 1 min to elute DNA. The isolated genomic DNA was stored at 4 ℃ until use.

PCR cloning of partial genomic DNA using degenerated primer

Due to unknown fMCHR1 and fMCHR2 nucleotide sequence, two degenerated primer sets were designed for PCR cloning of partial genomic DNA.

Before the PCR, *AccuPrep*® PCR PreMix (Bioneer) was prepared. For 20 ㎕ PCR reaction volume, it was composed 1 ㎕ of genomic DNA (30 ㎍/㎖) from flounder, 1 ㎕ of DGMR1-F (10 mM), 1 ㎕ of DGMR1-R primer (10 mM), and 17 ㎕ of distilled water per *AccuPrep*® PCR PreMix tube.

The PCR amplification for MCHR1 gene was performed using the following one-step cycle parameters : 40 cycles at 94 ℃ for 30 sec, 64 ℃ for 30 sec, 72 ℃ for 1 min. The PCR product was analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100bp size marker (Bioneer).

For 20 ㎕ PCR reaction volume, it was composed 1 ㎕ of genomic DNA (30 ㎍/㎖) from flounder, 1 ㎕ of DGMR2-F (10

mM), 1 ㎕ of DGMR2-R primer (10 mM), and 17 ㎕ of distilled water per *AccuPrep*® PCR PreMix tube.

The PCR amplification for MCHR2 gene was performed using the following one-step cycle parameters : 40 cycles at 94 ℃ for 30 sec, 57 ℃ for 30 sec, 72 ℃ for 1 min. The PCR product was analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100bp size marker (Bioneer).

Primer walking of genomic DNA

Genomic DNA of flounder were used as template. Primer walking was conducted using DNA Walking *SpeedUp*™ Premix Kit (Seegene) following the manufacturer's protocol. It was performed on ice in downstream of MCHR1 and upstream of MCHR2. Before the first PCR, PCR master mix prepared. For each 50 ㎕ PCR reaction, it was composed 43 ㎕ of 18 ㎕ of distilled water and 25 ㎕ of 2× SeeAmp™ ACP™ Master Mix II per reaction tube. 2 ㎕ of genomic DNA, 4 ㎕ of 2.5 pmol DW-ACP (one of DW-ACP 1, 2, 3, and 4) primers and 1 ㎕ of primer walking TSP 1 primer (10 mM) were added to each tube. The first PCR was performed using the following two-step cycle parameters : 1 cycle at 94 ℃ for 5 min, 42 ℃ for 1 min, 72 ℃ for 2 min and 30 cycles at 94 ℃ for 40 sec, 55 ℃ for 40 sec, 72 ℃ for 1.5 min. The first PCR products were purified using *AccuPrep*® PCR Purification Kit (Bioneer).

Before the second PCR, PCR master mix prepared. For

each 20 ㎕ PCR reaction, it was composed 15 ㎕ of 5 ㎕ of distilled water and 10 ㎕ of 2× SeeAmp™ ACP™ Master Mix II per reaction tube. 3 ㎕ of purified first PCR products, 4 ㎕ of DW-ACPN primer (10 mM) and 1 ㎕ of primer walking TSP 2 primer (10 mM) were added to each tube. The second PCR was performed using the following one-step cycle parameters : 35 cycles at 94 ℃ for 40 sec, 60 ℃ for 40 sec, 72 ℃ for 1.5 min. The second PCR products were purified using *AccuPrep*® PCR Purification Kit (Bioneer) and analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100bp size marker (Bioneer).

Before the third PCR, PCR Master Mix prepared. For each 20 ㎕ PCR reaction, it was composed 17 ㎕ of 7 ㎕ of distilled water and 10 ㎕ of 2× SeeAmp™ ACP™ Master Mix II per reaction tube. 1 ㎕ of purified second PCR products, 1 ㎕ of universal primer (10 mM) and 1 ㎕ of primer walking TSP 3 primer (10 mM) were added to each tube. The third PCR was performed using the following one-step cycle parameters : 30 cycles at 94 ℃ for 40 sec, 60 ℃ for 40 sec, 72 ℃ for 1.5 min. The second PCR products were purified using *AccuPrep*® PCR Purification Kit (Bioneer) and analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100bp size marker (Bioneer).

Isolation of RNA

Total RNA isolation was extracted from the olive flounder

brain using TRIzol reagent (Difco).

100 ㎎ of the fresh brain tissue was placed in liquid nitrogen and grinded to a fine powder with mortar and pestle under liquid nitrogen, placed them within 1 ㎖ of TRIzol reagent per 50 ㎎ of tissue. The lysate was incubated on ice for 5 min and 200 ㎕ of chloroform was added, mixed vigorously by vortex mixer for 15 sec and incubated on ice for 15 min. The sample was then centrifuged at 14,000 rpm for 15 min at 4 ℃. Equal volume of chloroform was added and mixed vigorously by vortex mixer for 15 sec and incubated on ice for 15 min. The sample was centrifuged at 14,000 rpm for 15 min at 4 ℃ and aqueous phase was transferred to a new tube. 500 ㎕ of isopropanol was added, mixed by vortexing and incubated for 15 min on ice. Chilled the sample tube on ice was centrifuged at 14,000 rpm for 5 min at 4 ℃ and the supernatant was discarded. The RNA pellet was washed with 1 ㎖ of 75% ice-cold ethanol and centrifuged at 14,000 rpm for 5 min at 4 ℃. The pellet was dried briefly for 10 min on ice and dissolved in 20 ㎕ of DEPC-treated water. The quality of RNA was estimated by electrophoresis on 1% formaldehyde-agarose gel. The isolated RNA was used for RT-PCR analysis.

Poly(A) RNA was isolated using a Micro-FastTrack 2.0™ (Invitrogen). 10 ㎕ (500 ㎍) of purified poly(A) RNA was aliquoted to a new microcentrifuge tube and 1 ㎖ of Micro-FastTrack 2.0™ lysis buffer was added. The mixed sample tube was heated at 65 ℃ for 5 min and placed immediately for 1 min on ice. 63 ㎕ of 5N NaCl was added, and mixed

well with the oligo-T cellulose for 2 min. The poly(A) RNA was isolated with a method described in Micro-FastTrack 2.0™ (Invitrogen) manual. The quality of poly(A) RNA was estimated by electrophoresis on 1% formaldehyde-agarose gel. The isolated poly(A) RNA was used to BD SMART™ RACE cDNA Amplification kit (Clontech) for MCH receptor gene.

First-strand cDNA

First-strand cDNA was constituted BD PowerScript Reverse Transcriptase (Clontech) following the manufacturer's protocol.

In 5'-RACE PCR of MCHR1 3 ㎍ of poly(A) RNA, each 12 ng of 5'-CDS primer and BD SMART II A oligonucleotide were added and sterile H_2O to a final volume 5 ㎕ to a nuclease-free microcentrifuge tube. In 3'-RACE PCR of MCHR1 3 ㎍ of poly(A) RNA, 12 ng of 3'-CDS primer was added and sterile H_2O to a final volume 5 ㎕ to a nuclease-free microcentrifuge tube. The each mixed contents were incubated at 70 ℃ for 2 min and quickly chilled on ice. After briefly spin down, 1 ㎕ dNTP mix (10 mM), 2 ㎕ of 5× First-Strand Buffer, 1 ㎕ DTT (20 mM) and BD PowerScript Reverse Transcriptase were added and mixed by gently pipetting. The each mixed contents were spined and incubated at 42 ℃ for 1.5 hr in a hot-lid thermal cycler. After the reaction was inactivated by heating at 72 ℃ for 7 min, the cDNAs was used as template for RACE reaction.

As previously stated, first-strand cDNA of MCHR2 was constituted following the MCHR1 first-strand cDNA protocol.

Rapid amplification of cDNA ends (RACE)

Before the primary PCR, PCR Master Mix prepared. For each 50 ㎕ PCR reaction, it was composed 41.5 ㎕ of 34.5 ㎕ of PCR-Grade Water (Clontech), 5 ㎕ of 10× BD Advantage 2 PCR Buffer, 1 ㎕ of dNTP (10 mM) and 1 ㎕ of 50× BD Advantage 2 Polymerase Mix per reaction tube. 2.5 ㎕ of each RACE-Raeady cDNA, 5 ㎕ of UPM (Universal primer Mix; 10 mM), 1 ㎕ of each RACE GSP1 primer (10 mM), and 41.5 ㎕ of the primary PCR master mix were added to each tube.

The primary PCR was performed using the following three-step cycle parameters; 5 cycles at 94 ℃ for 30 sec, 72 ℃ for 3 min, 5 cycles at 94 ℃ for 30 sec, 70 ℃ for 3 min, and 20 cycles at 94 ℃ for 30 sec, 68 ℃ for 30 sec, 72 ℃ for 3 min. The primary PCR product was analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100bp size marker (Bioneer).

Before the nested PCR, PCR Master Mix prepared and 5 ㎕ of the primary PCR products were diluted into 245 ㎕ of Tricine-EDTA buffer (Clontech). For each 50 ㎕ PCR reaction, it was composed 43 ㎕ of 36 ㎕ of PCR-Grade Water (Clontech), 5 ㎕ of 10× BD Advantage 2 PCR Buffer, 1 ㎕ of dNTP (10 mM) and 1 ㎕ of 50× BD Advantage 2

Polymerase Mix per reaction tube. 5 ㎕ of each diluted primary PCR product, 1 ㎕ of NUP (Nested Universal primer; 10 mM), 1 ㎕ of each RACE nested GSP primer (10 mM), and , 43 ㎕ of the nested PCR master mix were added to each tube.

The nested PCR was performed using the following one-step cycle parameters; 20 cycles at 94℃ for 30 sec, 68℃ for 30 sec, 72 ℃ for 3 min. The nested PCR product was analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100bp size marker (Bioneer).

End-to-end PCR reaction for full-length fMCHR1 and fMCHR2 cDNA synthesis

The first PCR reaction was performed by using the 5'- and 3'-RACE products without adding the primer. The 5'- and 3'-RACE fragments played two roles, namely, both as a template and a primer. The priming site was the overlapping region between the 5'- and 3'-RACE fragments. The full-length cDNA of fMCHR1 and fMCHR2 were synthesized by first PCR reaction.

For the first PCR reaction, it was composed with 1 ㎕ of diluted 5'-RACE product And 1 ㎕ of diluted 5'-RACE products, 1 ㎕ of Diluted 3'-RACE products, and 48 ㎕ of distilled water per *AccuPrep*® PCR PreMix tube.

The first PCR was performed using the following one-step cycle parameters; 1cycles at 94 ℃ for 3 min, 60 ℃ for 1

min, 72 ℃ for 5 min. The tube was chilled on ice for 2 min and spined briefly. The first PCR product was used for second PCR for full-length cDNA amplification and stored at -20 ℃ until use.

For the second PCR reaction, it was composed 45 ㎕ of distilled water per *AccuPrep*® PCR PreMix tube. 3 ㎕ of the first PCR products of fMCHR1, 1 ㎕ of SP1-F containing Hind Ⅲ restriction site and 1 ㎕ of SP1-R containing EcoR Ⅰ restriction site were added to reaction tube of fMCHR1. And 3 ㎕ of first PCR products of fMCHR2, 1 ㎕ of SP2-F containing Hind Ⅲ restriction site and 1 ㎕ of SP2-R containing EcoR Ⅰ restriction site were added to reaction tube of fMCHR2.

The second PCR was performed using the following one-step cycle parameters; 25 cycles at 94 ℃ for 30 sec, 57 ℃ for 30 sec, 72 ℃ for 3 min. The second PCR products were purified using *AccuPrep*® PCR Purification Kit (Bioneer) and analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100bp size marker (Bioneer).

The prepared full-length cDNA of fMCHR1 and fMCHR2 was cloned into pGEM T-easy vector (Promega).

Cloning with pGEM T-easy vector

PCR products were purified with *AccuPrep*® Gel Purification Kit (Bioneer) from 1.5% agarose gel and cloned into the pGEM T-easy vector (Promega). Purified PCR products were ligated into pGEM T-easy vector.

Ligation mixture (purified PCR product, 70 ng; pGEM T-easy vector, 40 ng; 2× ligation buffer, 5 ㎕; T4 DNA ligase, 1 ㎕; upto 10 ㎕ of distilled H_2O) was incubated at 16 ℃ for 4 hrs.

The ligate was used for *E. coli* XL1-Blue transformation.

Preparation of competent cell

The *E. coli* strain XLI-Blue [F'::Tn10 *pro*A^+B^+ *lac*I^q △(lacZ) M15/*recA1 endA1 gyrA96*(Nal^r) *thi hsdR17*(r_k^- m_k^+) *supE44 relA1 lac*] was incubated into a flask containing LB medium. The cell was incubated at 37 ℃ with moderate agitation until the cell density was OD_{600} = 0.5. The cultured cell was collected in to 50 ㎖ centrifuge tubes such as Falcon 2070 tubes and was chilled on ice for 10 min. The cell was pelleted by centrifugation at 1,000× g for 15 min at 4 ℃.

The pelleted cells were drained thoroughly by inverting the tubes on paper towels and rapping to remove any liquid. The cell pellet was resuspended by moderate vortex mixing with 1/3 volume of TB buffer (Table 2). The cells were incubated on ice for 15 min and were pellet again with same method

described above. The cells were resuspended with 1/10 volume of TB buffer and were incubated on ice for 15 min.

The cells aliquoted into chilled 1.5 ㎖ microcentrifuge tubes and flash freezed in liquid nitrogen and then placed at -70 ℃ Deep Freezer (Samwon).

Transformation

The tubes containing competent cell were removed from the Deep Freezer and were thawed at room temperature until the cell suspension was just liquefied and were placed on ice.

The DNA solution was added to the tubes and the tube was swirled to mix the DNA evenly with the cells. After the tubes were incubated on ice for 10 min, the cells were heat shocked by placing the tubes at 37 ℃ water bath for 55 sec and then chilled by returning the tubes immediately to ice. 900 ㎕ of LB medium was added and was incubated at 37 ℃ for 45 min. The cells were plated on LB agar plate containing ampicillin (60 ㎍/㎖) and the plate was incubated at 37 ℃ for 15~17 hr.

Table 2. TB buffer

Compound	Amount/liter	Final concentration
Pipes	3.0 g	10 mM
$MnCl_2$	10.9 g	55 mM
$CaCl_2$	2.2 g	15 mM
KCl	18.65 g	250 mM
Adjust to pH 6.7 with 10N KOH Sterilize by filtration (0.45 ㎛)		

Plasmid DNA extraction

Cloned plasmid DNA was purified with *AccuPrep*® Plasmid Extraction Kit (Bioneer). Cultured cell 1.5 ㎖ in LB broth containing ampicillin (60 ㎍/㎖) was centrifuged at 13,000 rpm for 1 min. Pellet was resuspended with 250 ㎕ of Resuspension buffer (RS) and mixed by vortexing.

The cell was dissolved in 250 ㎕ of Lysis buffer and mixed by gently inverting several times, and the lysate was incubated at room temperature for 5 min.

After neutralized with 350 ㎕ of Neutralization buffer, the lysate was mixed by gently inverting several times and centrifuged at 14,000 rpm, 4 ℃ for 10 min.

The supernatant was transferred to the binding column tube and centrifuged at 13,000 rpm for 1 min. The filtrate was poured out from collection tube and the column was washed with 700 ㎕ of 80% ethanol by centrifugation for 1 min and dried by additional centrifugation at 13,000 rpm for 1 min to completely remove ethanol. The binding column tube was transferred to sterilized 1.5 ㎖ microcentrifuge tube.

The plasmid DNA was eluted by adding 100 ㎕ of Elution Buffer to the binding column tube and centrifuged at 13,000 rpm for 1 min.

Purified DNA was digested with Hind III and EcoR I restriction enzymes (0.1 ㎍ of cloned plasmid DNA, 1 ㎕ of 10× restriction K buffer, each 0.5 ㎕ of Hind III and EcoR I, distilled water upto 10 ㎕) and analyzed on a 1.5% agarose gel stained with EtBr , along with DNA 100bp size marker

(Bioneer) and digested DNA of full-length fragment of fMCHR1 and fMCHR2 were purified using *AccuPrep*® Gel Purification Kit (Bioneer).

Sequence analysis

The clone was amplified with T7 and SP6 promoter primer located at pGEM T-easy vector and sequenced by ABI 3700 DNA analyzer from Genotech (Korea). The resulting sequences were aligned with GenBank database through the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Reverse transcription-polymerase chain reaction (RT-PCR)

In order to perform RT-PCR, total RNA was extracted from in brain, kidney, muscle, liver and embryo from mature olive flounder (size; 40 ± 10 ㎝, body weight; 1000 ± 200 g; 3 years old) using TRIzol reagent (Difco). *AccuPrep*® RT/PCR PreMix tube (Bioneer) was used.

Before RT-PCR, 1 ㎍ total RNA and 1 ㎕ of the reverse primer (RTMR1-R primer and of RTMR2-R primer) were mixed in a sterile tube and mixture was incubated at 70 ℃ for 5 min and chilled in ice.

For RT-PCR reaction, the incubated mixture and the forward primer (RTMR1-R primer and of RTMR2-R primer) were transferred to *AccuPrep*® PT/PCR PreMix tube (Bioneer)

, then filled up the reaction volume to 20 ㎕ with DEPC water.

The lyophilized blue pellet was dissolved by vortex mixer, and briefly spined down. cDNA synthesis was performed at 42 ℃ for 1 hr, and RTase inactivation was performed at 94 ℃ for 5 min.

Next, PCR step was performed using the following one-step cycle parameters; 35 cycles at 94 ℃ for 30 sec, 50 ℃ for 30 sec, 72 ℃ for 1 min. The PCR product was analyzed on a 1.5% agarose gel stained with EtBr, along with DNA 100bp size marker (Bioneer).

The preparation of probe and southern blot analysis

cDNA was synthesized from in liver, spleen, kidney, small intestine, muscle and brain tissues from olive flounder.

According to the manufacturer's protocol, the probes were prepared using Dig DNA labeling Mix (Roche).

Isolated cDNA (100 ㎍ per sample) was separated by electrophoresis on a 1.5% agarose gel with 1X TBE buffer. cDNA was transferred to a nylon membrane using XCell *SureLock*^{TM} Mni-Cell the with 1X TBE buffer for 1 hr with 200 mA, 12 V, and cross-linked using the UV Cross-Linker (Hoper) with preset condition (1.2 mJ/cm^2).

After UV cross-linking, the membrane was hybridized with the DNA fragment of fMCHR1 (and fMCHR2) coding region that were labeled with Digoxigenin-11-dUTP by PCR priming

using PCR Dig Probe Synthesis (Roch). Then, the membrane was washed twice at 68 ℃ for 10 min with shaking under 2× SSC, twice at 68 ℃ for 5 min with shaking under 2× SSC containing 0.1% SDS and twice at 68 ℃ in 0.1× SSC contining 0.1% SDS.

The membrane was washed briefly with shaking for 1 min under Buffer 1 (100 mM Tris-HCl; 150 mM NaCl; pH 7.5 at 20 ℃). Then, the membrane was incubated for 30 min with buffer 2 (1% blocking reagent in buffer 1) and for 30 min with diluted antibody conjugate solution (150 mU/㎖). Then, after localizing probe target with hybrids with Anti-Dig (anti-digoxigenin-alkaline phosphatase), the membrane was incubated in the dark with a freshly prepared color substrate solution containing NBT/BCIP stock solution overnight.

RESULTS AND DISCUSSION

Nucleotide sequences of MCH receptor genes in olive flounder

I have cloned two melanin-concentrating hormone receptor genes using a olive flounder brain cDNA as a template by polymerase chain reaction (PCR).

As shown in Figure 1, two PCR products, about 500bp (fMCHR1 fragment) and 375bp (fMCHR2 fragment) respectively obtained in genomic DNA of olive flounder owing to underexposed sequence database and cDNA library. fMCHR1 fragment was obtained using DGMR1-F and DGMR1-R primers and fMCHR2 was obtained using DGMR2-F and DGMR2-R primers. These primers were designed through alignments of MCH receptor genes of human, rat, fugu, and zebrafish. The nucleotide sequences of partial fMCHR1 and fMCHR2 were sequenced as shown in Figure 2, and analysed using the Genbank database.

Above partial MCHR1 and MCHR2 fragments size was short for RACE analysis, consequently as shown in Figure 3, two PCR products, more than 800bp of fMCHR1 fragment and about 800bp of fMCHR2 fragment were obtained using primer walking system (DNA Walking *SpeedUp*™ Premix Kit, Seegene) from olive flounder genomic DNA. As the result, about 200bp unknown sequence of fMCHR1 and 500bp unknown sequence of fMCHR2 were obtained.

As results of fragment sequences of MCHR1 and MCHR2 in

olive flounder, 5'- and 3'- Rapid amplification of cDNA ends (RACE) analysis performed using designed RACE GSP (gene specific primer) primer sets of fMCHR1 and fMCHR2.

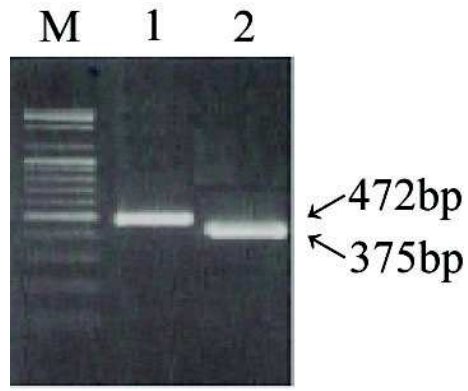
As shown in Figure 4, three PCR products, about 1.0Kb (5'-RACE product of fMCHR1), 1.2Kb (3'-RACE product of fMCHR1), and 1.2Kb (full-length cDNA of fMCHR1) were obtained from brain cDNA of olive flounder respectively.

Nucleotide and deduced amino acid sequence was obtained as shown in Figure 5 by sequencing service (Genotech).

Figure 6 shown three PCR products, about 1.2Kb (5'-RACE product of fMCHR2) and 0.9Kb (3'-RACE product of fMCHR2) and 1.3Kb (full-length cDNA of fMCHR2) respectively obtained from brain cDNA of olive flounder.

Nucleotide and deduced amino acid sequence was shown in Figure 7 by sequencing service (Genotech).

A



1.5% agarose gel
stained with EtBr

B

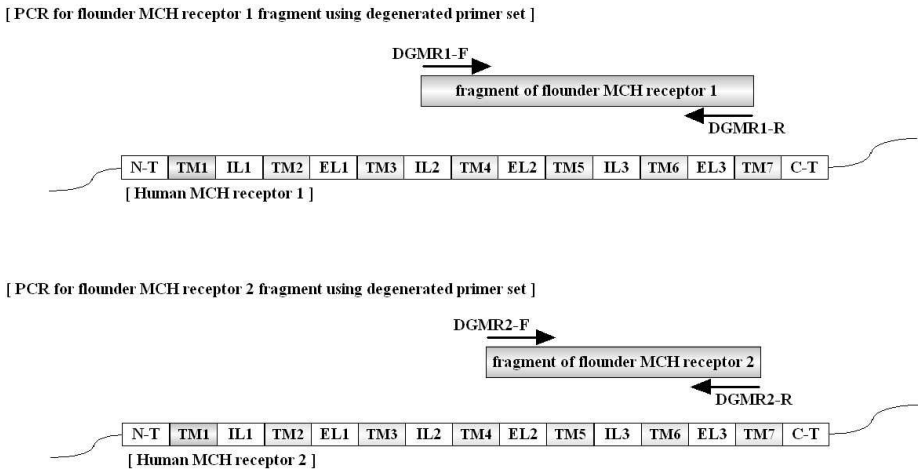


Figure 1. The result of PCR from partial genomic DNA using degenerated primer. (A) M: 100bp DNA ladder (Bioneer), 1: PCR products amplified with DGMR1-F and DGMR1-R in fMCHR1, and 2: PCR products amplified with DGMR2-F and DGMR2-R in fMCHR2. (B) Amplified regions of olive flounder MCH receptor gene comparing to human MCH receptor gene.

A. Partial nucleotide sequence of fMCHR1

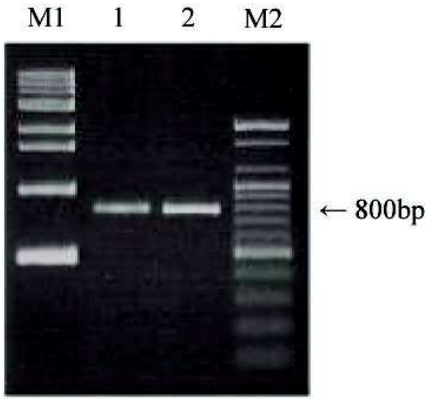
```
   1  CCCTTGACCGTTACTTGGCTACGGTCCATCCCATCCGCTTTAACTATGTCCGCACACCCT  60
  61  GTGTAGCAGCGCTGGTCATCGTCATTGTGTGGGGTCTGTCTTTCCTCACCATTATCCCTG 120
 121  TGTGGATGTATGCGGGCCTGATGCCTCTTCCAGATGGACTGGTTGCTTGTGCGCTCCTCC 180
 181  TGCCTGACCCAATTACCGACACATACTGGTTTACACTTTACCAGTTCTTTTTGGCCTTCG 240
 241  CCATGCCCTTGGTTATAATCTGCCTGGTGTTCTTCAAGATGCTCCAACACATGTCCAGCA 300
 301  GTGTGGCACCGCTGCCTCCACGGAGTCTGAGGGTGCGAACCAGGAAGGTGACCCGGATGG 360
 361  CGGTGGCCATCTGCCTTGCGTTCTTCATCTGCTGGGCTCCTTACTACATCCTTCAGCTGA 420
 421  TCCACCTTGGGGTGCAGAAGCCAACCCTTGCGTTCTCCTATGCGTACAACAT         472
```

B. Partial nucleotide sequence of fMCHR2

```
   1  ACCATCATGATCAACATACTAGTGTGGTTGGGCAGCTTCCTCCTCACCGTCCCTGTCATG  60
  61  ATGTACGCCAAGGTTGAGCGCAGGCAGCGTTTGGAGGTCTGCATGATGAACCTGGATGGG 120
 121  CCTGAGGACATGTACTGGTACACTTTCTACCAGTCCATCCTTGGCTACATCATCCCCTTC 180
 181  ATCATCATCAGCACCTTTTACTCGCTCACCCTCTACCACGTCTTCAGCTCCATCCGCCGG 240
 241  GTAAAACGCAAGCAGTCCGTCTGGGCTAAAAGAGCCACCAAGATGGTGCTGATGGTCATC 300
 301  GCATTGTTCCTGATCTGCTGGTCACCCTACCACGTCATCCAGGTGATCAACCTGAGCAAC 360
 361  AACACGCCGACCATC                                              375
```

Figure 2. Partial nucleotide sequence of MCH receptor gene in olive flounder genomic DNA

A



1.5% agarose gel stained
with EtBr

B

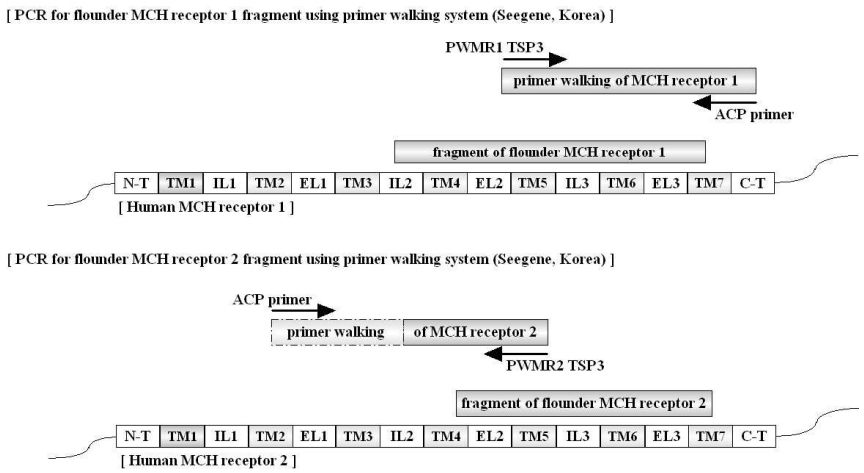
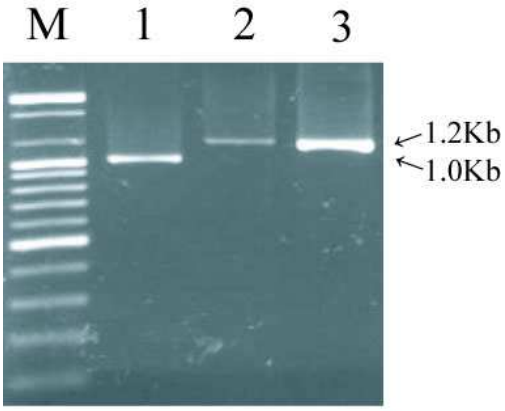


Figure 3. The result of primer walking from partial genomic DNA (A) M1: 1Kb DNA ladder, M2: 100bp DNA ladder (Bioneer), 1: primer walking products of fMCHR1 using ACP and PWMR1-TSP primers, and 2: primer walking products of fMCHR2 using ACP and PWMR2-TSP primers. (B) Amplified region of olive flounder MCH receptor gene comparing to human MCH receptor gene.

A



1.5% agarose gel stained with EtBr

B

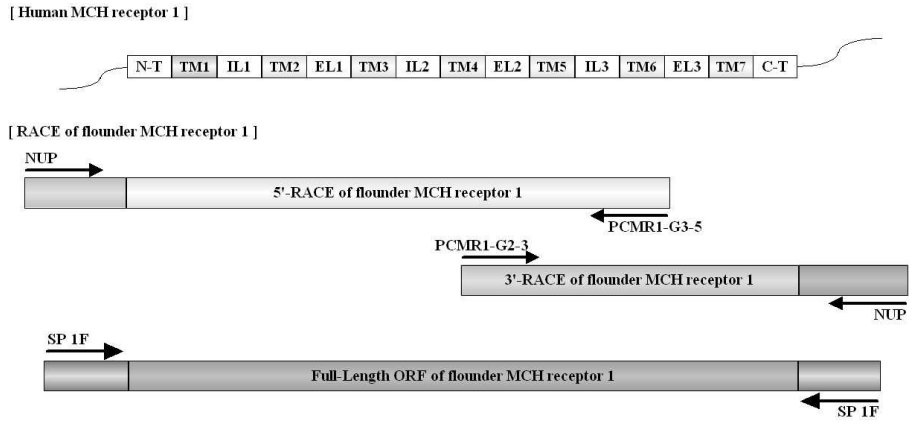


Figure 4. fMCHR1 RACE analysis from cDNA (A) M: 100bp DNA ladder (Bioneer), 1: 5'-RACE product (1.0Kb) in fMCHR1 using NUP and PCMR1-G3-5 primer, 2: 3'-RACE product (1.2Kb) in fMCHR1 using NUP and PCMR1-G2-3 primers. (B) Amplified region of olive flounder MCHR1 gene comparing to human MCH receptor 1 gene.

```
                                                                    acgcggggactacaggggcagcttcatcatcaccgagggagacacactcatctgctcacca
                                                                    tgattatttctaactgactcaaaaacaaaaacatctggaattaaagagtaataggtggatt
                                                                    cctttgttgactgcagctccagatgtttttcacttgaaaaacaggcacttcttaacttgga
                                                                    tacacctgatttttatttttacac

                                                                     M  D  F  H  N  D  S  N  F  S  V  S  H  T  N  S  T  T  T  A      20
                                                                1    atggatttccataacgattcaaatttttctgtctcacacactaattcaacaacaacagct
                                                                     V  Y  G  A  L  H  S  S  A  I  L  P  V  I  F  G  I  I  C  F      40
                                                               61    gtatatggggcccttcattccagtgccatcctccctgtcatcttcggcatcatctgtttt
                                                                     L  G  I  L  G  N  C  I  V  M  Y  T  I  I  K  K  T  K  C  R      60
                                                              121    cttggtatcttggggaactgcatcgttatgtacaccatcataaagaagaccaagtgccgc
                                                                     A  K  Q  T  V  P  D  I  F  I  L  N  V  S  I  V  D  L  L  F      80
                                                              181    gccaagcagactgttccggacatctttatcttaaacgtgtcgattgttgacctcctcttt
                                                                     L  L  G  M  P  F  L  I  H  Q  L  L  G  N  G  S  W  H  F  G     100
                                                              241    ctccttgggatgccgttcctcatccaccagttgctgggcaatggcagttggcactttgga
                                                                     A  T  M  C  T  V  I  T  A  L  D  S  N  S  Q  I  V  S  T  Y     120
                                                              301    gccacaatgtgtacagtcatcactgcgctcgactccaacagccagatagtcagtacttac
                                                                     I  L  T  A  M  T  L  D  R  Y  L  A  T  V  H  P  I  R  F  N     140
                                                              361    atcctcactgctatgaccccttgaccgttacttggctacggtccatcccatccgctttaac
                                                                     Y  V  R  T  P  C  V  A  A  L  V  I  V  I  V  W  G  L  S  F     160
                                                              421    tatgtccgcacaccctgtgtagcagcgctggtcatcgtcattgtgtggggtctgtctttc
                                                                     L  T  I  I  P  V  W  M  Y  A  G  L  M  P  L  P  D  G  L  V     180
                                                              481    ctcaccattatccctgtgtggatgtatgcgggcctgatgcctcttccagatggactggtt
                                                                     A  C  A  L  L  L  P  D  P  I  T  D  T  Y  W  F  T  L  Y  Q     200
                                                              541    gcttgtgcgctcctcctgcctgacccaattaccgacacatattggtttacactttaccag
                                                                     F  F  L  A  F  A  M  P  L  V  I  I  C  L  V  F  F  K  M  L     220
                                                              601    ttctttttggccttcgccatgcccttggttataatctgcctggtgttcttcaagatgctc
                                                                     Q  H  M  S  S  S  V  A  P  L  P  P  R  S  L  R  V  R  T  R     240
                                                              661    caacacatgtccagcagtgtggcaccgctgcctccacggagtctgagggtgcgaaccagg
                                                                     K  V  T  R  M  A  V  A  I  C  L  A  F  F  I  C  W  A  P  Y     260
                                                              721    aaggtgacccggatggcggtggccatctgccttgcgttcttcatctgctgggctccttac
                                                                     Y  I  L  Q  L  I  H  L  G  V  Q  K  P  T  L  A  F  S  Y  A     280
                                                              781    tacatccttcagctgatccaccttggggtgcagaagccaacccttgcgttctcctatgcg
                                                                     Y  N  I  A  I  S  M  G  Y  A  N  S  C  I  N  P  F  L  Y  I     300
                                                              841    tacaacatagccattagcatgggctacgctaacagttgcatcaacccatttctctacatc
                                                                     I  L  S  E  T  F  K  R  Q  F  L  R  A  V  R  P  V  N  R  K     320
                                                              901    atcctcagtgagactttcaagaggcagtttctcagagccgtacgtccggtcaacagaaag
                                                                     F  R  V  N  P  S  T  T  D  G  G  S  V  S  M  R  M  D  L  K     340
                                                              961    ttccgcgtgaacccgagcaccacggatggtggcagcgtgagcatgcgaatggacctgaag
                                                                     G  A  R  Q  E  P  A  P  R  E  M  I  P  S  N  V  A  P  Q  *     359
                                                             1021    ggggctcggcaggagccggcccctcgggagatgataccatccaatgtggcgccacaatga

                                                                    atcagaggcaaagaatcaaataattactccatcaccatcttatctgcaccaagtttggaca
                                                                    ccagacacaaacaataatgtctgcacatctacttcaatcaagcatcttgtttaaacagcat
                                                                    gtctgatatttttctgcccaaaccagatacaatttttcttttcccattcttcataaattta
                                                                    aaacagttagagctcctgaatgggcttggacacatttttatttttagtgattgaaacctgcg
                                                                    aatcataacattcttaataacagggagctcatactccataatttcaaatc
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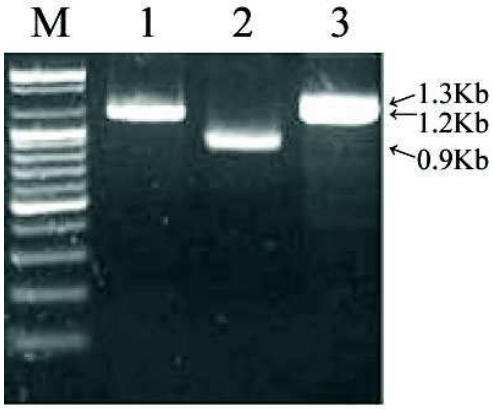
Figure 5. The nucleotide of deduced amino acid sequences of cDNA encoding fMCHR1 in olive flounder.

A



1.5% agarose gel stained with EtBr

B

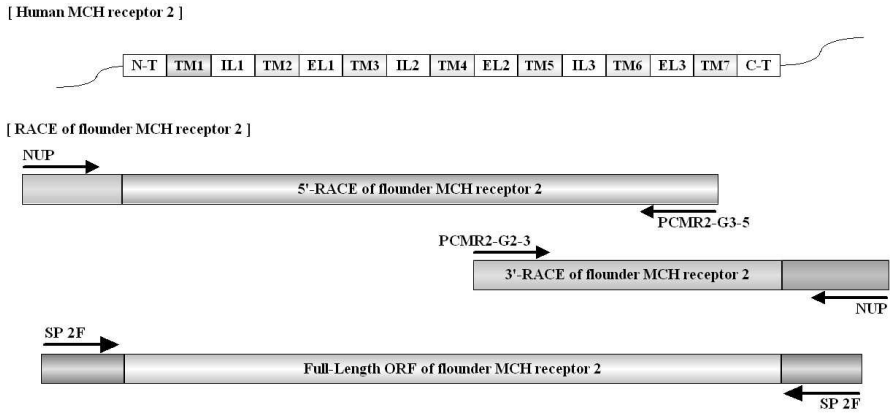


Figure 6. fMCHR2 RACE analysis from cDNA (A) M: 100bp DNA ladder (bioneer), 1: 5'-RACE product (1.2Kb) in fMCHR2 using NUP and PCMR2-G3-5 primer, 2: 3'-RACE product (0.9Kb) in fMCHR2 using NUP and PCMR2-G2-3 primers. (B) Amplified region of olive flounder MCHR2 gene comparing to human MCH receptor 1 gene.

```
gagaagaactccaggaagaaagacgcgctcctgcgctcttggagagtgcgtgaatttgctg
cctgaaacttttggagagcgtccgcttcaaatccatccacccagggagctgatttctgaga
ggagccgaag

      M  G  D  T  G  T  F  C  N  Q  T  A  N  L  T  D  P  A  C  L      20
   1  atgggcgacacgggcacattctgcaaccaaacagccaacctgacagacccggcgtgtctg
      N  S  T  R  P  S  Y  S  H  I  D  I  T  T  F  M  H  I  F  P      40
  61  aactcaacccgcccgtcgtacagccacatagacatcaccacgttcatgcacatattcccc
      T  I  Y  G  I  L  C  S  V  G  V  I  A  N  G  L  V  I  Y  A      60
 121  accatctacggcatcctgtgctcggttggagttattgccaacggactggtcatctacgcg
      V  A  A  C  K  K  K  M  V  S  D  I  Y  V  L  N  L  A  I  A      80
 181  gtggcggcatgcaaaaagaaaatggtctccgacatctacgtgctgaacttggccatagcg
      D  M  L  F  L  L  V  M  P  F  N  I  H  Q  L  V  R  D  R  Q     100
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      W  V  F  G  N  F  M  C  K  A  V  V  V  D  V  S  N  Q  F  T     120
 301  tgggtgttcgggaactttatgtgcaaagcggtggtggtggacgtgagcaaccagttcacc
      T  V  G  I  V  T  V  L  C  I  D  R  Y  I  A  I  V  H  P  T     140
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      S  E  K  R  T  I  H  W  T  I  M  I  N  I  L  V  W  L  G  S     160
 421  tcggagaaaaggaccatccactggaccatcatgatcaacatactagtgtggttgggcagc
      F  L  L  T  V  P  V  M  M  Y  A  K  V  E  R  R  Q  R  L  E     180
 481  ttcctcctcaccgtccctgtcatgatgtacgccaaggttgagcgcaggcagcgtttggag
      V  C  M  M  N  L  D  G  P  E  D  M  Y  W  Y  T  F  Y  Q  S     200
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      I  L  G  Y  I  I  P  F  I  I  I  S  T  F  Y  S  L  T  L  Y     220
 601  atccttggctacatcatccccttcatcatcatcagcacctttactcgctcaccctctac
      H  V  F  S  S  I  R  R  V  K  R  K  Q  S  V  W  A  K  R  A     240
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      T  K  M  V  L  M  V  I  A  L  F  L  I  C  W  S  P  Y  H  V     260
 721  accaagatggtgctgatggtcatcgcattgttcctgatctgctggtcaccctaccacgtc
      I  Q  V  I  N  L  S  N  N  T  P  T  I  T  F  V  Y  A  Y  H     280
 781  atccaggtgatcaacctgagcaacaacacgccgaccatcaccttcgtctatgcctaccac
      I  S  I  C  L  S  Y  S  H  S  C  I  N  P  L  M  L  L  I  F     300
 841  atcagcatctgtctcagctactctcacagctgcatcaacccactcatgctgctcatcttc
      A  Q  N  Y  R  D  R  L  C  R  R  N  M  L  N  S  S  Q  H  S     320
 901  gcccagaactatcgcgaccgcctttgccgcagaaatatgctcaacagttcccagcattca
      S  K  L  T  V  V  K  T  D  G  S  S  I  T  N  N  P  N  Y  R     340
 961  tccaagctcacagtcgtcaaaacagatggttccagtataaccaataaccccaactaccgc
      C  T  V  V  *                                                  344
1021  tgtactgtcgtataa

tcgcaaagtgtgtgtccttttgtagatacacatgtaaatgttctgctgctcacctcgaaag
gaagtagctcgatgaatggtgttgtccgtgaaataacattccccagcattttcataactgg
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gaaataggtgaaatacaggtcatatcaaaggctttcaacagagagtgcaagaaaaagatct
gcagaggagagcaagccggccaaaaaaaaaaaaaaaaaaaaaaaa
```

Figure 7. The nucleotide of deduced amino acid sequences of cDNA encoding fMCHR2 in olive flounder.

Amino acid sequence identity, phylogenetic tree and the structure of MCH receptors on membrane

Deduced amino acid sequences of fMCHR1 and fMCHR2 were respectively analyzed for multiple alignment, phylogenetic tree and deduced structure MCH receptor on cell membrane.

The multiple alignment, phylogenetic tree, and structure of flounder MCHR1 on cell membrane were shown in Figure 8, 9, and 10 using a deduced amino acid sequence of cDNA encoding fMCHR1. Figure 10 described N-linked glycosylation site (Asn^{15}), ligand binding site (Asn^{15}, Asp^{111}) (Saito *et al.*, 2003), induction site of signal transduction (Arg^{143}) (Saito *et al.*, 2005), potent phosphorylation site (Thr^{317}) (Saito *et al.*, 2004). The multiple alignment, phylogenetic tree, and structure of flounder MCHR2 on cell membrane were shown in Figure 11, 12, and 13 using a deduced amino acid sequence of cDNA encoding fMCHR2.

As shown in Figure 14, comparative analysis of MCHR1 and MCHR2 amino acids was numerical difference among flounder, fugu, mouse, and human. A numerical difference of flounder MCHR1 amino acids was shown in N- and C-terminus region. A numerical difference of flounder MCHR2 amino acids was shown in N- and C-terminus, transmembrane 3, intra-loop 2 and 3, and extra-loop 3 region. Figure 15 described alignment of the amino acid sequences of MCHR1 and MCHR2 from olive flounder. fMCHR1 and fMCHR2 was shown to positive 48.5% and identity of 29.2% respectively.

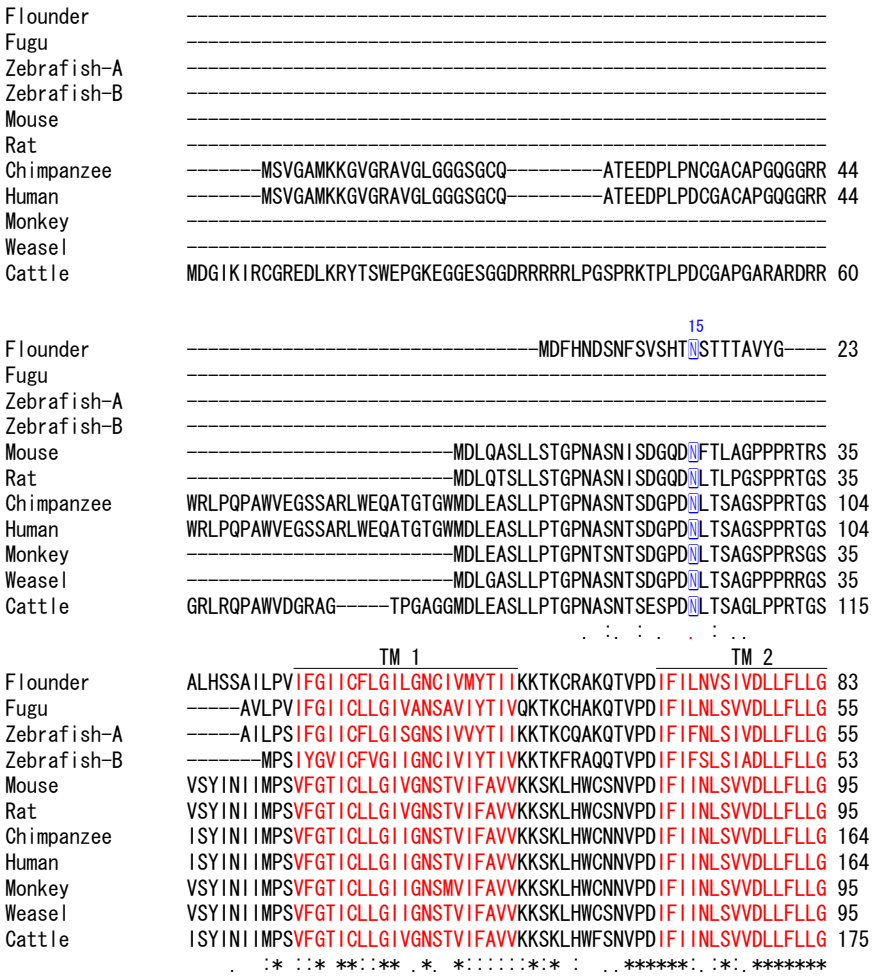

Figure 8. Multiple alignment of the deduced amino acid sequence of fMCHR1 with known MCHR1 protein sequence taken from GenBank. The lines above sequences are the predicted transmembrane domains; boxes are the predicted functional amino acids (ligand binding: Asn^{15}, Asp^{111}; N-linked glycosylation: Asn^{15}; signal transduction: Arg^{143}; potent phosphorylation: Thr^{317}, Ser^{325}, Thr^{342}; forming disulfate bonds: Cys^{104}, Cys^{182}) based on the published rat MCH receptor 1 analysis (Saito *et al.*, 2003, 2004, 2005).

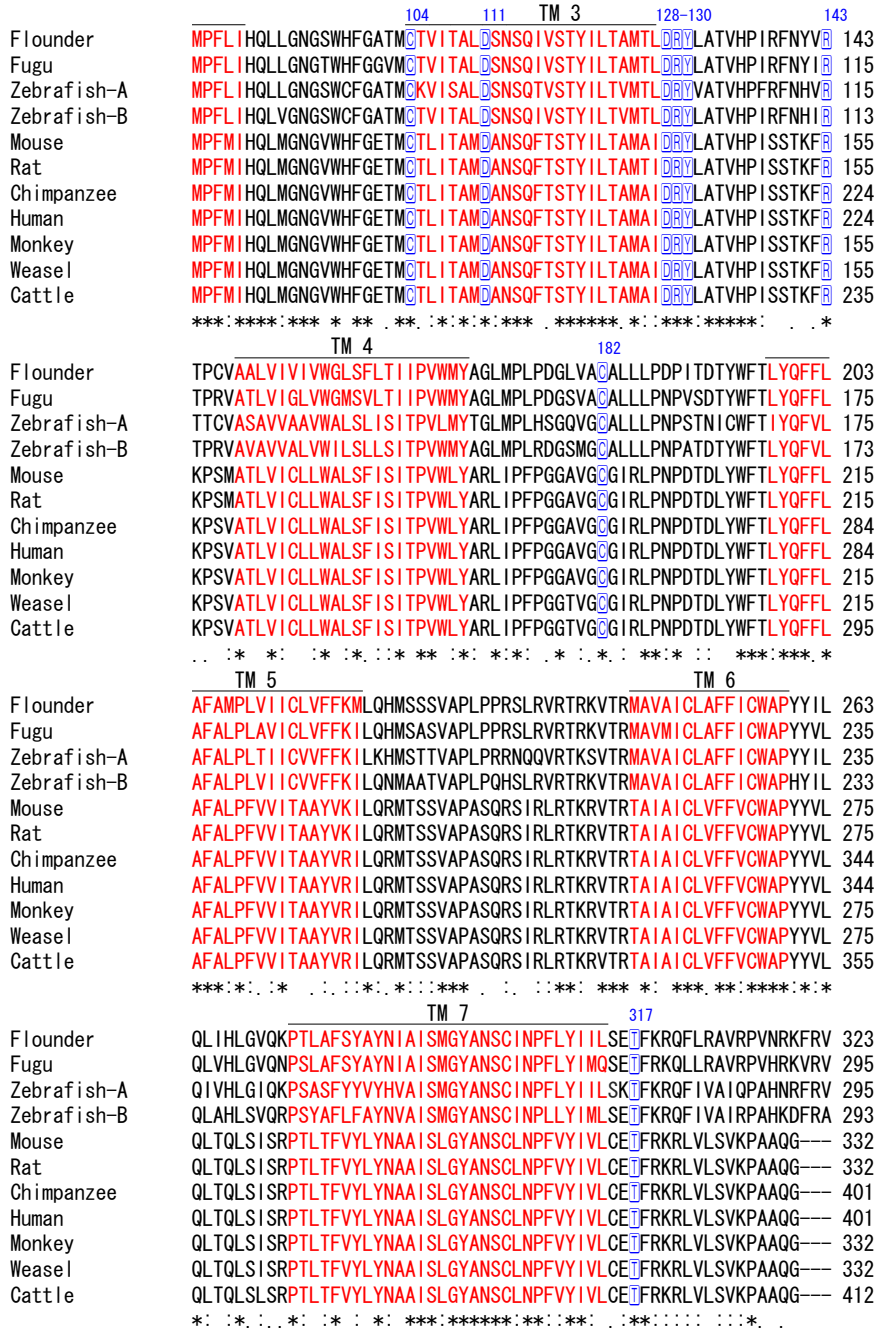

Figure 8. continued, the conserved Asp-Arg-Tyr(DRY) motif at the cytoplasmic side of TM3.

```
Flounder          NPSTTDGGSVSMRMDLKGARQEPAPREMIPSNVAPQ 359
Fugu              NPSTTDEGSVRVRMVPQEPQQDEASGEMRPSNVGSE 331
Zebrafish-A       NPSS-TEATVSLRLATDCQRHVPADNSE--------- 322
Zebrafish-B       DP---ADGSVSLRLAPDVAQQSQSSRELLPVTVAVH 326
Mouse             -----QLRTVSNAQTADEERTESKGT---------- 353
Rat               -----QLRTVSNAQTADEERTESKGT---------- 353
Chimpanzee        -----QLRAVSNAQTADEERTESKGT---------- 422
Human             -----QLRAVSNAQTADEERTESKGT---------- 422
Monkey            -----QLRAVSNAQTADEERTESKGT---------- 353
Weasel            -----QLRAISNAQTADEERTESKGT---------- 353
Cattle            -----QLRAVSNAQTADEERTESKGA---------- 433
                  ..:::   ::      .  :     .  . . .
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Figure 8. continued.

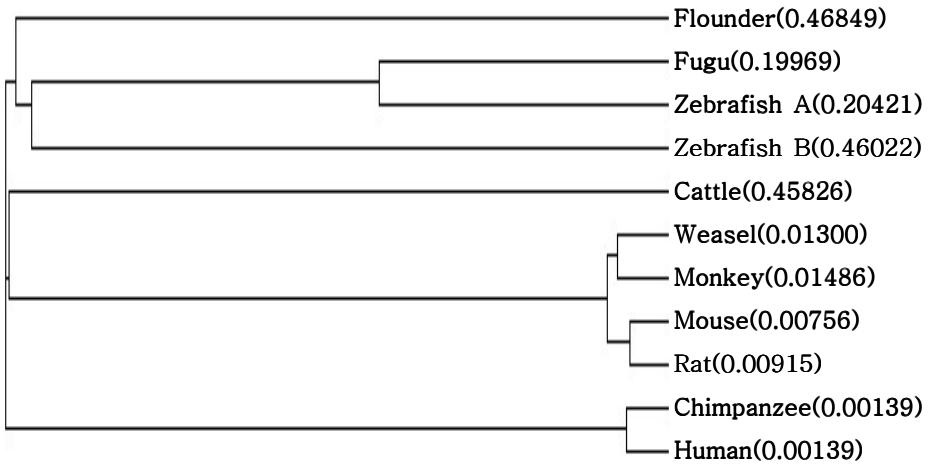


Figure 9. A molecular phylogenetic tree of Melanin-concentrating hormone receptor 1 based on the Neighbor-joining method. Numbers in parentheses are branch lengths.

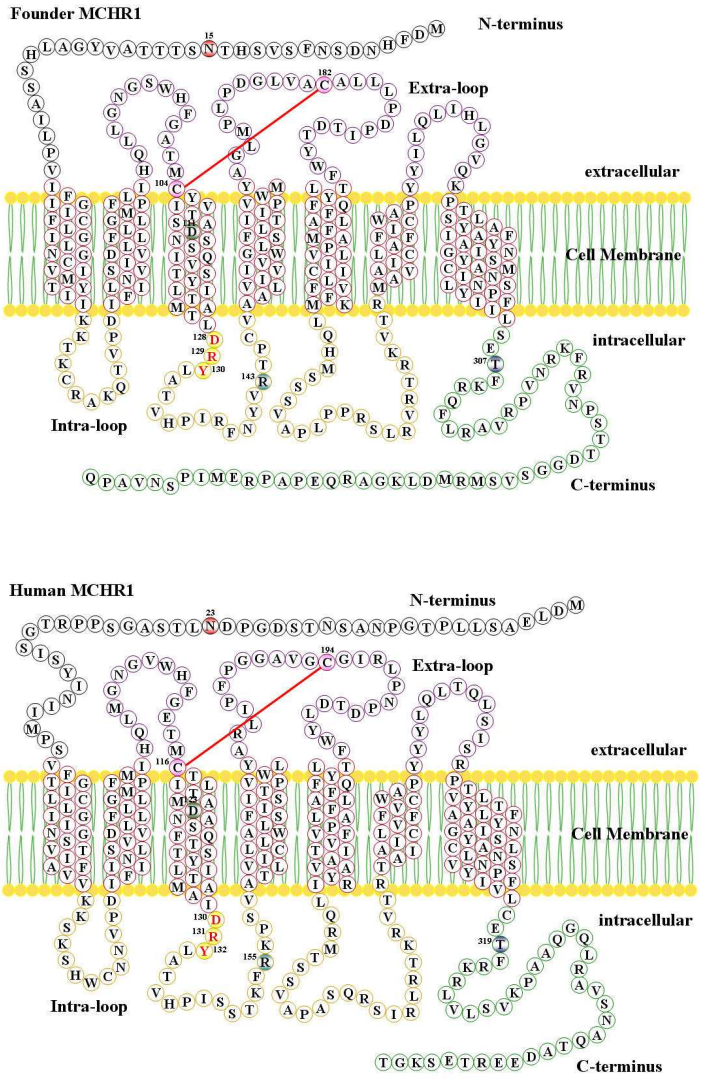


Figure 10. Structure of the flounder MCHR1 according to the model proposed by Alex *et al*. Structure of the deduced amino acid sequence of flounder MCHR1 with known human MCH receptor protein structure

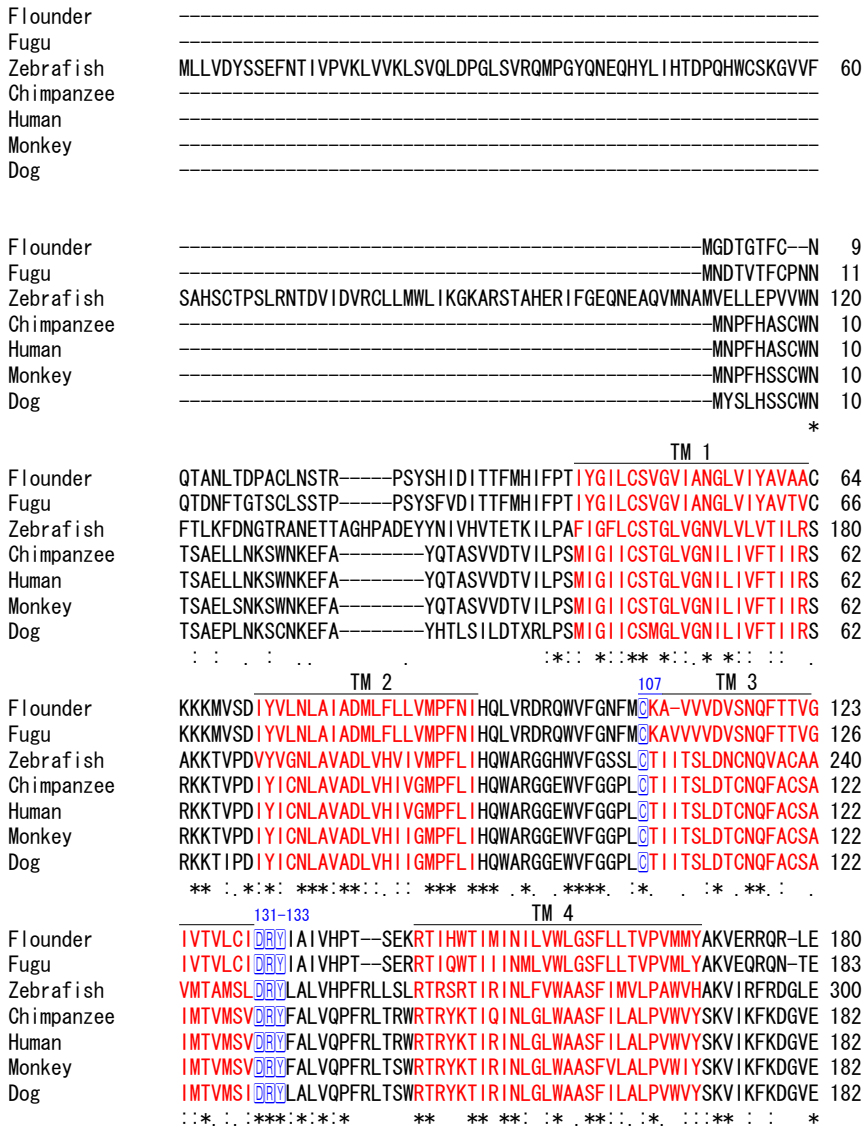


Figure 11. Multiple alignment of the deduced amino acid sequence of fMCHR2 with known MCHR2 protein sequence taken from GenBank. The lines above sequences are the predicted transmembrane domains; forming disulfate bonds: Cys^{104}, Cys^{182}); the conserved Asp-Arg-Tyr(DRY) motif at the cytoplasmic side of TM3.

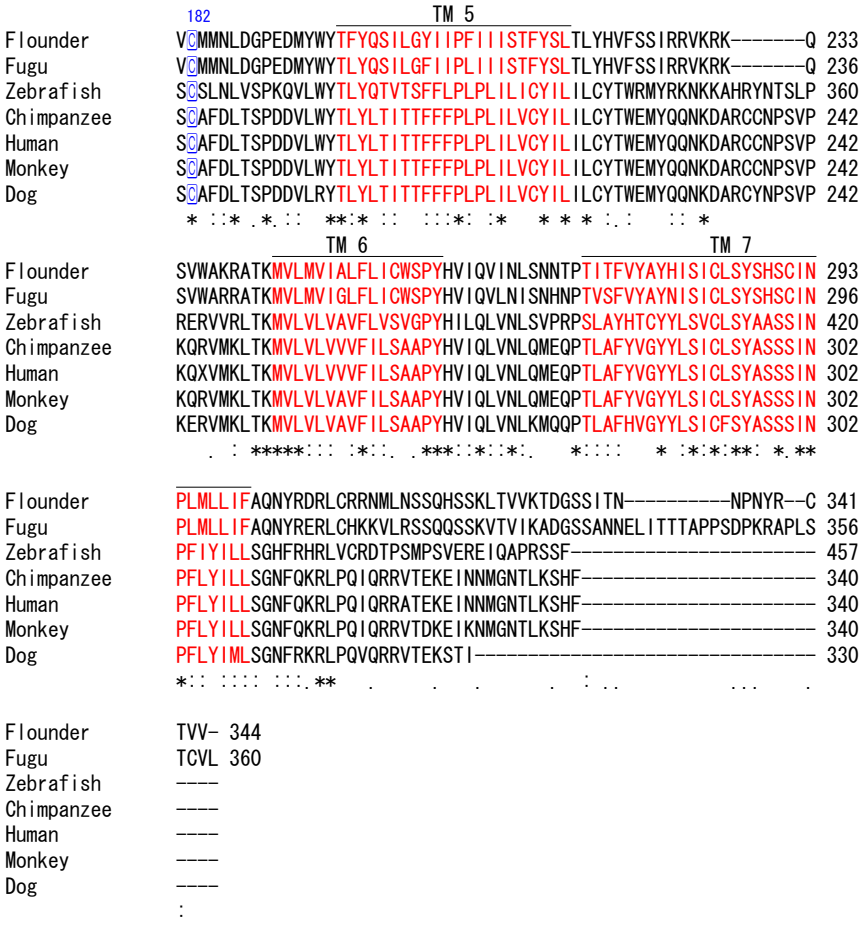


Figure 11. continued.

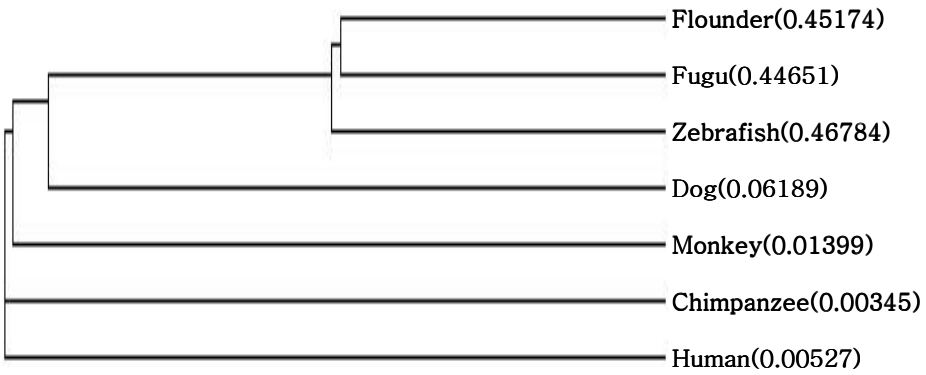


Figure 12. A molecular phylogenetic tree of Melanin-concentrating hormone receptor 2 based on the Neighbor-joining method. Numbers in parentheses are branch lengths.

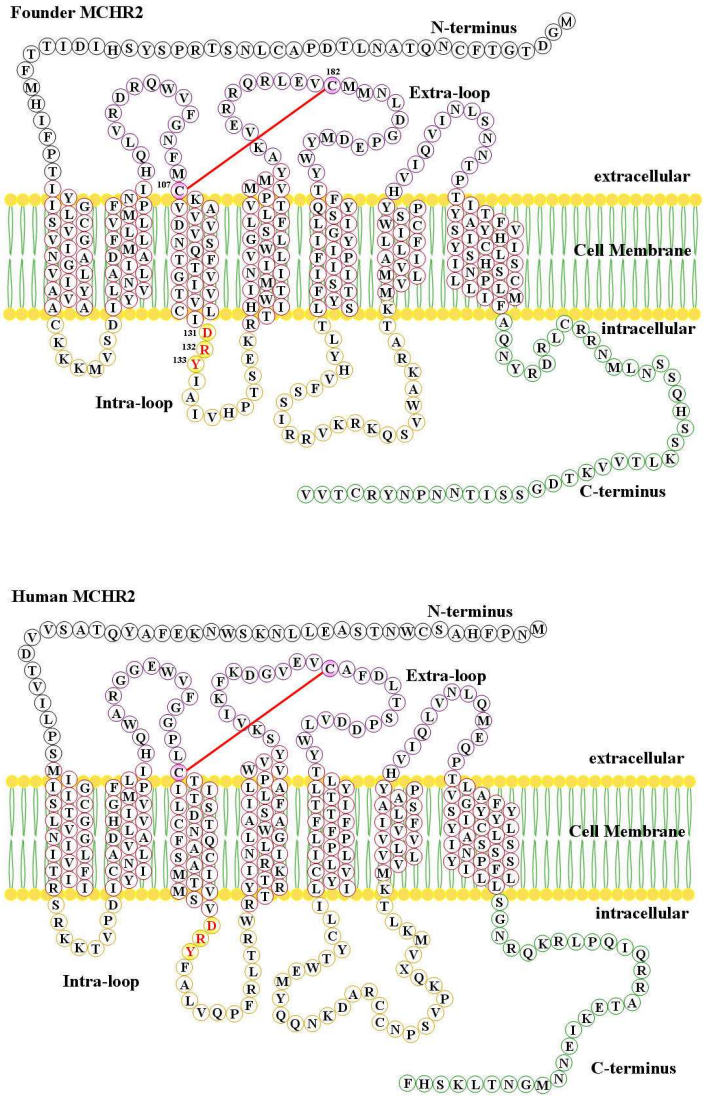


Figure 13. Structure of the flounder MCHR2 according to the model proposed by Alex *et al*. Structure of the deduced amino acid sequence of flounder MCHR2 with known MCH receptor protein structure.

MCHR1	N-term.	TM1	IL1	TM2	EL1	TM3	IL2	TM4	EL2	TM5	IL3	TM6	EL3	TM7	C-term.
Flounder	33	21	13	21	15	24	20	22	28	22	25	15	13	30	57
Mouse	45														39
Human	114														39

MCHR2	N-term.	TM1	IL1	TM2	EL1	TM3	IL2	TM4	EL2	TM5	IL3	TM6	EL3	TM7	C-term.
Flounder	41	22	8	21	15	23	13	27	25	22	25	16	13	29	44
Fugu	43					24									57
Human	39						15		26		32				31

N-terminus, TM; transmembrane, IL; intra-loop, EL; extra-loop, C-terminus

[The number is amino acid counts in each region]

Figure 14. Comparative analysis of flounder, mouse and human MCHR1 and MCHR2. MCHR1; A numerical difference of amino acid was shown in N- and C-terminus region. MCHR2; A numerical difference of amino acid was shown in N- and C-terminus, transmembrane 3, intra-loop 2 and 3, and extra-loop 3 regions.

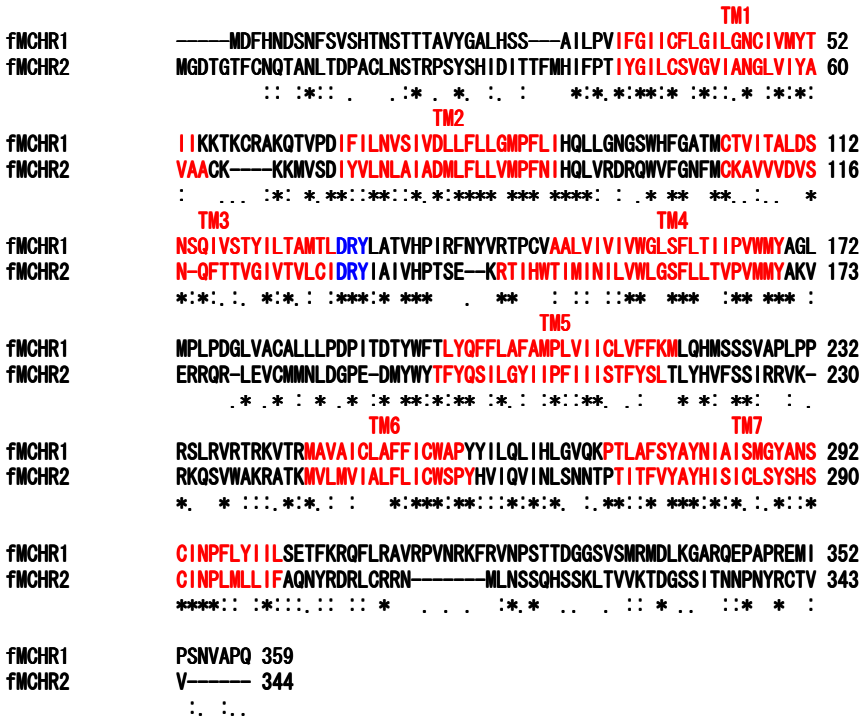


Figure 15. Alignment of the amino acid sequences of MCHR1 and MCHR2 from olive flounder. Identical amino acids in all sequences are asterisk marked. fMCHR1 and fMCHR2 was shown to positive 48.5% and identity of 29.2% respectively.

Tissue distribution of MCHR1 and MCHR2 genes

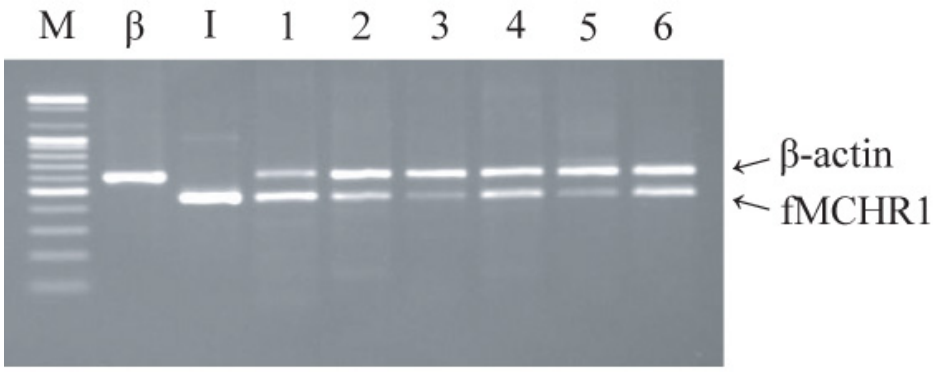
In order to determine the expression of the MCH receptor genes at the transcription level, total RNA was isolated from flounder liver, spleen, kidney, small intestine, muscle, and brain tissues.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the above RNAs as a template. The resulting RT-PCR products were analyzed on 1.5% agarose gel electrophoresis. As shown in Figure 16 A, approximately 450bp DNA fragment of MCHR1 gene was amplified from all total RNAs extracted from the liver, spleen, kidney, small intestine, muscle, and brain tissues, indicating that MCHR1 gene was expressed in all tissues examined.

However, as shown in Figure 17 A, approximately 500bp DNA fragment of MCHR2 gene was amplified from all total RNAs extracted from the liver, spleen, kidney, small intestine, muscle, and brain tissues, indicating that MCHR2 gene was only expressed in brain.

Also, the tissue-specific expression of MCHR1 and MCHR2 genes was confirmed by Southern blot analysis. The results of Southern blotting were shown in Figure 16 B and Figure 17 B.

A



B

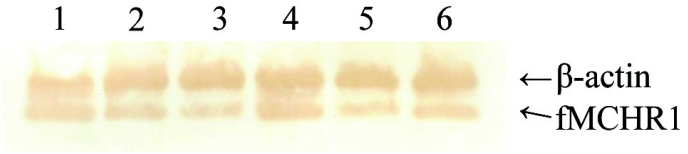
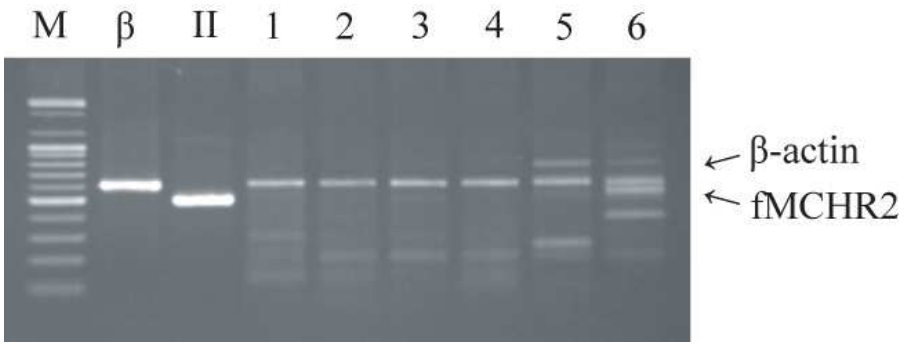


Figure 16. RT-PCR and Southern blot analysis of fMCHR1 (A) RT-PCR analysis of β-actin and fMCHR1 mRNA expression in flounder tissues. M: 100bp DNA ladder (bioneer), β: positive control amplified with pβ-actin cDNA, I: positive control amplified with pfMCHR1 cDNA, 1: liver, 2: spleen, 3: kidney, 4: small Intestine, 5: muscle, 6: brain. (B) Southern blot analysis of β-actin and fMCHR1.

A



B

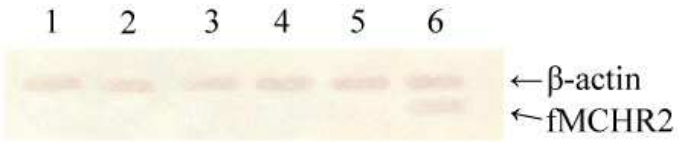


Figure 17. RT-PCR and Southern blot analysis of fMCHR2 (A) RT-PCR analysis of β-actin and fMCHR2 mRNA expression in flounder tissues. M: 100bp DNA ladder (bioneer), β: positive control amplified with pβ-actin cDNA, I: postive control amplified with pfMCHR1 cDNA, 1: liver, 2: spleen, 3: kidney, 4: small intestine, 5: muscle, 6: brain. (B) Southern blot analysis of β-actin and fMCHR2.

국문 초록

양식 넙치의 멜라닌 농축 호르몬 수용체 유전자 특성

정 인 영

부경대학교 대학원 미생물학과

대표적인 양식어종인 넙치 (*Paralichthys olivaceus*)의 멜라닌 농축 호르몬 (melanin-concentrating hormone; MCH) 는 19개의 아미노산으로 구성된 환형의 구조를 지니는 시상하부 호르몬으로서, 이 호르몬은 경골어류 표피조직 내의 색소 변화 조절하는 것으로 확인되었다.

MCH는 포유동물에서도 확인되었는데, 포유동물에서는 섭식과 에너지 항상성 조절을 하는 것으로 나타났다.

최근 여러 그룹의 연구에서 reverse pharmcology 기법을 이용하여 orphan G protein-coupled receptor (oGPCR)인 GPR24, GPR245가 MCH에 대한 특이적인 receptor임을 확인하는 보고가 있었다.

본 논문에서는 넙치의 멜라닌 농축 호르몬 수용체 (melanin-concentrating hormone receptor; MCHR) 유전자 특성을 연구하였고, 두 가지 종류의 MCHR1과 MCHR2를 확보하였다.

MCH receptor 유전자를 cloning 하기 위하여 인간 및 Zebrafish, Fugu등의 MCHR1의 부분 염기서열에서 특이적인 프라이머 조합을 합성하고 넙치 뇌로부터 rapid amplification of cDNA ends (RACE)를 실시하여 1077bp, 359개 아미노산을 암호화하는 open reading frame (ORF)를 확보하였다. MCHR2도 마찬가지로 부분 염기서열에서 특이적인 프라이머 조합을 합성하고 넙치 뇌로

부터 rapid amplification of cDNA ends (RACE)를 실시하여 1032bp, 344개 아미노산을 암호화하는 open reading frame (ORF)를 확보하였다.

넙치의 MCHR1은 인간의 MCHR1과 58%의 아미노산 상동성을 나타내었고, 넙치의 MCHR2는 인간의 MCHR2와 38%의 아미노산 상동성을 나타내었다. 이들 수용체는 G protein-coupled receptor 중에 rhodopsin과 유사한 Class I 에 속한다. 넙치의 MCHR2는 넙치의 MCHR1에 29.2%의 아미노산 상동성을 나타내었다.

간, 비장, 신장, 소장, 근육, 뇌에서 추출된 전체 RNA에서 증폭한 MCHR1 유전자는 간, 비장, 신장 소장, 근육, 뇌, 등의 모든 조직에서 발현되는 것으로 나타났다. 그러나 MCHR2 유전자는 위의 모든 조직에서 발현정도를 분석 하였으나 뇌에서만 발현 되는 것으로 확인되었다.

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길게만 느껴졌던 3년의 시간을 뒤로하고 마무리를 하려합니다. 약간은 뒤늦은 마무리를 하는 이 순간까지 항상 변함없이 격려해 주시고 따가운 채찍질도 함께 주신 지도교수 송영환 교수님께 깊이 감사드립니다. 또한 바쁜 시간 속에서도 저의 부족한 논문을 읽고 수정해 주시고 챙겨주신 김군도 교수님과 양식학과 김종명 교수님께 감사드립니다. 학부 4년과 석사 3년간 깊은 가르침과 사랑으로 대해주신 이원재 교수님, 김진상 교수님, 이명숙 교수님, 이훈구 교수님, 김영태 교수님, 최태진 교수님 감사드립니다.

세심한 관심으로 가르쳐주시며 지금까지 옆에서 많은 가르침을 주신 전정민 선배님께 감사드리며, 실험실에 들어 올 때 흔쾌히 반겨주시고 공부하게끔 자리를 잡아주시고, 지금은 멀리서 도움을 주신 이종규 선배님, 가끔이지만 급할 때 SOS 메시지를 날리면 언제나 내 동생처럼 도와주신 수산과학원 위생팀 유홍식 선배님, 부산대학 병원 김종숙 선배님 깊이 감사드립니다. 생화학 실험실의 이재형 선배님, 대전에서 물심양면으로 많은 관심을 가지고 도와주신 최용운, 강지희 선배님께도 깊이 감사드립니다.

실험실 들어왔을 때 처음으로 피펫을 잡고 실험을 가르쳐줬던 혜은이, 같이 고생하며 1년 먼저 졸업한 나의 영원한 친구 정환이, 지금은 다른 곳에서 열심히 공부를 하고 있는 동생인 승표와 재기에게도 너무 고맙다고 전합니다. 그리고 지금 같이 실험실에서 생활하는 후배들과 7325에서 졸업한 많은 학부생들, 이름을 모두 나열하지 않았지만 그 후배들에게도 고맙다는 말 전합니다.

오랫동안 7호관에서 동거 동락한 대성이형, 용배, 윤숙이, 미영이, 영주, 근호에게도 고맙다는 말 전하고, 같이 졸업하게 된 재경이, 경규, 경우, 다영 에게도 고맙다는 말 전합니다. 중요한 시간 멀리서 응원을 아끼지 않았던 종호, 가끔 같이 놀며 고민도 많이 나누었던 준이, 열심히 최선을 다하고 있는 재성이, 종오, 영감이라고 놀리면서도 응원을 아끼지 않았던 지현(임)이 호선(천)이, 그리고 많은 미생물학과 선후배님들께 감사드립니다.

마지막으로, 힘든 살림에도 언제나 변함없이 나의 결정과 그 길을 믿고 응원과 함께 사랑을 주시고 힘든 시간 무릎 꿇지 않게 언제나 힘을 주신 아버지, 몸이 편찮으시지만 장남을 챙겨주시느라 아픈 것도 잊고 사시는 어머님께 감사드리며 이제 곧 결혼하는 둘째, 박사과정을 밟는 막내에게도 고맙다는 말 전합니다.