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Molecular Cloning and Characterization of the Rod Opsin Gene in Olive Flounder *Paralichthys olivaceus*

Jong-Myoung Kim*, Sung-Wan Kim and Sung Koo Kim[1]

Departments of Aquaculture, and [1]Biotechnology and Bioengineering, Pukyong National University, DaeYeon-3-Dong, NamGu, Busan 608-737, Korea

Rhodopsin, a dim-light receptor, is a model system for the study of G protein-coupled receptors that transduce extracellular signals into cells. To study the molecular mechanisms of visual systems in fish, the rod opsin gene of olive flounder *Paralichthys olivaceus* was characterized. The full-length *P. olivaceus* opsin gene was obtained by PCR amplification of genomic DNA, as well as cDNA synthesis. A comparison of clones obtained from both methods indicated that the olive flounder rod opsin gene lacks introns. Sequence analysis of the opsin gene indicated that it contains a 1,056-bp open reading frame encoding 352 amino acids. The deduced amino acid sequence contains features of typical rod opsins, such as sites for Schiff's base formation (K296) and its counterion (E113), disulfide formation (C110 and C187), and palmitoylation (C322 and C323). An opsin sequence alignment showed the highest similarity between *P. olivaceus* and *Solea solea* (95.1%), followed by *Hippoglossus hippoglossus* (94.5%). An opsin phylogenetic tree revealed a close relationship between olive flounder and teleost rod opsins.

Key words: *Paralichthys olivaceus*, Olive flounder, Rod opsin, G protein-coupled receptor (GPCR)

Introduction

G protein-coupled receptors (GPCRs) are among the largest membrane receptor families and play an important role in transmitting extracellular signals into the cell interior (reviewed by Khorana et al., 2002). Upon stimulation by extracellular signals such as hormones, neurotransmitters, or physiological (olfactory, gustatory, and visual) stimuli, conformational changes in GPCRs initiate a signal transduction cascade by activating heterotrimeric GTP/GDP-binding proteins. This in turn activates or inhibits secondary effectors, leading to physiological responses. While GPCRs recognize a diverse array of signals, all GPCRs share a common structural topology of seven transmembrane helices. This, together with some conserved sequences, suggests that there may be a common activation mechanism in GPCRs (Kim et al., 2005), possibly by a similar helical movement upon agonist activation.

Most vertebrates possess two types of photoreceptor cells, i.e., rod cells for scotopic vision and cone cells for photopic vision (Khorana, 2000). Rhodopsin, a dim-light photoreceptor, consists of a polypeptide, opsin, and retinal chromophore. Upon light activation, isomerization of 11-*cis*-retinal into *all-trans*-retinal induces structural changes in the transmembrane domain. This leads to conformational changes in the cytoplasmic loops, initiating interaction with G proteins and rhodopsin kinase. Rhodopsin is a model system for the study of GPCRs mainly due to its expression level, which is much higher than that of most other GPCRs, and its accessibility for biophysical assays.

Fish live in aquatic environments with varied light intensity. Their visual systems are adapted to different photic environments and differ in visual receptor cell content or absorption maxima of photoreceptors. Many teleosts have two types of photoreceptor cells, while cephalopods contain a single type of rhodopsin. Some fishes, such as American and European eels, can change the expression pattern of their rhodopsins during migration from a river to the deep sea to adjust to the new environment (Archer et al., 1995; Hope et al., 1998). Rhodopsin chromophores with different absorption maxima are tuned by a change in the primary amino acid structure of the opsin proteins

*Corresponding author: jongkim@pknu.ac.kr

(Yokoyama, 1995). Various amino acid changes resulting in rhodopsin absorption maxima shifts have been examined (Nakayama and Khorana, 1991; Yokoyama and Radlwimmer, 1998). These characters make fish a good model system for examining the molecular basis of visual photoreceptors.

The olive flounder *Paralichthys olivaceus* belongs to the family Paralichthyidae in the order Pleuronectiformes. This marine flat fish is cultured in coastal areas of Korea key, Japan, and China and is the most commonly cultured marine fish in Korea (http://fs.fips.go.kr/index3_1.jsp). The olive flounder lives in benthic environments after spending the fry stage in the pelagic zone. It is interesting to study the visual systems of vertically migrating fish as they adapt to changes in the light environment. Here we isolated and characterized the rod opsin gene of the olive flounder to characterize its visual photoreceptors and the evolutionary relationships between fish species.

Materials and Methods

Materials

Restriction endonucleases, T4 DNA ligase, and calf intestine endonucleases were obtained from New England Biolabs (Beverly, MA). TriReagent™ was obtained from Sigma (Saint Louis, MO). A PolyATtract RNA isolation system, reverse transcriptase, and a cDNA synthesis kit were purchased from Promega (Madison, WI). Oligonucleotides used for the amplification of the opsin gene were obtained from Genotech (DaeJeon, Korea). An AccuPrep Genomic DNA extraction kit was purchased from Bioneer Corporation (DaeJeon, Korea). Kits used for plasmid isolation, gel extraction, and PCR purification were purchased from Nucleogen (Seoul, Korea). DNA walking *Speed UP™* Premix Kit™ and pMD18-T vector were obtained from Seegene (Seoul, Korea) and Takara (Shiga, Japan), respectively. Olive flounder tissue was provided by Dr. Gang-Woong Kim at the National Fisheries Research and Development Institute (NFRDI), Korea.

Cloning of the opsin gene

Genomic DNA was isolated from the blood of olive flounder using the AccuPrep Genomic DNA Extraction Kit according to the manufacturer's instructions. Oligonucleotides (F1: 5'-GCAAGAATTC ATGAACGGCACAGAGGGACC, R1: 5'-ATTTGC GGCCGCTT ATGCAGGGGACACAGAG) used for PCR amplification of the opsin gene were deduced from the conserved regions of zebrafish, Japanese medaka, and Atlantic salmon opsin genes (Philp et al., 2000). In addition, oligonucleotides F1 and R1 contained *Eco*RI and *Not*I recognition sequences, respectively, at their 5' ends to facilitate cloning into the corresponding pMT4 site (Oprian et al., 1987). PCR was conducted under the following conditions: denaturation at 95°C for 5 min, 30 cycles of amplification including denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 3 min. Amplified DNA was digested with *Eco*RI and *Not*I as recommended by the enzyme supplier for cloning into pMT4 (Oprian et al., 1987).

DNA walking was used to obtain DNA fragments near the 5'- and 3'-flanking regions of the opsin gene. Three oligonucleotides corresponding to the antisense strand (TSPF1: GAGGGCATGCAGTGCTC, TSPF2: TGTGCTGTCAAGGAGGCTGC, TSPF3: GTGTG GCCTGGTATATCTTC) near the 5' end and the three oligonucleotides corresponding to the sense strand (TSPR1: GAGCACTGCATGCCCTC, TSPR2: ACCGCAAGGTTCAGAAGGATG, TSPR3: GAAA CATATAGGCACCCAGG) near the 3' end of the gene were designed. PCR was conducted with these primers, together with the universal primers in the DNA walking *Speed UP™* Premix Kit, to amplify the flanking sequences according to the manufacturer's instructions. Upon agarose gel electrophoresis followed by purification, amplified DNA was cloned into the pMD18-T vector.

RNA isolation and cDNA synthesis

Total RNA was extracted from the eye of olive flounder using TriReagent according to the manufacturer's protocol. Isolation of mRNA was carried out using PolyAT Tract mRNA isolation systems according to the manufacturer's instructions. Reverse transcription of mRNA was carried out with the ImProm-II Reverse Transcription System, using oligo (dT) as a primer, according to the manufacturer's protocol. RT-PCR was conducted with F1 and R1 to detect the opsin transcript in the cDNA as described above. RACE-PCR was also used to obtain the DNA sequence at the 3'-terminus of the gene.

DNA fragments amplified by PCR or DNA walking were cloned either directly into pMD18-T vector or into pMT4 after digestion with *Eco*RI or *Not*I. Ligated DNA was transformed into *E. coli* DH5α as described by Inoue et al., 1990). Plasmids were isolated by the alkaline-SDS lysis method as described by Sambrook and Russell (2001).

Sequencing and phylogenetic analysis

Plasmids were subjected to DNA sequence analysis using the M13F or M13R primers for the constructs

cloned into pUC19 and pMD-18T plasmids. Constructs cloned into pMT4 were sequenced using primers specific to the vector. The sequence of the selected clones was analyzed in both directions to avoid errors.

The identified sequence was subjected to homology analysis with sequences from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). ClustalW (Thompson et al., 1994) was used to align the sequences. Phylogenetic analysis was performed with Mega 3.1 (Molecular Evolutionary Genetics Analysis) program (http://www.megasoftware.net/mega.html) and the neighbor-joining method. An opsin phylogenetic tree was constructed with 1000 bootstrap replicates.

Results and Discussion

Most vertebrates possess two types of photoreceptor cells, i.e., rod cells for scotopic vision and cone cells for photopic vision. Fish live in environments with varied light intensity, thus they possess photoreceptor cells adapted to their habitats. In addition, fish migrating from the pelagic zone to a benthic habitat, or vice versa, during ontogeny can change their visual receptors in different developmental stages (Helvik et al., 2001). Therefore, fish are a good model system for studying visual systems adapted to specific light environments.

Rhodopsin is a model system for studying GPCRs and visual signal transduction. To study the molecular characteristics of rhodopsin in fish, we isolated the rod opsin gene from olive flounder, a major cultured marine fish in Korea. Amplification of the rod opsin gene was carried out using PCR. High molecular weight genomic DNA was isolated from the blood of olive flounder (data not shown). Oligonucleotides F1 and R1 were designed to contain conserved sequences corresponding to the 5' and 3' ends, respectively, of the opsin genes of fishes including Atlantic salmon, zebrafish, Japanese medaka, and *Plecoglossus*. In addition, primers F1 and R1 contain *Eco*RI and *Not*I recognition sequences, respectively, at their 5' ends to facilitate cloning into pMT4 (Oprian et al., 1987), which has been used for the expression of bovine opsin and other GPCRs. PCR was conducted with the annealing temperature ranging from 40°C to 55°C to find the optimum condition for amplification, as their primers contain degenerated sequences. DNA fragments of approximately 1 kb were detected when the annealing temperature was 42°C (data not shown). This fragment was cloned either directly into the pMD18-T vector or into a 5.3-kb *Eco*RI/*Not*I fragment of pMT4 after digestion with the enzymes. DNA sequence analysis of the fragments (Fig. 1) shows that olive flounder opsin has the highest similarity to the *Hippoglossus hippoglossus* rod opsin. In order to confirm whether the opsin gene contains introns, the opsin gene was also obtained from cDNA synthesis. For this, total RNA was isolated from the retina of olive flounder using TriReagent. The integrity of the isolated RNA was confirmed by RNA gel electrophoresis (data not shown). Isolation of mRNA was carried out using a biotinylated oligo (dT) probe followed by streptavidin-coupled paramagnetic particles. Single-stranded cDNA was synthesized from the retinal mRNA using the oligo (dT) primer and reverse transcriptase. To detect whether the opsin transcript was present in mRNA, PCR was conducted using the single-stranded cDNA as a template and opsin gene-specific primers F1 and R1 as described above. Detection of a 1-kb DNA fragment (Fig. 2) suggests that the olive flounder opsin gene lacks introns, as do those of other teleosts.

DNA walking was carried out to confirm the DNA sequences of the flanking regions that overlapped with primers F1 and R1 and to identify the flanking sequences. Six oligonucleotides corresponding to the regions near the 5' or 3' end were designed, as described in the Materials and Methods. These, together with universal primers, were used to amplify DNA fragments around the 5' and 3' ends of the gene. DNA fragments of up to 1.5 kb were obtained depending on the universal primers used (data not shown). Based upon DNA sequence analysis of the fragments, we compiled the complete coding sequence of the rod opsin gene (Fig. 1). It consists of a 1,056-bp structural gene encoding 352 amino acids, together with the flanking sequences including putative poly(A) adenylation sites (AATAAA) located 319 and 414 nucleotides downstream from the stop codon (Fig. 1). The predicted open reading frame showed a high degree of homology to rod opsins previously identified. This indicates that the isolated olive flounder gene belongs to the rod opsin group.

A hydrophobicity plot of the deduced amino acid sequence generated using the Kyte-Doolittle method indicated the presence of seven putative transmembrane helices (data not shown) around the regions corresponding to the transmembrane regions of bovine rhodopsin (Fig. 1). Analysis of the amino acid sequence showed that the functionally important rhodopsin residues are conserved. The olive flounder sequence contains a lysine (K296) residue in the putative seventh transmembrane domain that attaches to the chromophore by Schiff's base linkage (Wang et

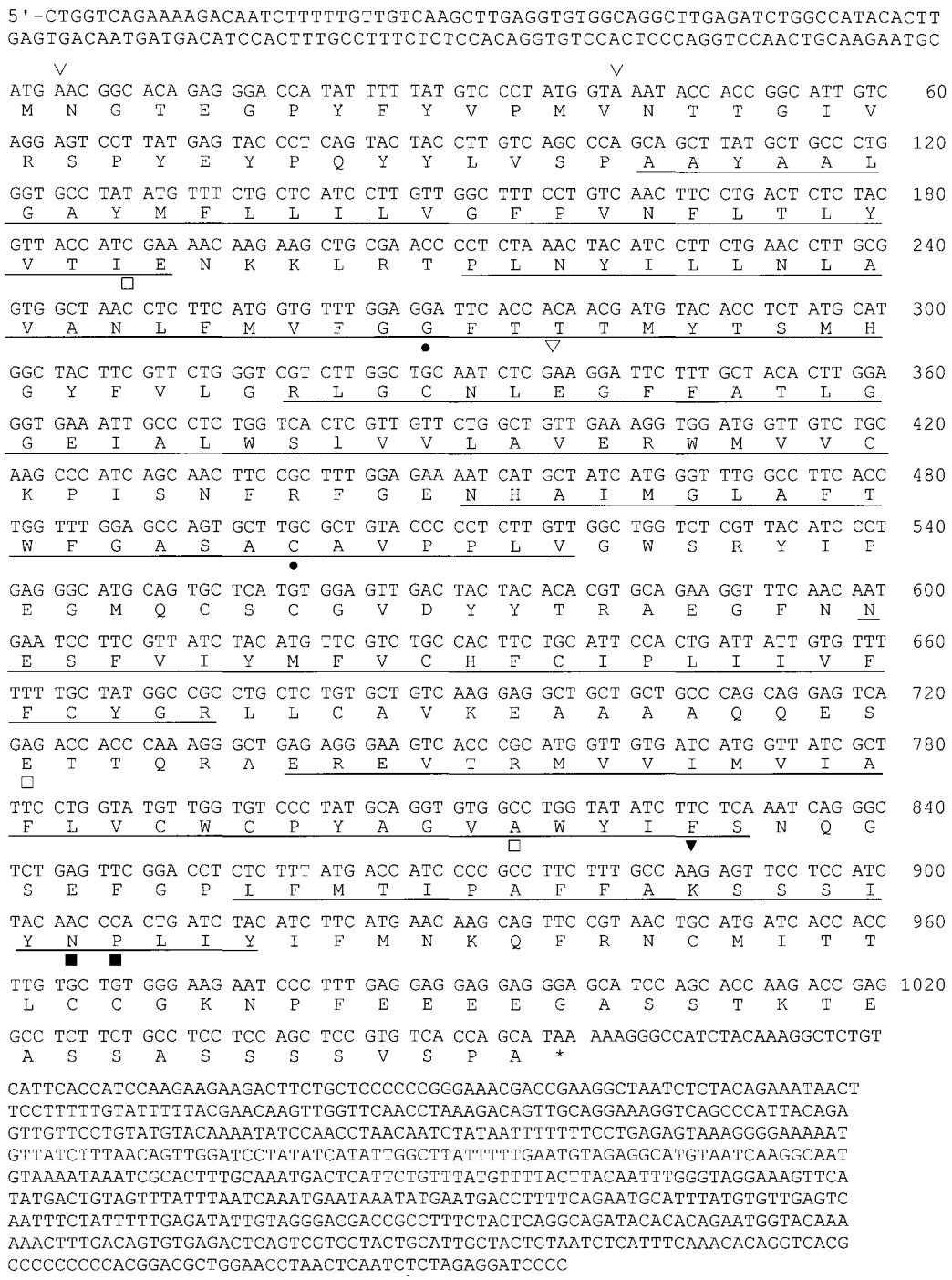

Fig. 1. The rod opsin gene of olive flounder *Paralichthys olivaceus*. The complete sequence of the opsin gene open reading frame, including the 5'-end regulatory sequence and the deduced amino acid sequence, is shown. The stop codon is indicated by an asterisk (*), and the seven transmembrane domains are underlined. Amino acid residues involved in Schiff's base formation and its counterion (▼K296 and ▽E113), disulfide bond formation (● C110, C187) glycosylation (N2, N15), and palmitoylation (■C322, C323) are indicated. Amino acids implicated in the spectral tuning of rhodopsin are indicated by squares (□N83, F261, A292).

al., 1980) and a counterion glutamate residue at position 113 in the predicted transmembrane domain III (Sakmar et al., 1989). Two cysteine residues found at positions 110 and 187 may form a disulfide bridge

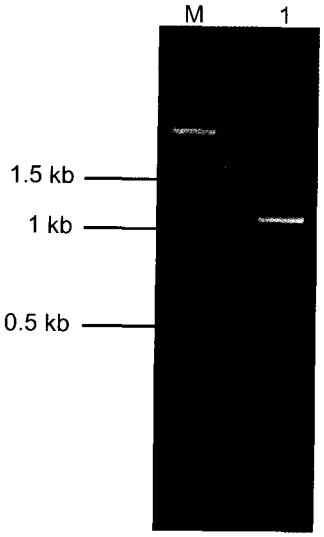

Fig. 2. RT-PCR of rod opsin cDNA using mRNA isolated from olive flounder retina. PCR products (lane 1) together with a molecular weight marker (M) were subjected to 1% agarose gel electrophoresis followed by staining with ethidium bromide. The sizes of the molecular weight markers are indicated on the left.

Table 1. Percent identity between rod opsins isolated from various fish species

Species	*Paralichthys olivaceus*	
	Accession number	Rod opsin (%)
Solea solea	Q9YGZ5	95.1
Hippoglossus hippoglossus	AAM17918	94.5
Liza aurata	Q9YGZ6	93.5
Mugil cephalus	Q9YGZ9	93.3
Mullus surmuletus	Q9YH01	93.3
Oryzias latipes	BAD99136	92.9
Pseudopleuronectes americanus	AAT72123	92.6
Takifugu rubripes	AF201474	92.4
Tilapia rendalli	BAC02626	86.2
Danio rerio	AAD14679	83.8
Cyprinus carpio	CAA96518	82.9

that is critical for the conformation of functional opsin protein (Karnik et al., 1988). It also contains asparagines at positions 2 and 15 where glycosylation is important for the targeting and folding of rhodopsin (Kaushal et al., 1994). Potential phosphorylation sites (Ohguro et al., 1994) such as Ser and Thr, required for desensitization, and two palmitoylations sites at positions 322 and 323, believed to be important in anchoring rhodopsin in the cell membrane by palmitic acid esterification (Ovchinnikov et al., 1988), were found in the C-terminus. In addition, glutamate

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halibutrod      MNGTEGPYFYVPMVNTTGIVRSPYDYPQYYLVNPAAYAALGAYMFLLILVGFPVNFLTLY 60
flounderrod     MNGTEGPYFYVPMVNTTGIVRSPYEYPQYYLVSPAAYAALGAYMFLLILVGFPVNFLTLY 60
nilerod         MNGTEGPFFYVPMVNTTGIVRSPYEYPQHYLVSPAAYAALGAYMFFLILAGFPINFLTLY 60
torafugurod     MNGTEGPFFYVPMVNTTGVVRSPYDYPQYYLVNPAAYAALGAYMFLLILLGFPINFLTLY 60
zebrafishrod    MNGTEGPAFYVPMSNATGVVRSPYEYPQYYLVAPWAYGFVAAYMFFLIITGFPVNFLTLY 60
humanrod        MNGTEGPNFYVPFSNATGVVRSPFEYPQYYLAEPWQFSMLAAYMFLLIVLGFPINFLTLY 60
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halibutrod      VTLEHKKLRTPLNYILLNLAVANLFMVFGGFTTTMYTSMHGYFVLGRLGCNLEGFFATLG 120
flounderrod     VTIENKKLRTPLNYILLNLAVANLFMVFGGFTTTMYTSMHGYFVLGRLGCNLVGFFATLG 120
nilerod         VTIEHKKLRTPLNYILLNLAVADLFMVFGGFTTTMYTSMHGYFVLGRLGCNLEGFFATLG 120
torafugurod     VTIEHKKLRTPLNYILLNLAVADLFMVFGGFTTTMYTSMHGYFVLGRLGCNLEGFFATLG 120
zebrafishrod    VTIEHKKLRTPLNYILLNLAIADLFMVFGGFTTTMYTSLHGYFVFGRLGCNLEGFFATLG 120
humanrod        VTVQHKKLRTPLNYILLNLAVADLFMVLGGFTSTLYTSLHGYFVFGPTGCNLEGFFATLG 120
                **:::***************:*:****:****:*:***:*****:*  **** *******

halibutrod      GEIGLWSLVVLAVERWMVVCKPISNFRFGENHAIMGLGWTWIGASACAVPPLVGWSRYIP 180
flounderrod     GEIALWSLVVLAVERWMVVCKPISNFRFGENHAIMGLAFTWFGASACAVPPLVGWSRYIP 180
nilerod         GEIGLWSLVVLAVERWLVVCKPISNFRFGENHAIMGLAFTWLAALSCAGPPLVGWSRYIP 180
torafugurod     GEIGLWSLVVLAIERWVVVCKPISNFRFGENHAIMGLAFTWIMASACAVPPLVGWSRYIP 180
zebrafishrod    GEMGLKSLVVLAIERWMVVCKPVSNFRFGENHAIMGVAFTWVMACSCAVPPLVGWSRYIP 180
humanrod        GEIALWSLVVLAIERYVVVCKPMSNFRFGENHAIMGVAFTWVMALACAAPPLAGWSRYIP 180
                **:.* ******:**::*****:**************:.:**. * :** ***.*******

halibutrod      EGMQCSCGVDYYTRAEGFNNESFVIYMFVCHFLIPLTIVFFCYGRLLCAVKEAAAAQQES 240
flounderrod     EGMQCSCGVDYYTRAEGFNNESFVIYMFVCHFCIPLIIVFFCYGRLLCAVKEAAAAQQES 240
nilerod         EGMQCSCGVDYYTRAEGFNNESFVIYMFICHFLIPLTVVFFCYGRLLCAVKEAAAAQQES 240
torafugurod     EGMQCSCGVDYYTRAEGFNNESFVIYMFVCHFLIPLIIIFFCYGRLLCAVKEAAAAQQES 240
zebrafishrod    EGMQCSCGVDYYTRTPGVNNESFVIYMFIVHFFIPLIVIFFCYGRLVCTVKEAARQQQES 240
humanrod        EGLQCSCGIDYYTLKPEVNNESFVIYMFVVHFTIPMIIIFFCYGQLVFTVKEAAAQQQES 240
                **:*****:****    .**********: ** **: ::*****:*: :*****  ****
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Fig. 3. Alignment of rod opsins from olive flounder (flounderrod), Atlantic halibut (halibutrod), nile tilapia (nilerod), torafugu (torafugurod), zebrafish (zebrafishrod), and human (humanrod). Asterisks (*) indicate identical amino acids across species.

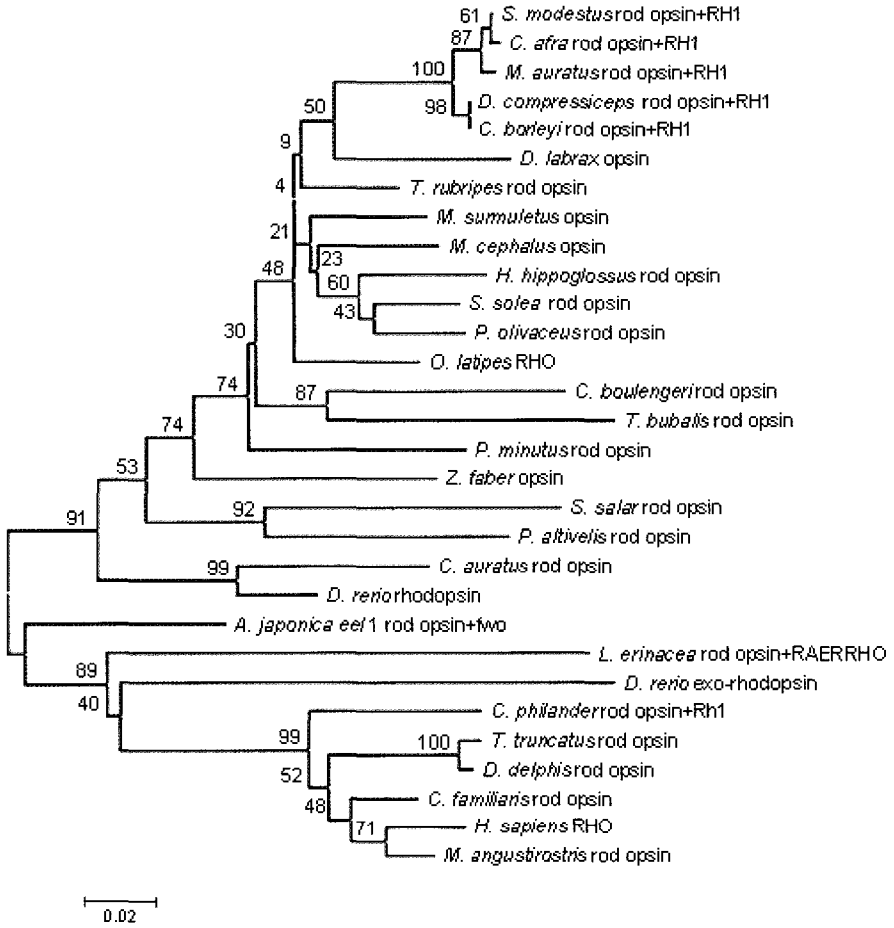

Fig. 4. Phylogenetic analysis of various rhodopsins using Mega 3.1 and the neighbor-joining method. The phylogenetic trees were constructed with 1000 bootstrap replicates. Scale bars indicate genetic distance. *Carassius auratus* (goldfish, GenBank accession number AAA49191), *Mugil cephalus* (flathead mullet, CAA77250), *Mullus surmuletus* (striped red mullet, CAA77248), *Salmo salar* (Atlantic salmon, AAF44620), *Leucoraja erinacea* (little skate, AAC60251), *Pomatoschistus minutus* (sand goby, CAA44275), *Solea solea* (common sole, CAA77254), *Zeus faber* (John Dory, CAA74832), *Danio rerio* (zebrafish, AAD24751), *Danio rerio* (zebrafish, BAA88958), *Homo sapiens* (human, P08100), *Oryzias latipes* (Japanese medaka, P87369), *Takifugu rubripes* (Fugu rubripes, AAF44621), *Dicentrarchus labrax* (European sea bass, CAA77255), *Dimidiochromis compressiceps* (Malawi eye-biter, AAY26022), *Copadichromis borleyi* (red fin, AAY26036), *Canis familiaris* (dog, CAA50502), *Caluromys philander* (bare-tailed woolly opossum, AAQ82903), *Tursiops truncatus* (bottlenosed dolphin, AAC12940), *Mirounga angustirostris* (northern elephant seal, AAP13021), *Plecoglossus altivelis* (ayu, BAC00857), *Anguilla japonica* (Japanese eel, CAB56646), *Delphinus delphis* (common dolphin, AAC12761), *Cottinella boulengeri* (short-headed sculpin, AAB61727), *Taurulus bubalis* (long-spined bullhead, AAB61729), *Stigmatochromis modestus* (AAY26037), *Melanochromis auratus* (golden cichlid, AAY26030), *Cynotilapia afra* (dogtooth cichlid, AAY26033), *Hippoglossus hippoglossus* (Atlantic halibut, AAM17918)

and arginine at positions 134 and 135, which are important for the activation of rhodopsin (Franke et al., 1990), are conserved, although there is a Trp residue at position 136 in place of Tyr.

Molecular cloning of opsins has been done in both freshwater (Minamoto and Shimizu, 2003; Matsumoto et al., 2006) and marine fishes (Bellingham et al., 1998; Hunt et al., 2001). The deduced amino acid sequence of the olive flounder rod opsin was compared to opsins isolated from various species (Fig. 3). Sequence alignment showed that most amino acids reflecting the opsin characteristics described above were identified. The amino acid identities between opsins were calculated from pairwise alignments (Table 1). Olive flounder rod opsin had 95.1, 94.5, 92.6, 92.4, and 83.8% amino acid identity with opsins

isolated from *Solea solea*, *H. hippoglossus*, *Pseudopleuronectes americanus*, *Takifugu rubripes*, and *Danio rerio,* respectively. Higher amino acid identity was found with opsins isolated from species of the order Pleuronectiformes (*S. solea* and *H. hippoglossus*), although the *P. americanus* opsin had a lower identity than *Mugil cephalus* and *Mullus surmuletus*, of the order Perciformes. Opsins isolated from Cypriniformes (*D. rerio* and *Cyprinus carpio*) showed the lowest identity with olive flounder opsin. A phylogenetic tree was constructed to examine the evolutionary relationships of fish opsins (Fig. 4). The rod opsins from various species of Pleuronectiformes were closely related. They were more distantly related to rod opsins from elasmobranches such as the skate, which has an exclusively rod retina (O'Brien et al., 1997), and octopus. In general, teleost rod opsins were clearly distinct from mammal opsins.

Opsin amino acid residues affect the absorption maximum of rhodopsin. Opsin sequences from various organisms and their absorption maxima were studied to determine the relationship between the amino acid sequence and λ_{max} of visual pigments (Nakayama and Khorana, 1991; Imai et al., 1997; Yokoyama and Radlwimmer, 1998). Three amino acid residues at positions 81, 261, and 292 have been implicated in the spectral tuning of rhodopsin. Most fishes living near the water surface have Asp, Tyr, Ala, or Asp, Phe, Ala at these three positions, respectively. In contrast, fishes living at depths of 400-5000 m have Asn, Ser, Ala at these positions. Olive flounder rod opsin has Asn83, Phe261, and Ala292 at the corresponding positions. This suggests that olive flounder is more adapted to the zone above 500 m, consistent with its benthic habitat.

Acknowledgments

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