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Cloning of Rod Opsin Genes Isolated from Olive Flounder *Paralichthys olivaceus*, Japanese Eel *Anguilla japonica*, and Common Carp *Cyprinus carpio*

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G Protein-coupled receptors (GPCRs) mediating wide ranges of physiological responses is one of the most attractive targets for drug development. Rhodopsin, a dim-light photoreceptor, has been extensively used as a model system for structural and functional study of GPCRs. Fish have rhodopsin finely-tuned to their habitats where the intensity and the wavelength of lights are changed depending on its water-depth. To study the detailed molecular characteristics of GPCR architecture and to understand the fishery light-sensing system, genes encoding rod opsins were isolated from fishes living under different photic environments. Full-length rod opsin genes were obtained by combination of PCR amplification and DNA walking strategy of genomic DNA isolated from olive flounder, *P. olivaceus*, Japanese eel, *A. japonica,* and Common carp *C. carpio*. Deduced amino acid sequences showed a typical feature of rod opsins including the sites for Schiff's base formation (Lys296) and its counter ion (Glu113), disulfide formation (Cys110 and Cys187), and palmitoylation (Cys322 and Cys323) although Cys322 is replaced by Phe in Japanese eel. Comparison of opsins by amino acid sequence alignment indicated the closest similarity between *P. olivaceus* and *H. hippoglossus* (94%), *A. japonica and A. anguilla* (98%), *and C. carpio* and *C. auratus* (95%), respectively.

Key words: Rhodopsin, Visual receptor, G protein-coupled receptor, GPCR, Spectral tuning

Introduction

G protein coupled receptors (GPCRs) transduce a wide variety of extracellular signals ranging from neurotransmitters, hormones, ions, to physiological stimuli including smell and light into inside the cells. Upon recognition of extracellular signals, conformational changes in GPCRs initiate a series of signal transduction cascades through the activation of GTP/GDP-binding proteins. While GPCRs recognize signals of diverse chemical structures, all GPCRs have a common structural topology, seven transmembrane helices. This, together with some conserved sequences, suggests GPCRs share a common activation mechanism as well as the determinants endowing specificity. It is important to obtain information about the detailed structural changes occurred in GPCRs to develop a structure-based drug modulating a specific GPCR signaling (Rosenbaum et al. 2009) although only a few crystal structures of GPCRs at the atomic level has been obtained so far (Palczewski et al. 2000 and Cherezov et al, 2007).

Visual signal transductions are mediated by two types of photoreceptor cells in which rod cell is responsible for scotopic vision and the cone cell is responsible for photopic vision. Rhodopsin, a dim-light photoreceptor composed of an apoprotein, opsin, and 11-*cis*-retinal, has been used as a prototype for the study of GPCRs partly due to its higher expression level and easier accessibility for preparation and bio-physical analysis (Khorana, 2000). Absorption of a photon by rhodopsin causes isomerization of 11-*cis*-retinal to all-*trans* form and induces a series of conformational changes in rhodopsin leading to visual signal transduction pathway through the activation of G protein (transducin). Different forms of rhodopsin chromophores are distinguished by UV/Vis spectral analysis of its absorption maximum. The level of rhodopsin chromophore depending on its light activations can also be used to define amino acid

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residues required to stabilize the structures of rhodopsin and its light-activated intermediates (Menon, 2001).

Fish live in the habitats with various salt concentrations changing from freshwater to sea water, temperatures changing from the freezing arctic zone to near hot spring, and light environments from the top of water directly exposed to the sunlight to the deep sea where almost no sunlight penetrated. Fish living under various photic environments possess photoreceptors with its absorption maximum adapted to the habitats (Lythgoe, 1979; Hunt et al., 2001). In addition, fish migrating from the pelagic zone to a benthic habitat or in the other way during ontogeny could change their visual receptors depending on their developmental stages (Helvik et al., 2001). American and European eels were known to change the pattern of their expression when the eel migrates from a river to the deep sea to adapt the new photic environments (Archer et al., 1995; Hope et al., 1998). Fishes examined in this study are olive flounder, Japanese eel, and Common carp that live under different environments and are among the most commercially important fish species in Korea. Olive flounder is one of the major mariculture species in Korea and lives in benthic environments after spending the fry stage in the pelagic zone. While Japanese eel spawn in the sea, small eels ascend the rivers in schools and grow in the freshwater. Common carp inhabited in freshwater with temperature ranges between 3 and 35°C exhibiting tolerance to a wide variety of conditions. It generally favors large water bodies with slow flowing or standing water and soft bottom sediments but could also thrive in large turbid rivers.

Rhodopsin is a good model system for studying structural and functional study of rhodopsin and GPCR as change in amino acid sequences was known to cause a shift of their absorption maxima (Yokoyama, 1995; Kim et al., 2007). Therefore, UV/Vis spectral analysis of rhodopsin provide information about how subtle differences in the primary sequence of opsin affect the tertiary structure of rhodopsin and GPCRs In this study, genes encoding rod opsins were isolated from fishes living under different environments to examine the characteristics of rhodopsin enabling to sensitize the light in their watery environments and maintaining its structural stability in the freezing to ambient water temperatures.

Materials and Methods

Materials

T4 DNA ligase, 1 kb ladder, and AccuPreP® Genomic DNA extraction kit were purchased from Bioneer (Daejeon, Korea). Wizard® Plus Maxipreps DNA Purification System and pGEM®-T Easy Vector system were purchased from Promega Corporation (Madison, WI, USA). Various restriction endonucleases were purchased from Bioneer (Daejeon, Korea) and New England Biolabs (Beverly, MA, USA). Plasmid Purification mini Kit, Gel Extraction Kit and PCR Purification Kit were purchased from NucleoGen (Seoul, Korea). DNA Walking SpeedUp™ PremixKit II was obtained from Seegene (Seoul, Korea). Oligonucleotides (Table 1) and 5× HiQ-PCR mix, and DNA sequencing analysis were obtained from Genotech (Daejeon, Korea). MyCycler™ Thermal Cycler used for PCR reaction was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Genomic DNA extraction and cloning of rod opsin gene

Genomic DNA was isolated from 100 μL blood by using AccuPrep® Genomic DNA Kit according to the manufacturer's instruction. Isolated DNA was confirmed by agarose gel electrophoresis followed by staining with ethidium bromide as described (Sambrook and Russell, 2001). Oligonucleotides F1 and R1 (Table 1) were designed to contain the conserved nucleotide sequences corresponding to the 5'- and 3'-termini of opsin genes of zebrafish, Japanese medaka, and Atlantic salmon (Philp et al., 2000). At the 5'-end of each primer, sequence corresponding to restriction endonucleases *EcoR*I and *Not*I recognition sites was also included to facilitate the cloning into expression vector, pMT4 (Oprian et al., 1987). PCR amplification was carried out with 50 μL reaction containing 0.3 μg of genomic DNA, 0.5 μM each of primers, and 10 μL of 5×HiQ-PCRmix. PCR reaction was carried out with an initial denaturation at 95°C for 3 min, together with 30 cycles of reactions comprised of denaturation at 94°C for 1 min, annealing either at 48°C (Common carp) or 50°C (olive flounder, Japanese eel) for 30 sec, and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. PCR products identified upon agarose gel electrophoresis (~1 kb) were purified by gel extraction and ligated into pGEM-TEasy vector according to the manufacturer's protocol. Recombinant DNA was transformed into *E.coli* DH5α competent cell prepared as described

Table 1. List of oligonucleotides and sequences used for the amplification of opsin genes

Primers	Sequence	Comment
F1	5'-GCAAGAATTCATGAACGGCACAGAGGGACC-3'	PCR
R1	5'-ATTTGCGGCCGCTTATGCTTATGCAGGGGACACAGAG-3'	
HCBOpF1	5'-CGAGAGGTGGATGGTTGTC-3'	
HCBOpF2	5'-C/TGAGACCACCCAGAGGGC-3'	
HCBOpF3	5'-TGCCAGCGTGGCCTGGTA-3'	
HCOpR1	5'-GACAACCATCCACCTCTCG-3'	
HCOpR2	5'-CGACGTGTACATCGTCGTGGTG-3'	
HCOpR3	5'-GGAAGAACATGTACGCGGCCAGG-3'	
OpsBgF1	5'-GAAGGCATGCAGTGTTC-3'	
OpsBgF2	5'-C/TGAGACCACCCAGAGGGC-3'	
OpsBgF3	5'-TGCCAGCGTGGCCTGGTA-3'	DNA
OpsBgR1	5'-GAACACTGCATGCCTTC-3'	Walking/PCR
OpsBgR2	5'-CGACGTGTACATCGTAGTGGTG-3'	
OpsBgR3	5'-GCGTGAACATGTAGGCAGCCAGG-3'	
OpsF1	5'-GAGGGCATGCAGTGCTC-3'	
TSPFCF2	5'-TGTGCTGTCAAGGAGGCTGC-3'	
TSPFCF3	5'-GTGTGGCCTGGTATATCTTC-3'	
OpsFCR1	5'-GAGCACTGCATGCCCTC-3'	
TSPFCR2	5'-ACCGCAAGGTTCAGAAGGATG-3'	
TSPFCR3	5'-GAAACATATAGGCACCCAGG-3'	

(Inoue et al., 1990). Plasmids were isolated in a small scale by alkaline lysis according to manufacturer's instructtions, confirmed by restriction endonuclease digestions followed by agarose gel electrophoresis, and then subject to DNA sequencing analysis.

Genomic DNA walking PCR was carried out to obtain the sequences around the regions corresponding to the primers used for PCR amplification. For this, template-specific primers were designed to contain sequences complimentary to the 5' -regions of the opsin genes obtained from Common carp (HCBOpF1, HCBOpF2, HCBOpF3), Japanese eel (OpsBgF1, OpsBgF2, OpsBgF3), and olive flounder (OpsF1, TSPFCF2, TSPFCF3). Other sets of oligonucleotides corresponding to the 3'-sense strands of opsin gene from Common carp (HCOpR1, HCOpR2, and HCOpR3), Japanese eel (OpsBgR1, BgOpR2, and BgOpR3), and olive flounder (OpsFCR1, TSPFCR2, and TSPFCR3) were also included (Table 1). DNA walking PCR was performed with universal primers supplied by DNA walking speedUP™ Premix Kit together with template-specific primers according to manufacturer's manual. PCR products obtained from the second PCR reactions were resolved upon agarose gel electrophoresis, purified by Gel Extraction Kit, and then cloned into the pGEM-T Easy Vector. Recombinant DNA was transformed into *E. coli* and then analyzed by restriction digestion and DNA sequencing analysis as described above.

Sequence alignment and phylogenetic analysis

Obtained DNA sequences were first compared with opsin genes in NCBI database using BlastN and BlastX followed by multiple amino acid sequence alignment by using ClustalW (Thompson et al., 1994). Accession numbers of opsin amino acid sequences searched from NCBI Gen Bank were of Zebrafish (*Danio rerio*, BC045288.1), Japanese medaka (*Oryzias latipes*, AB180742.1), goldfish (*Carassius auratus*, P32309), European eel (*Anguilla anguilla*, deep water form, Q90214, fresh water form, Q90215), Japanese eel (*Anguilla japonica*, deep sea form, AJ 249203), White spotted conger (*Conger myriaster*, fresh water form, BAB21487), Common sole (*Solea solea*, CAA77254), Flat head mullet (*Mugil cephalus*, CAA77250), Atlantic halibut (*Hippoglossus hippoglossus*, AAM17918), Nile tilapia (*Oreochromis niloticus*, AAY26023), *Labeotropheus fuelleborni* (AAY26028), *Paralabidochromis cyaneus* (AAV-93304), human (*Homo sapiens*, P08100), striped red mullet (*Mullus surmuletus*, CAA77248), torafugu (*Takifugu rubripes*, AAF44621), saddle back dolphin (*Delphinus delphis*, AAC12761), dog (*Canis lupus familiaris*, CAA50502), and bare-tailed woolly opossum (*Caluromys philander*, AAQ82903).

Phylogenetic tree was constructed by neighbor-joining method using MEGA v 4.0. Various opsins exhibiting higher homology with predicted rod opsins of Japanese eel, olive flounder, and Common carp were selected using the BlastP from NCBI. Assessing tree reliability was tested using a bootstrap with 1000 replicates.

Results and Discussion

Rhodopsin has been used as a prototype for the structural and functional analysis of GPCR mainly for its easier biophysical analysis using UV/Vis spectroscopy. Analysis of rhodopsin in fishes living under different water environments are of focus in this study to examine the molecular determinants critical for adopting the structural motif common to all GPCR as well as the one endowing the specificity. Genes encoding rod opsins were isolated from fishes living under different environments and accounting for major production in aquaculture and capture fishery in Korea.

In order to examine the fish-specific molecular characteristics of rod opsins, genomic DNA was isolated from the whole blood of olive flounder, Japanese eel, and Common carp. After confirming high molecular weight by agarose gel electrophoresis (data not shown), genomic DNA was used as a template for the PCR amplification of the opsin genes which was known to lack introns in bony fish (Fitzgibbon, 1995). Oligonucleotide primers F1 and R1 (Table 1) were designed from the conserved sequences corresponding to the 5'- and 3'- ends, respectively, of the opsin genes isolated from other fishes (Philp et al., 2000) as described above. Approximately 1 kb PCR products were obtained from PCR reactions by using annealing temperatures of 48°C (Common carp) and 50°C (olive flounder, Japanese eel, data not shown). Sequence analysis of the cloned genes by using BlastN and BlastX showed the highest similarity to rod opsins which had been previously reported (Helvik, 2001; Philp et al., 2000; Kim et al., 2007). DNA walking was carried out to acquire information about the flanking untranslated regions outside the 5'- and 3'-ends of the opsin gene and, in particular, to confirm the correct sequences corresponding to the primers used for PCR amplification. For this, target-specific primers were designed to contain sequences corresponding to the sense- or anti-sense sequences of the opsin genes obtained from above PCR (Table 1). Products of 250 bp and 800 bp (olive flounder), 460 bp and 300 bp (Japanese eel), and 800 bp and 850 bp (Common carp) corresponding to the 5'- and 3'-end of the opsin genes, respectively, were obtained from DNA walking PCR. Based upon DNA sequence analysis of fragments obtained from PCR and DNA walking, sequences containing the full-length opsin genes with its flanking sequences were compiled. Combined sequences should be reconfirmed by PCR with primers which land outside of the full ORF. The results showed that rod opsin genes consist of 1,056-bp structural genes encoding 352 amino acids in olive flounder (Fig. 1) and Japanese eel (Fig. 2) and 1,062 bp structural gene encoding 354 amino acids in Common carp (Fig. 3). Rod opsin gene isolated from Japanese eel turned out to be identical to the deep-sea form of rod opsin (Zhang et al. 2000). The predicted open reading frame showed a high amino acid sequence similarity to previously identified rod opsins indicating that the isolated genes actually belong to the opsin group.

Amino acid sequences of isolated opsins were also compared each other to analyze the characteristics of rod opsins in fish (Fig. 4). Amino acid sequences conserved include a lysine (K296) residue within the putative transmembrane domain VII that attaches to 11-*cis*-retinal by a Schiff's base linkage (Wang et al., 1980) and a counterion, glutamic acid (E113) in the predicted third transmembrane domain (Sakmar et al., 1989). Two cysteine residues were found in positions corresponding to Cys110 and Cys187 of bovine rhodopsin that may form a disulfide bridge critical for maintaining the conformation of functional opsin and GPCRs (Karnik et al., 1988). Two asparagine residues were found at positions 2 and 15 and believed to be the glycosylation sites important for targeting and folding of rhodopsin (Kaushal et al., 1994). Several serine and threonine residues were also found in the carboxyl terminal regions where the potential phosphorylation might occur (Ohguro et al., 1994). Amino acid residues known to be important for the activation of rhodopsin (Franke et al., 1990), *e.g.* Glu134 and Arg135 are also conserved while the Trp is substituted for Tyr at position 136 position in most of fishery rod opsins examined in this study. In addition, two cysteine residues, Cys322 and Cys323 which might be required for anchoring rhodopsin in the cell membrane by palmitic acid esterification (Ovchinnikov et al., 1988) were found in the C-terminus although one of the cysteines, Cys322, was replaced by Phe322 in opsin isolated from Japanese eel. Absence of a cysteine probably affecting the sensitization and desensitization processes of signal transduction was also found in other eel species including white spotted conger, European eels, and lamprey (Davies, 2007) but not in other migrating fish including Atlantic salmon, *Salmo salar* (Philp, 2000). This suggests that the absence of a cysteine may be a structural characteristic of rod opsin in *Anguillidae* but not in all of the anadromous fish.

Amino acid residues in opsin were known to affect the absorption maximum of rhodopsin. Analysis of

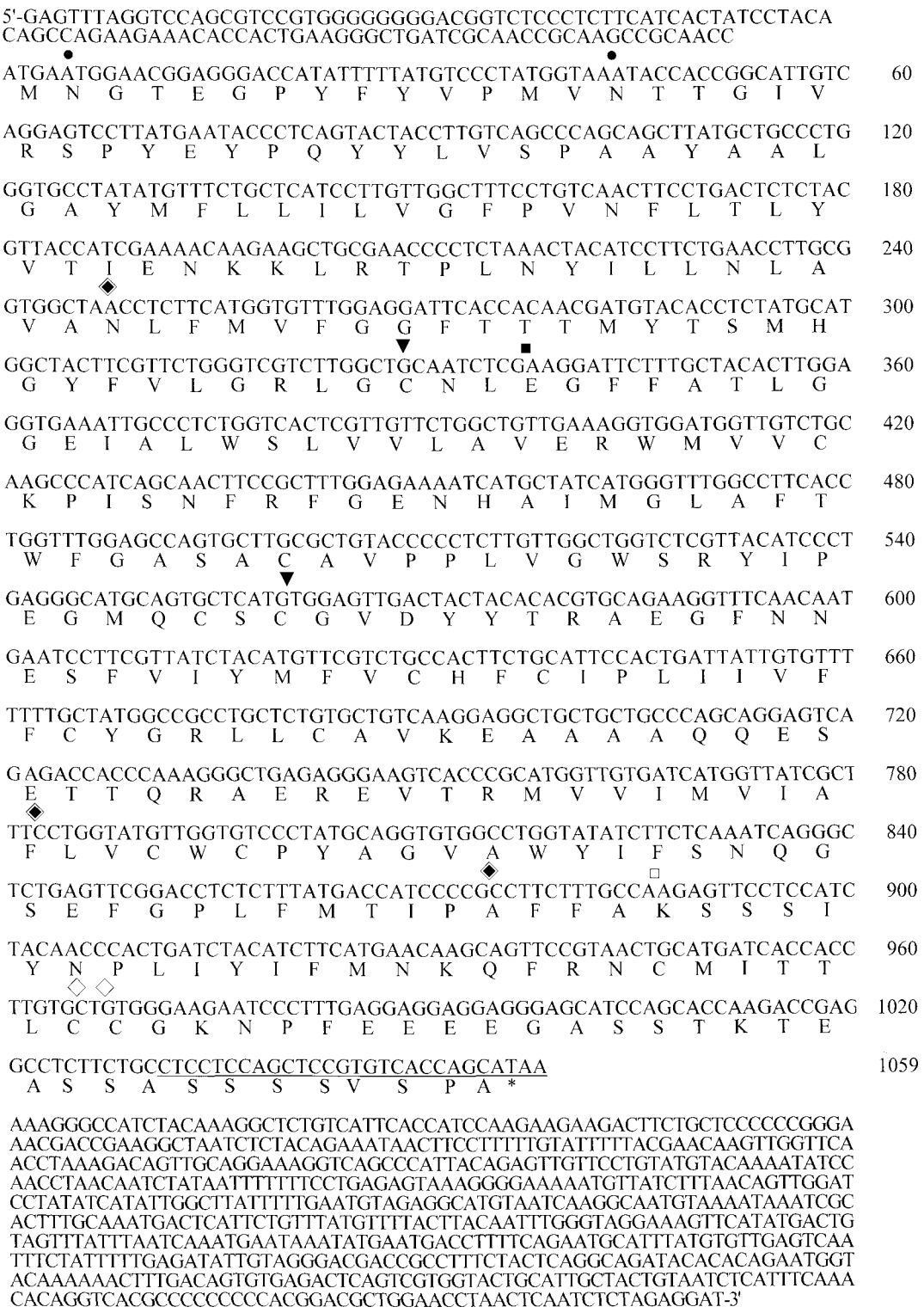

Fig. 1. Nucleotide and deduced amino acid sequence of the rod opsin gene isolated from olive flounder, *Paralichthys olivaceus*, together with 5'- and 3'- flanking sequences. Sites involved in Schiff's base formation and its counterion site (□K296 and ■E113), disulfide bond formation (▼C110, C187), glycosylation (●N2, N15) and palmitoylation (◇C322, C323) are indicated together with residues implicated in the spectral tuning of rhodopsin (◈N83, F261, A292). The stop codon is indicated by an asterisk (*). Sequences corresponding to the primers F1 and R1 are marked as underlines, respectively.

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5'-GAGTTTAGGTCCAGCGTCCGTGGGGGGGGTCGACCTGGTGCAGGATTACCTGGAACAGAGTG
AGAAAGAAAGAGAGAAAAACGGCCCAGAAAGCACTGGGGCTTCCTTATTATAGGGTTTACCCCCA
GGTGCTCTCAATTAGAAAGGGGTGCACCGCACACACTGGCCGACTCCTGTAACTACAATAAAAAT
AAAACAATATTAGAGACAGTCGTCACATCTGTTTCCAGTATCAGTGCATCACTCTGCTGTTTGAAG
TGTAAACACCTCACAGCTAGACGAGACAACACTTCTGAAGGACTGATCGAAAAACGCAGCC
      •                                                    •
ATGAACGGCACAGAGGGCCCTAATTTCTACATCCCTATGTCCAATATCACTGGAGTGGTG      60
 M  N  G  T  E  G  P  N  F  Y  I  P  M  S  N  I  T  G  V  V

AGGAGCCCCTTCGAATACCCACAGTACTACCTAGCCGAACCATGGGCCTACACGATCCTG     120
 R  S  P  F  E  Y  P  Q  Y  Y  L  A  E  P  W  A  Y  T  I  L

GCTGCCTACATGTTCACGCTGATTCTCCTGGGCTTCCCCGTCAACTTTCTCACTCTCTAC     180
 A  A  Y  M  F  T  L  I  L  L  G  F  P  V  N  F  L  T  L  Y

GTCACCATCGAGCACAAGAAGCTGAGGACCCCCTTAAATTACATCCTTCTCAACCTGGCT     240
 V  T  I  E  H  K  K  L  R  T  P  L  N  Y  I  L  L  N  L  A
          ◈
GTGGCCAATCTCTTCATGGTCTTCGGCGGCTTCACCACTACGATGTACACGTCGATGCAT     300
 V  A  N  L  F  M  V  F  G  G  F  T  T  T  M  Y  T  S  M  H
                          ▼           ■
GGCTACTTTGTCTTCGGTGAAACAGGCTGCAACCTAGAAGGATACTTTGCTACCCTCGGC     360
 G  Y  F  V  F  G  E  T  G  C  N  L  E  G  Y  F  A  T  L  G

GGTGAAATTTCGCTCTGGTCTCTGGTTGTCCTGGCTATCGAGAGGTGGGTGGTTGTCTGC     420
 G  E  I  S  L  W  S  L  V  V  L  A  I  E  R  W  V  V  V  C

AAGCCAATGAGCAACTTCCGATTTGGTGAGAACCACGCCATCATGGGCTTGGCATTTACC     480
 K  P  M  S  N  F  R  F  G  E  N  H  A  I  M  G  L  A  F  T

TGGATCATGGCCAATACATGTGCTTTGCCTCCTCTGTTTGGATGGTCCAGGTACATCCCA     540
 W  I  M  A  N  T  C  A  L  P  P  L  F  G  W  S  R  Y  I  P
                      ▼
GAAGGCATGCAGTGTTCATGCGGGGTTGACTATTACACCCTCAAGCCTGAAGTCAACAAT     600
 E  G  M  Q  C  S  C  G  V  D  Y  Y  T  L  K  P  E  V  N  N

GAGTCTTTCGTCATCTACATGTTCATAGTTCACTTCTCCATCCCCCTCACCATTATCTCC     660
 E  S  F  V  I  Y  M  F  I  V  H  F  S  I  P  L  T  I  I  S

TTCTGCTACGGCCGACTGGTGTGCACCGTCAAGGAGGCTGCCGCCCAGCAGCAGGAGTCC     720
 F  C  Y  G  R  L  V  C  T  V  K  E  A  A  A  Q  Q  Q  E  S

GAGACTACCCAGAGGGCAGAGCGGGAGGTCACCCGCATGGTGGTCATCATGGTCATCGCA     840
 E  T  T  Q  R  A  E  R  E  V  T  R  M  V  V  I  M  V  I  A
 ◈
TTCCTGGTCTGCTGGATCCCCTATGCCAGCGTGGCCTGGTACATCTTCACCCACCAGGGA     900
 F  L  V  C  W  I  P  Y  A  S  V  A  W  Y  I  F  T  H  Q  G
                                  ◈           □
AGCACATTTGGGCCTGTCTTCATGACAGTACCCTCCTTCTTTGCCAAGAGCTCGGCAATC     960
 S  T  F  G  P  V  F  M  T  V  P  S  F  F  A  K  S  S  A  I

TACAACCCCCTGATCTACATCTGCCTGAACAGTCAGTTCCGCAACTGCATGATCACCACC    1020
 Y  N  P  L  I  Y  I  C  L  N  S  Q  F  R  N  C  M  I  T  T
    ◇  ◇
TTGTTCTGCGGGAAGAATCCCTTTCAGGAGGAAGAGGGAGCATCCACCACTGCCTCCAAG    1059
 L  F  C  G  K  N  P  F  Q  E  E  E  G  A  S  T  T  A  S  K

ACCGAGGCCTCCTCAGTGTCCTCTGTGTCTCCCGCATAA
 T  E  A  S  S  V  S  S  V  S  P  A  *

GGCACCGACCCACCACGCCCCCCCCACGGACGCTGGACCTAAACTCA - 3'
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Fig. 2. Nucleotide and deduced amino acid sequences of opsin gene isolated from Japanese eel, *Anguilla japonica*. Sites responsible for Schiff's base formation and its counterion (□K296 and ■E113), disulfide bond formation (▼C110, C187), glycosylation (●N2, N15) and palmitoylation (◇F322, C323) are indicated together with the stop codon (*). Amino acids implicated in the spectral tuning of rhodopsin are also indicated (◈N83, F261, S292). Sequences corresponding to the primers F1 and R1 are marked as underlines, respectively.

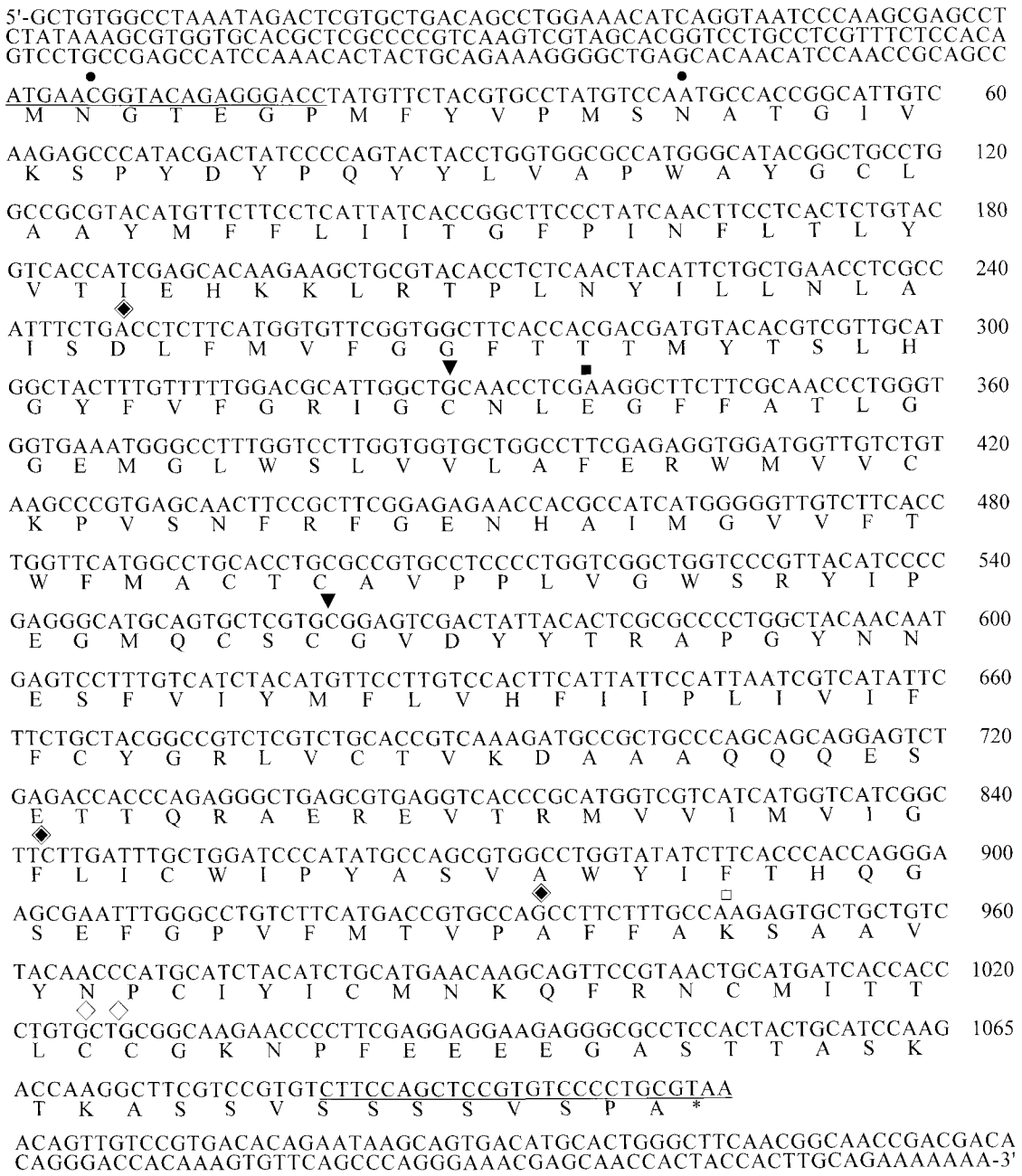

Fig. 3. Nucleotide and deduced amino acid sequences encoding the complete open reading frame of opsin gene isolated from Common carp, *Cyprinus carpio*. Sites responsible for Schiff's base formation and its counterion (□K296 and ■E113), disulfide bond formation (▼C110, C187), glycosylation (●N2, N15) and palmitoylation (◇C322, C323) are indicated together with the stop codon (*). Amino acid residues implicated in the spectral tuning of rhodopsin are indicated (◈D83, F261, A292). Sequences corresponding to the primers F1 and R1 are marked as underlines, respectively.

visual pigments have been examined to find the relationship between opsin sequence and its tertiary structure reflected in λmax of the chromophore (Nakayama and Khorana, 1991; Imai et al., 1997; Yokoyama and Radlwimmer, 1998). Amino acid sequences of rod opsins have been analyzed also in marine (Archer et al., 1995) and freshwater (Hunt et al., 2001) species living at different water-depths. Three amino acids residues (position 83, 261, and 292) in rod opsin were identified to be important for

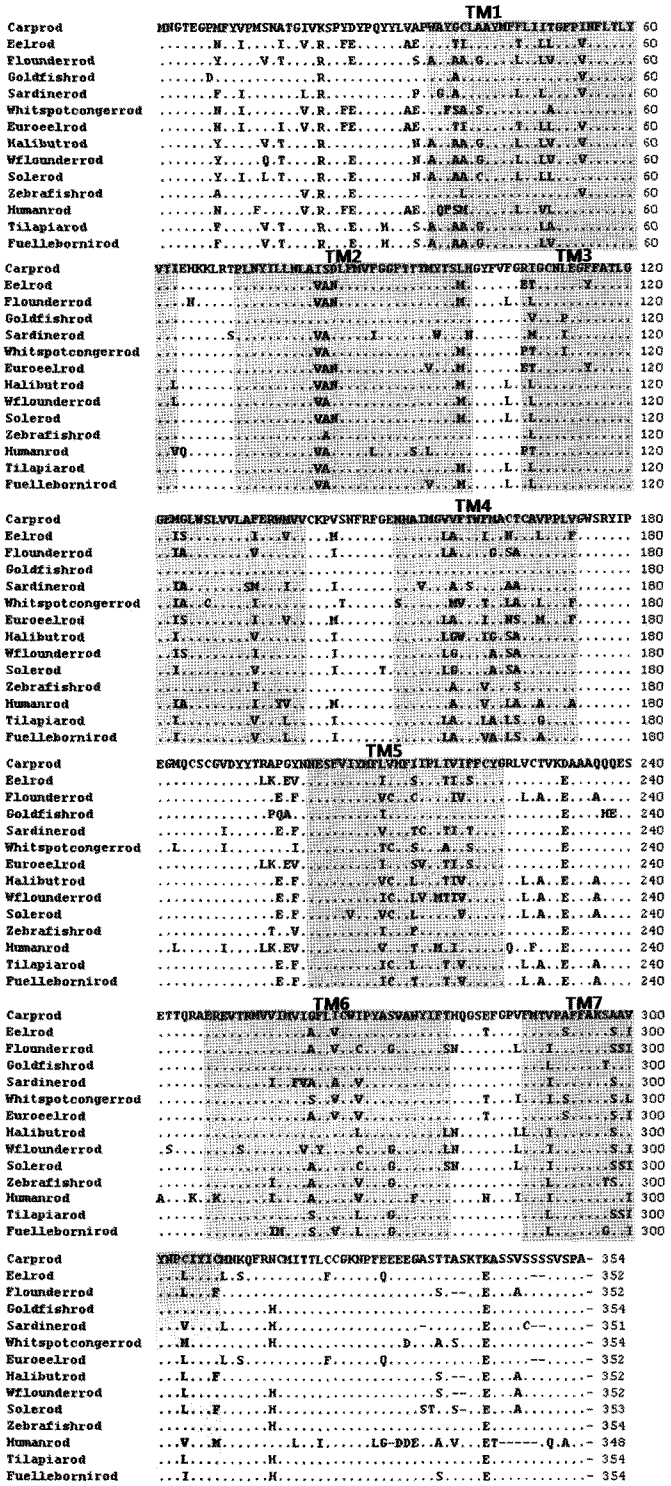

Fig. 4. Multiple amino acid alignment of rod opsins. Sequences compared include opsins from *Cyprinus carpio* (Carprod), *Paralichthys olivaceus* (Flounderrod), *Anguilla japonica* (Eelrod), *Carassius auratus* (Goldfishrod), *Sardina pilchardus* (Sardinerod), *Conger myriaster* (Whitspotcongerrod), *Anguilla anguilla* (Euroeelrod), *Hippoglossus hippoglossus* (Halibutrod), *Solea solea* (Solerod), *Pseudopleuronectes americanus* (Wflounderrod), *Danio rerio* (Zebrafishrod), *Homo sapiens* (Humanrod), *Oreochromis niloticus* (Tilapiarod), and *Labeotropheus fuelleborni* (Fuellebornirod). Dots show identical amino acids among species and shadow boxes indicate the regions corresponding to the putative seven transmembrane helices.

Table 2. Percent identity between amino acid sequences of rod opsins isolated from olive flounder and common carp to those of opsins from other species

Paralichthys olivaceus		Cyprinus carpio	
Species (accession number)	a.a. sequence identity (%)	Species (accession number)	a.a. sequence identity (%)
Hippoglossus hippoglossus (AF156265)	94	Carassius auratus (P32309)	95
Solea solea (Y18672)	94	Danio rerio (AB087811)	92
Pseudopleuronectes americanus (AY631036)	93	Oryzias latipes (AB180742.1)	86
Liza aurata (Y18671)	93	Poecilia reticulata (DQ912023.1)	86
Liza saliens (Y18670)	93	Zosterisessor ophiocephalus (Y18678.1)	85
Mugil cephalus (Y18668)	93	Tetraodon nigroviridis (Q9DGG4)	85
Chelon labrosus (Y18669)	92	Takifugu rubripes (NM_001078631.1)	85
Lithognathus mormyrus (Y18667)	91	Gobius niger (Q9YGZ2)	84
Dicentrarchus labrax (Y18673)	90	Sardina pilchardus (Q9YGZ0)	84
Danio rerio (AB087811)	85		

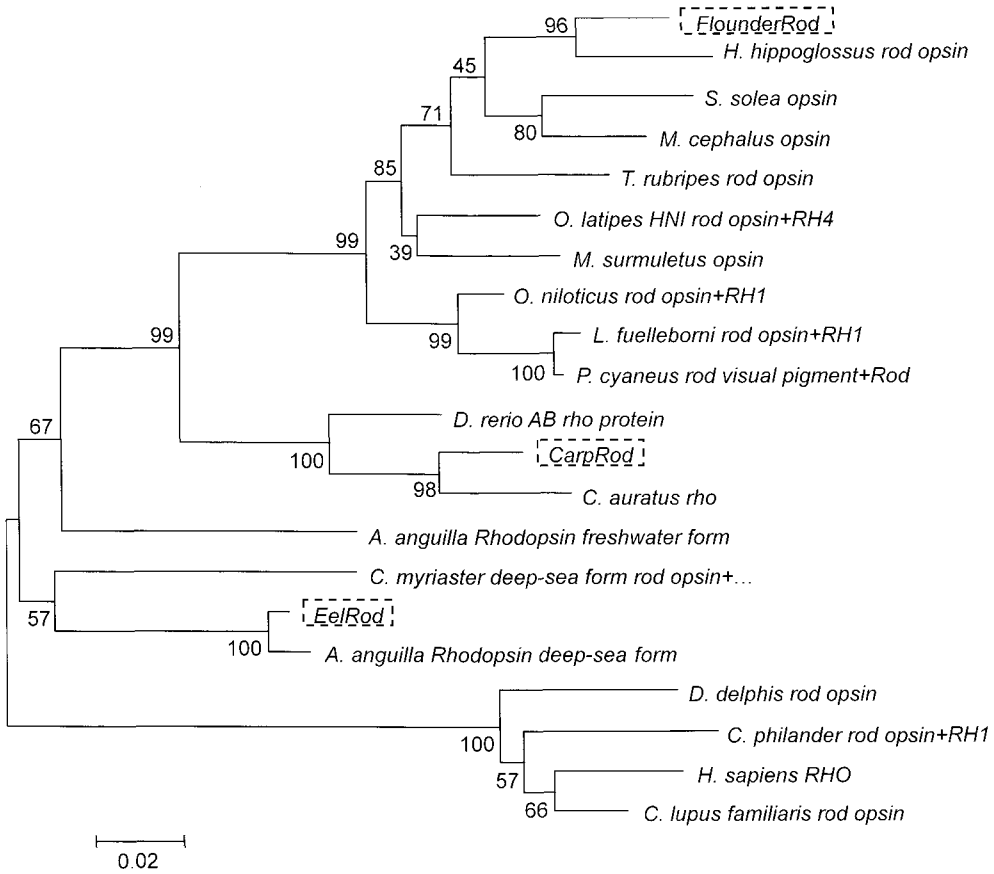

Fig. 5. Phylogenetic tree constructed from the comparison of vertebrate rod opsins by neighbor-joining method using MEGA v 4.0. Node values represent an analysis of 1000 bootstrap trials. Sequences compared include opsins of olive flounder (Flounderrod, *Paralichthys olivaceus)*, common carp (Carprod, *Cyprinus carpio*), Japanese eel (*Anguilla japonica*), zebrafish (*Danio rerio,* BC045288.1), Japanese medaka (*Oryzias latipes*, AB180742.1), goldfish (*Carassius auratus,* P32309), European eel (*Anguilla anguilla*, deep water form, Q90214, fresh water form, Q90215), White spotted conger (*Conger myriaster*, fresh water form, BAB21487), common sole (*Solea solea*, CAA77254), Flat head mullet (*Mugil cephalus*, CAA77250), Atlantic halibut (*Hippoglossus hippoglossus*, AAM17918), Nile tilapia (*Oreochromis niloticus*, AAY26023), *Labeotropheus fuelleborni* (AAY26028), *Paralabidochromis cyaneus* (AAV93304), human (*Homo sapiens*, P08100), Striped red mullet (*Mullussur muletus*, CAA77248), torafugu (*Takifugu rubripes*, AAF44621), Saddle back dolphin (*Delphinus delphis*, AAC12761), Dog (*Canis lupus familiaris*, CAA50502), and bare-tailed woolly opossum (*Caluromys philander*, AAQ82903) were included for the comparison. Opsins isolated in this study are marked by dotted boxes.

spectral tuning (for review see Bowmaker & Hunt, 1999). For fishes living near surfaces, typical amino acid residues known to be important for rod opsin tuning were Asp, Tyr, Ala or Asp, Phe, Ala in the positions. The corresponding amino acid residues in both marine and freshwater species living at depths of 400-500 m were Asn, Ser, Phe. While rod opsins of Japanese eel and olive flounder have amino acids Asn, Phe, Ser, and Asn, Phe, Ala, respectively, opsin of Common carp has Asp, Phe, Ala at the positions. This implies that Japanese eel and olive flounder have rhodopsin adapted more to the deep water-depth zone around (- 500 m) as consistent with their benthic habitats.

Amino acid sequences deduced from opsin genes isolated in this study were compared to those of other species (Fig. 4). Predicted seven transmembrane helices were found in all opsins isolated. Table 2 showed the percent of identity between rod opsins by using Clustal W program. Opsin isolated from olive flounder showed 94%, 94%, 93%, 85%, and 73% of amino acid identity with *Hippoglossus hippoglossus* (Helvik et al., 2001), *Solea solea, Pseudopleuronectes americanus, Danio rerio* and *Homo sapiens,* respectively (Table 2, Thompson et al., 1994). Japanese eel showed 98%, 90%, 85% and 78% amino acid identity with opsin isolated from *Anguilla anguilla, Conger myriaster, Danio rerio*, and *Homo sapiens* (data not shown). Common carp showed 95%, 92%, 83%, 81%, and 76% of identity with opsins isolated from *Carassius auratus, Danio rerio, Astyanax mexicanus, Sardina pilchardus* and *Homo sapiens* (Table 2). The results are consistent with their taxonomic classification as the highest identity was found in the species known to be closely related under the current classification system. Phylogenetic trees were constructed using neighbor-joining method (Fig. 5) to analyze the phylogenetic relationships of olive flounder, Japanese eel, and Common carp. Various vertebrate opsins ranging from human to teleost were included for comparison and the results showed that opsins were grouped into five main branches, reflecting each fish's opsin belong to these five classes. Analysis of the specificity and sensitivity of rhodopsin chromophores provide information for the detailed aspects of GPCR structure in addition to be helpful for designing the light-device in fishing and aquaculture.

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