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Molecular Cloning and Tissue-specific Expression of the Melanocortin 4 Receptor Gene from Olive Flounder, *Paralichthys olivaceus*

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G protein-coupled receptors (GPCR) constitute the largest superfamily of cell membrane receptors, mediating diverse signal-transduction pathways. The melanocortin 4 receptor (MC4R) has been of interest for its physiological role and size, one of the smallest among the GPCRs, which makes it a good model system for the structural study of GPCRs. To study the molecular structure and tissue-specific expression of MC4R in olive flounder (*Paralichthys olivaceus*), the full-length MC4R gene was obtained using PCR amplification of genomic DNA as well as cDNA synthesis. Sequence analysis of the gene indicates that 978 bp of the MC4R gene encodes 325 amino acids without introns. Sequence alignment with the MC4Rs from other fish shows the highest degree of identity (96%) between *Paralichthys olivaceous* and *Verasper moseri,* followed by *Takifugu rubripes* and *Tetraodon nigroviridis* (89%). RNA was isolated from various tissues to examine the tissue distribution of MC4R by using RT-PCR. The results showed major expression of MC4R in the liver, brain, and eye, which is consistent with the expression pattern in other fish belonging to the order Pleuronectiformes.

Key words: G protein-coupled receptor, Melanocortin 4 receptor, Expression, Olive flounder

Introduction

G protein-coupled receptors (GPCRs) which contitute one of the largest superfamilies of membrane receptors, participate in various signaling cascades that couple external signals to internal cellular processes. Upon recognition of a diverse array of extracellular ligands, including neurotransmitters and hormones, and upon receipt of physiological stimuli, such as light, odorants, and some tastants, GPCRs undergo conformational changes, initiating signal transduction cascades through the activation of heterotrimeric G-proteins (Gether, 2000). This in turn activates or inhibits secondary messengers, leading to a variety of physiological responses. Although GPCRs recognize many diverse ligands, all GPCRs share a common topology: seven-transmembrane (7TM) helices, together with some conserved amino acid sequences. GPCRs have been regarded as one of the most attractive targets for drug development, as more than 30% of drugs on the market are known to be effective by acting on GPCR signaling. There has been intense research on the structural differences between GPCRs, with and without bound ligand (for reviews, see Smith, 2010; Rosenbaum et al., 2009). Although some structural information at the atomic level has been obtained for the inactive state of GPCRs (Lodowski et al., 2009), detailed information about the structural organization, particularly for the activated state, is still lacking.

Melanocortin receptors (MCRs) belong to the rhodopsin subfamily of GPCRs, one of the largest subfamilies. MCRs are activated by the ligands α-, β-, and γ-melanocyte stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH), derived from the precursor pro-opiomelanocortin (POMC), and mediate a variety of signaling pathways. Five subtypes of melanocortin receptors (MC1R–MC5R) have been identified in mammals (Desarnaud et al., 1994; Gantz et al., 1993a; Gantz et al., 1993b; Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994; Mountjoy et al., 1992). Melanocortin 1 receptor (MC1R), expressed mainly in melanocytes, is involved in skin- and hair-color determination, in addition to having anti-inflammatory action in

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immunocytes such as leukocytes. MC2R is activated by ACTH and mediates physiological reactions in the adrenal gland. MC3R and MC4R are involved in food intake and energy balance in animals; this was shown by MC3R and MC4R knock-out mice exhibiting hyperphagia, hyperinsulinemia, and maturity-onset obesity that corresponded with agouti obesity syndrome (Huszar et al., 1997; Butler et al., 2000). Administration of MCR agonists, such as MSHs and natural and synthetic compounds, including melanotan II (MT-II), into mice caused a loss of appetite and lean phenotype (Chen et al., 2000). Injection of antagonistic ligands of MC3R and MC4R blocked this effect and lead to weight gain. In addition, MC4R is the most common monogenic determinant of obesity (Leibel, 1997; Leibel et al., 1997) and, therefore, has been regarded as one of the main targets for the treatment of anorexia and obesity. MC5R is distributed throughout the peripheral tissue, although its physiological function is not yet clear (Schiöth, 2001).

Olive flounder, *Paralichthys olivaceus*, belongs to the family Paralichthyidae of the order Pleuronectiormes. It is one of the major cultured marine fishes in Korea; in 2008, approximately 46,000 tons were produced, according to *The Status of Aquaculture of Marine Fishes* (MOMAF, 2009). The physiology of olive flounder is an active area of research due to interest in its mass production. The MC4R gene of olive flounder is of particular interest, as it may be involved in the control of body weight and meat quality. In this study, the MC4R gene was isolated by PCR amplification of genomic DNA and cDNA isolated from olive flounder tissues. The tissue-specific expression of MC4R was also analyzed.

Materials and Methods

Materials

T4 DNA ligase and the *AccuPrep*® Genomic DNA Extraction Kit were purchased from Bioneer (Daejeon, Korea). TRI REAGENT™ was obtained from Sigma (Saint Louis, MO). The PolyATract® mRNA Isolation System, ImProm-II™ Reverse Transcription System and Wizard® *Plus* Maxipreps DNA Purification System were purchased from Promega (Madison, WI). Restriction endonucleases were purchased from Bioneer (Daejeon, Korea) and New England Biolabs (Beverly, MA). Kits used for plasmid purification, gel extraction, and PCR purification were purchased from NucleoGen (Seoul, Korea). The DNA Walking *SpeedUp*™ Premix Kit II was obtained from Seegene (Seoul, Korea). The pGEM®-T Easy Vector System was obtained from Promega Corporation (Madison, WI). Oligonucleotides and 5X HiQ-PCR mix were obtained from Genotech (DaeJeon, Korea). The PCR primers used are listed in Table 1.

Isolation of genomic DNA and RNA

Olive flounder genomic DNA was extracted from 100 μL of whole blood using the *AccuPrep*® Genomic DNA Extraction Kit according to the manufacturer's instructions. Total RNA was extracted from various internal organs including the liver and brains using TRI REAGENT™ according to the manufacturer's protocol. RNA was analyzed by agarose gel electrophoresis and UV/Vis spectrophotometry, then stored at -80°C until use. The isolation of mRNA was carried out using the PolyATract® mRNA Isolation System according to the manufacturer's protocol.

Table 1. List of oligonucleotide sequences used for the experiment

Primer	Sequence (5'→3')	Comment
MC4RF1	ATC AAG AGC ATG GAC AAC GT	Partial MC4R
MC4RR1	ATC ATG AGG ATG AGG TGG AG	
MCDWF1	CGC TTG CAC ATG AAG CGC AT	DNA walking PCR
MCDWF2	AG(G/C) GCG CCA ACA TGA AGG	
MCDWF3	GGT GTT CGT GGT GTG CTG G	
MCDWR1	AAG ATG GTG ATG TAG CGG TCG	
MCDWR2	AGC AGG CTG CAG ATG GAC	
MCDWR3	GGA GCT GCA GAT CAT AGA GTC A	
RTMC4RF	CTG GAG AAC ATC CTG GTG GTC G	RT-PCR
RTMC4RR	GAA GAT GGT GAT GTA GCG GTC G	
actinF	GCA GGT CAT CAC CAT CGG	β-actin
actinR	GAG TAT TTG CGC TCA GGT G	

Cloning of the melanocortin 4 receptor gene

The PCR reaction mixture used for the amplification of partial regions of the MC4R gene contained 0.5 μg genomic DNA, 0.5 μM MC4RF1 and MC4RR1 primers, and 1X HiQ-PCR Mix. The PCR reaction was carried out with an initial denaturation at 95°C for 5 min, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, and 2 min at 72°C, with a final extension at 72°C for 5 min. To obtain the 5'- and 3'-ends of the olive flounder MC4R gene, DNA walking PCR was conducted using the DNA Walking *SpeedUp*™ Premix Kit II. Three forward (pMCDWF1, pMCDWF2, pMCDWF3) and three reverse (pMCDWR1, pMCDWR2, pMCDWR3) primers, corresponding to the sense and anti-sense strands (Table 1) of the MC4R gene, were used to amplify the 5'- and 3'-end regions of the gene, respectively (Table 1). The amplified PCR products were cloned into the pGEM®-T Easy Vector, transformed into *E. coli,* and then analyzed by restriction digestion and DNA sequencing. Amplification of the MC4R gene was performed using the first-strand cDNA as a template.

Tissue-specific expression of the MC4R gene analyzed by RT-PCR

To examine the tissue-specific expression of MC4R in olive flounder, RNA was extracted from various internal organs, including the kidney, gut, gill, liver, intestine, spleen, heart, eye, and brain, as described above. The isolation of mRNA was carried out using the PolyATract® mRNA Isolation System according to the manufacturer's protocol. First-strand cDNA was synthesized using mRNA as a template and oligo$(dT)_{13}$ as a primer using the ImProm-II™ Reverse Transcription System. RT-PCR was performed using the first-strand cDNA as a template and specific primers (RTMC4RF, RTMC4RR) derived from the MC4R sequence of olive flounder, as determined in this study. As an internal positive control, PCR amplification of the β-actin gene was conducted using primers corresponding to the β-actin gene (actinF and actinR). RT-PCR was performed with a cycle of 3 min incubation at 95°C followed by 25 cycles of 40 s at 94°C, 40 s at 60°C, and 40 s at 72°C, with a final extension of 5 min at 72°C.

Sequence alignment and phylogenetic analysis

From the full-length MC4R gene derived from PCR, using the genomic DNA and cDNA as a template, a compiled amino acid sequence was aligned with other known MCR sequences using Clustal W (Thompson et al., 1994). The accession numbers of melanocortin 4 receptor sequences searched in the NCBI GenBank were as follows: *Homo sapiens* MC4R (NM005912), *Mus musculus* MC4R (NM016977), *Gallus gallus* MC4R (AB012211), *Squalus acanthias* MC4R (AY169401), *Danio rerio* MC4R (NM173278), *Oncorhynchus mykiss* MC4R (AY534915), *Takifugu rubripes* MC4R (AY227794), *Tetraodon nigroviridis* MC4R (AY332240), *Carassius auratus* MC4R (AJ534337), and *Verasper moseri* MC4R (AB287975).

A phylogenetic tree was constructed by the neighbor-joining method using MEGA v 4.0 (Tamura et al., 2007). Accession numbers of other melanocortin receptor sequences selected using BLASTp were *Homo sapiens* MC3R (NM019888), MC5R (NM005913), *Mus musculus* MC3R (NM008561), MC5R (013596), *Gallus gallus* MC3R (AB017137), MC5R (AB012868), *Squalus acanthias* MC3R (AY560605), MC5R (AY562212), *Danio rerio* MC3R (NM180972), MC5Ra (AY078990), MC5Rb (AY078991), *Takifugu rubripes* MC5R (AY227796), and *Tetraodon nigroviridis* MC5R (AY332241). Tree reliability was assessed using a bootstrap with 1000 replicates. The outgroup was the human melanocortin 1 receptor (AF153431).

Results and Discussion

Cloning of the MC4R gene

MC4R belongs to the largest subfamily of GPCRs, the rhodopsin subfamily, which mediate a diverse array of signal transduction pathways. Among GPCRs, MC4R has been a focus of intense research for its association with obesity. It is also regarded as a model system for the structural study of GPCRs, as MC4R is one of the smallest GPCRs, which may favor a relatively stable structure during crystallization. The MC4R gene in olive flounder, one of the major aquaculture species in Korea, is of interest because it is implicated in body-weight control.

Amplification of the MC4R gene was initially carried out using genomic DNA as a template because no introns were found in the coding region of the MC4R gene in other fishes (Klovins et al., 2004). For this purpose, genomic DNA was isolated from the whole blood of olive flounder, as described in the Materials and Methods. A high molecular weight of the genomic DNA, determined by agarose gel electrophoresis, confirmed the integrity of the genomic DNA (data not shown) suitable for amplification. PCR primers (Table 1) were designed from the conserved regions of all known fish MC4R genes (rainbow trout, spiny dogfish, spotted Japanese

pufferfish, goldfish, zebrafish, and fugu) (Metz et al., 2006). Amplification of the MC4R gene using genomic DNA and the oligonucleotides MC4RF1 and MC4RR1 resulted in a 450 bp fragment (data not shown), as expected from the size of the corresponding regions conserved in other MC4Rs. Sequence analysis of the DNA, as well as the amino acid sequence translated using BlastN and BlastX, revealed a high similarity with other MC4R genes (data not shown). To obtain the full length of the MC4R gene through DNA walking, oligonucleotides corresponding to the sense and antisense regions of the target gene were designed (Table 1). Cloning and sequence analysis of the clones obtained from DNA walking showed amplification of 715-bp and 634-bp fragments, corresponding to the 5'- and 3'-end regions of the gene, respectively (Fig. 1). DNA sequence information based on the analysis of the partial gene products amplified by PCR and DNA walking experiments were compiled to obtain the full-length MC4R gene. The results showed that the full-length MC4R gene in olive flounder consists of 978 base pairs encoding 325 amino acids (Fig. 1). A comparison of the PCR products obtained by amplification of the genomic DNA with those obtained by amplification of the cDNA prepared by using RNA isolated from brain tissue, showed similar sizes within 1 kb, as determined by agarose gel electrophoresis (data not shown). The DNA base sequence of the MC4R gene, as well as the absence of any introns, was confirmed by analysis of the MC4R clones obtained from cDNA amplification.

The amino acid sequence of MC4R was deduced from the full length MC4R gene. Sequence alignment of its amino acids against other known MC4Rs using the Clustal W program showed the highest similarity (96%) to MC4R of barfin flounder (*V. moseri*), followed by fugu (89%), zebrafish (80%), and human (67%). The amino acid sequence was also compared with that of other species by the percentage of identity (Fig. 2 and Table 2). The MC4R amino acid sequence is highly conserved even between fish and mammalian orthologs (65-70%), in contrast to that of other subtypes of melanocortin receptors, such as MC1R and MC2R (52-53%) (Schiöth et al., 2005). Hydropathy analysis of its amino sequence predicted seven transmembrane regions, one of the characteristics of GPCRs (Fig. 2). Other characteristics of the MC4R gene of olive flounder included the lack of introns in the putative coding region and the presence of an ERY/DRY motif at the end of the TM3, consistent with all known MC4R genes. Putative N-glycosylation sites were also identified at Asn^{2}, Asn^{15}, Asn^{95}, and Asn^{109}. Alignment of the MC4R genes also indicated the presence of the characterized PMY motif in the first intracellular loop, a lack of Pro in TM5 (Ringholm et al., 2002; Cerdá-Reverter et al., 2005), and short extracellular and intracellular loops. In general, the regions corresponding to the seven transmembrane helices were more conserved relative to the N- and C-terminals, which showed more variability. Concerning the DPXXY motif present at the end of the 7TM region of MC4R, most of the species compared in Fig. 3 exhibited a DPLIY motif, although a DPIIY motif was present in the MC4R of olive flounder, barfin flounder, fugu, and Japanese pufferfish. Teleost fishes appeared about 150 MYA in the late Jurassic period and diverged from the lineage leading to mammals about 400-450 MYA (Schiöth, 2006). Differences in the melanocortins and their receptors have been used to trace the detailed evolutionary relationships amongst the teleosts. A close evolutionary relationship among the teleost species is reflected in the phylogenetic tree (Fig. 3) constructed by comparing MCR sequences using the neighbor-joining method. Moreover, olive flounder MC4R contains fifteen cysteine residues in all ORF regions, corresponding to the same number and residue locations in the MC4Rs of barfin flounder and fugu (Kobayashi et al., 2007). Human MC4R has a similar pattern composed of 15 cysteine residues at similar locations, except for Cys^{172} in human MC4R (residue 172 of the MC4R of barfin flounder, fugu, and olive flounder is serine), which corresponds to Cys^{175} in the fish species. It is possible that these cysteine residues play an important role in the functioning of MC4Rs, similar to the crucial role played by a disulphide bridge between the EC loops of MC5R in its pharmacology (Schiöth, 2001).

Tissue-specific expression of the MC4R gene in olive flounder was examined using reverse transcription PCR, as shown in Fig. 4. As an internal positive control in the RT-PCR experiments, primers corresponding to the β-actin gene were included (Fig. 4B). Expression of the MC4R gene was detected in the liver, eye, and brain of olive flounder. Interestingly, expression of the MC4R gene was reported only in the liver from barfin flounder, in which a higher level of expression was observed in fasting fish than in fed fish (Kobayashi et al., 2007). The results indicate that olive flounder and barfin flounder, both of which belong to the order Pleuronectiformes, showed abundant expression in the liver. It is possible that reducing the feeding caused an increase in the expression of the MC4R in the liver because the MC system is associated with liver functions (Yada et al.,

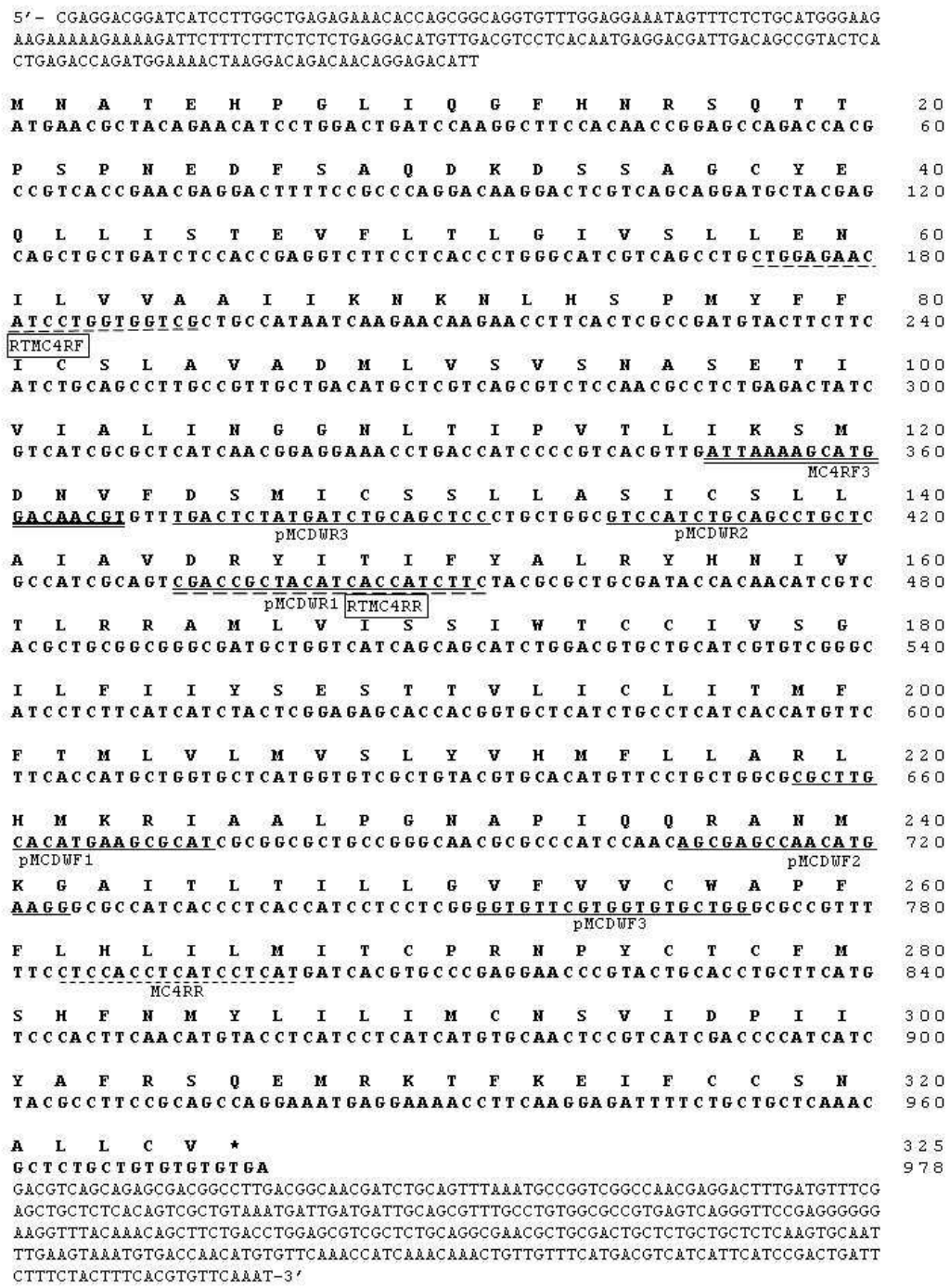

Fig. 1. Nucleotide and predicted amino acid sequences of the MC4R gene isolated from olive flounder, *Paralichthys olivaceus*, together with 5'- and 3'- flanking sequences (GenBank Accession number HQ230046). The complete open reading frame sequence of MC4R was indicated as bold letters together with the stop codon as indicated by an asterisk (*).

2000; Kobayashi et al., 2007). Other tissues showing expression of the MC4R gene included the eye and brain, but little expression occurred in the intestine, spleen, kidney, heart, gut, or gill. While most mammalian MC4R expression was detected only in the brain (Adan et al., 2006), others reported expression of MC4R in the peripheral tissues of rainbow trout (Haitina et al., 2004) and in the chicken

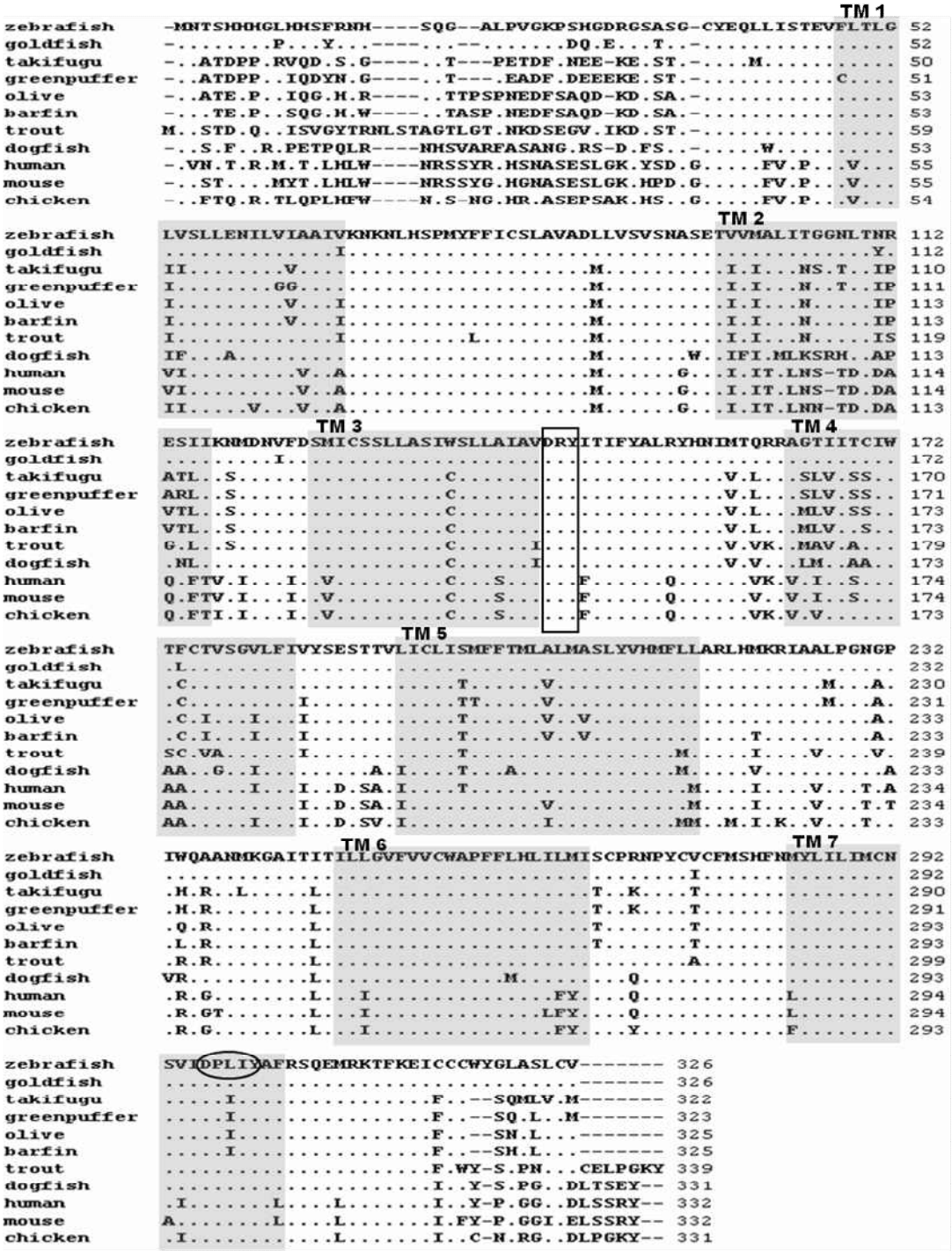

Fig. 2. Alignment of MC4Rs from olive flounder together with zebrafish, goldfish, fugufish, greenpufferfish, barfin flounder, rainbow trout, spiny dogfish, human, mouse, chicken. Conserved sequences indicate as dots. Regions corresponding to the seven transmembranes are indicated by shadow boxes. DRY motif is indicated as square box and DPXXY motif is indicated as oval shape.

(Ringholm et al., 2002; Takeuchi et al., 1998; Teshigawara et al., 2001). However, the putative MC4R of lamprey, an extant jawless fish, was expressed in the skin, liver, heart and skeletal muscle, but not in the brain (Haitina et al., 2007). Thus, the expression pattern of the MC4R gene in fish may suggest

Table 2. Percentage identity of the MC4R amino acid sequences among different species. hMC: human MC4R, mMC: mouse MC4R,. cMC: chicken MC4R, dMC: spiny dogfish MC4R, zMC: zebrafish MC4R, tMC: rainbow trout MC4R, fMC: fugu MC4R, pMC: pufferfish MC4R, gMC: goldfish MC4R, oMC: olive flounder MC4R, bMC4: barfin flounder MC4R

	hMC	mMC	cMC	dMC	zMC	tMC	fMC	pMC	gMC	oMC	bMC
hMC	100	93	87	70	69	68	66	65	70	67	68
mMC		100	85	67	70	69	65	66	69	68	67
cMC			100	67	68	71	65	65	67	68	68
dMC				100	77	74	73	72	73	74	73
zMC					100	78	76	78	96	80	80
tMC						100	78	79	78	82	80
fMC							100	94	76	89	88
pMC								100	77	89	88
gMC									100	78	79
oMC										100	96
bMC											100

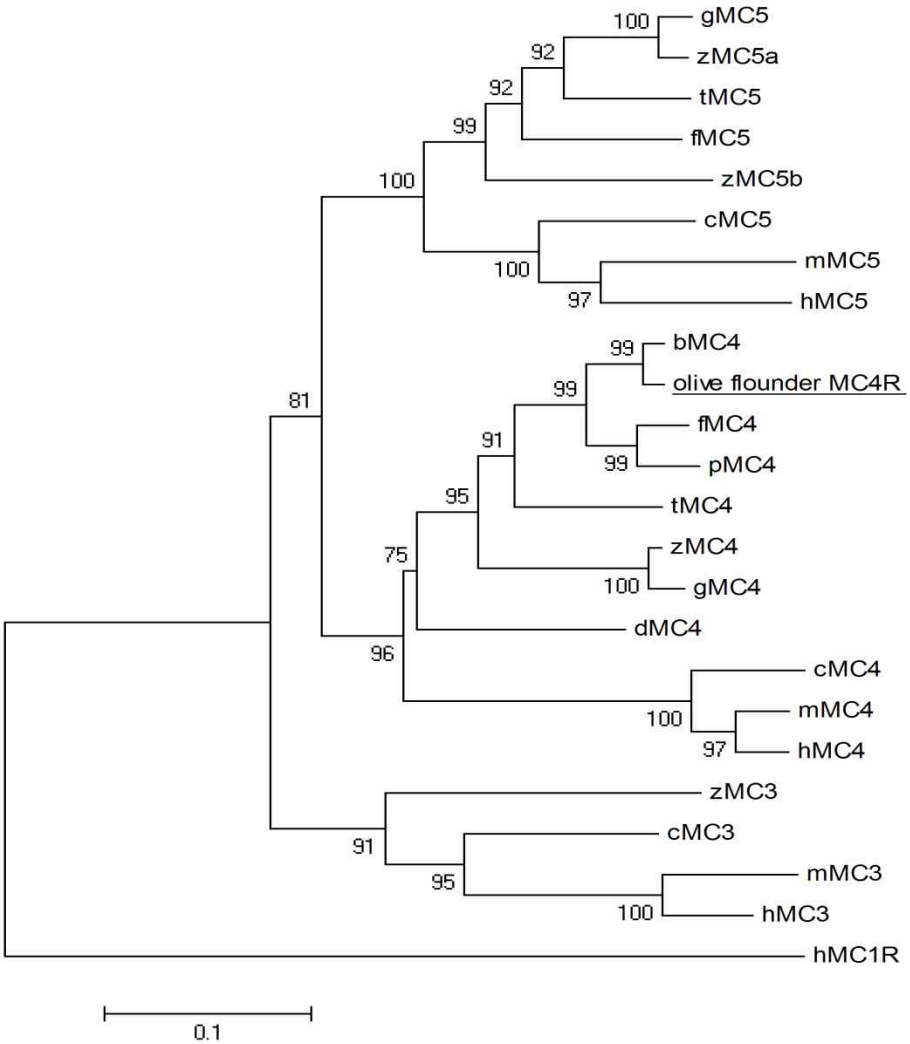

Fig. 3. Phylogenetic tree for the MC receptors using comparison of the full-length amino acid sequences. hMC: human MCR, mMC: mouse MCR, cMC: chicken MCR, fMC: fugu MCR, pMC: pufferfish MCR, zMC: zebrafish MCR, gMC: goldfish MCR, tMC: rainbow trout MCR, dMC: spiny dogfish MCR, bMC4: barfin flounder MC4R.

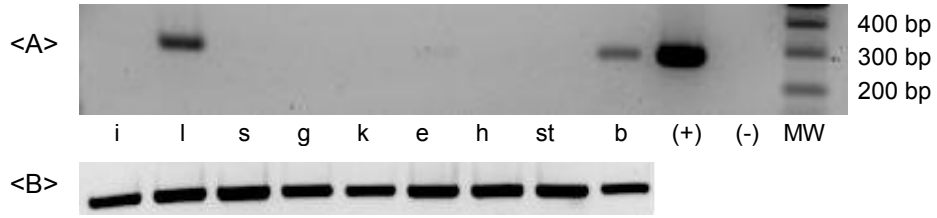

Fig. 4. Analysis of MC4R <A> and β-actin <B> gene expression in various tissues of olive flounder (*Paralichthys olivaceus*) by using RT-PCR as described in Methods. RNAs were isolated from various tissues as follows : i; intestine, l; liver, s; spleen, g; gill, k; kidney, e; eye, h; heart, st; stomach, b; brain, (+); positive control, olive flounder genomic DNA, (-); negative control, water. MW; DNA 100 bp ladder.

a broader role for the receptor during early vertebrate evolution. It seems that the specific role of MC4R in the central nervous system has undergone evolutional development. This also suggests an evolutionary cleavage in the central function of MCRs as the agnathans and gnathostomes diverged.

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