The Journal of Experimental Biology 216, 1041-1052

doi:10.1242/jeb.078980

RESEARCH ARTICLE

Three cone opsin genes determine the properties of the visual spectra in the Japanese anchovy, *Engraulis japonicus* (Engraulidae, Teleostei)

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SUMMARY

A complement of cone visual pigments was identified in the Japanese anchovy *Engraulis japonicus*, one of the engraulid fish species that has a retina specialized for polarization and color vision. The nature of the chromophore bound to opsin proteins was investigated using high performance liquid chromatography. The opsin genes were then cloned and sequenced, and the absorption spectra of different types of cones were obtained by microspectrophotometry. Two green (EJ-RH2-1, EJ-RH2-2) and one red (EJ-LWS) cone opsin genes were identified and are presumably related to the vitamin A1-based visual pigments (i.e. rhodopsins) with λ_{max} values of 492, 474 and 512 nm, respectively. The long and short cones from the ventro-temporal retinal zone consisted of a pure population of RH2 class gene-based pigments (λ_{max}=492 nm). The long and short cones from other retinal areas and the lateral components of the triple cones possessed a mixture of RH2 and LWS class gene-based pigments that exhibited a λ_{max} of ~502 nm. The central component of the triple cones contained only RH2 class gene-based pigments (λ_{max}=474 nm). Thus, *E. japonicus* possesses a middle-wave range of spectral sensitivity and acquires different color vision systems in distinct visual fields.

Key words: fish, HPLC, microspectrophotometry, triple cones, gene expression, visual pigment, color vision, polarization vision.

Received 9 August 2012; Accepted 19 November 2012

INTRODUCTION

The aquatic environment is characterized by a great variety of light conditions. It has been shown in many studies and comprehensive reviews (Chiao et al., 2000; Kusmić and Gualtieri, 2000; Lythgoe, 1979; Temple, 2011) that aquatic animals, particularly fish, exhibit numerous adaptations and specializations of their visual systems to match the ambient light properties of the environment. In fish, as in other lower vertebrates studied thus far, specialized mechanisms have been developed at the periphery of the visual system (i.e. the eye and retina) that are highly effective in the selection and processing of visual stimuli, which are relevant to different forms of visually guided behavior (Collin, 1997; Ewert, 1997).

In addition to the changes in light intensity that occur as a result of dawn and sunset, variations in the spectral properties of the water media are diverse and important, as they influence many life strategies of fishes, including breeding behavior, camouflage and feeding behavior. That is why most diurnal fish have color vision based on several types of cone photoreceptor cells in the retina, which exhibit different visual pigments with diverse spectral properties. The expression of these pigments is encoded by four opsin genes: short-wavelength-sensitive (UV) type 1 (SWS1), short-wavelength-sensitive (blue) type 2 (SWS2), rhodopsin-like (green) (RH2), and middle- and long-wavelength-sensitive (red) (LWS) (Bowmaker, 2008; Ebrey and Koutalos, 2001; Yokoyama, 2000). A light-sensitive chromophore, such as 11-*cis*-retinal (in a 'family' of A1 pigments, rhodopsins) or 11-*cis*-3, 4-dehydroretinal (in A2 pigments, porphyropsins), is bound to the opsin protein in a visual pigment molecule. The specific spectral properties of a visual pigment are characterized by the maximum absorbance value (λ_{max}), which is determined by the interplay between the chromophore and the amino acid residues of the opsin (for a review, see Bowmaker, 2008).

The specialization of color vision in fish is expressed at the morphological, biochemical and physiological levels of organization and is realized in fish behavior (Collin, 1999). The spectral absorbance of the cones has only been determined in three anchovy species. The long and short cones of the Japanese anchovy, *Engraulis japonicus*, and the European anchovy, *E. encrasicolus*, have the same spectral sensitivity, with λ_{max} values of 502 and 492 nm in different retinal zones (Kondrashev et al., 2012; Zueva and Govardovskii, 1991); additionally, the bay anchovy, *Anchoa mitchilli*, possesses long and short cones with a λ_{max} of ~540 nm (Novales Flamarique and Hárosi, 2002). Besides long and short cones, several species of anchovy also possess another type of photoreceptor: triple cones composed of three equal or non-equal units (members) with well-developed inner and outer segments (Zueva and Govardovskii, 1991). In several dozen species of fish, these triple cones are distributed usually over the whole retinal area randomly, in a small number (Hess, 2009). In contrast, the triple cones of anchovies are numerous regular elements of the photoreceptor mosaic located in relatively extended dorsal and ventral zones of the retina. It was discovered that in two species, *E. japonicus* and *E. encrasicolus*, these cones add 'color to the scene' because the absorbance of different members of the triple cones exhibit λ_{max} values of 475 and 502 nm (Kondrashev et al., 2012).

The anchovy long and short cones possess a remarkable and unique morphology in that the orientation of the photoreceptive lamellae in the outer segments is different from the orientation found not only in fish but also in all other vertebrates. The planes of the lamellae of the long and short cones run parallel to the cell axis in the most retinal areas and the lamellar planes of both types of cones are orthogonal to each other, providing a structural basis for detection of polarized incidental light (Awaiwanont et al., 2001; Fineran and Nichol, 1978; Novales Flamarique and Hárosi, 2002; Novales Flamarique, 2011; Novales Flamarique and Hawryshyn, 1998; Zueva, 1981). The triple cones, potentially involved in color vision, apparently lack polarization sensitivity because their lamellae in the outer segments are in a normal transverse position relative to the cell axis (Zueva and Govardovskii, 1991; Hess, 2009; Novales Flamarique, 2011). Thus, anchovies possess color and polarization vision in the same retina, and these two forms of vision are enabled by different combinations of visual pigments.

There are no clear data on the complement of visual pigments in anchovies. It has been recently shown that the spectral absorbance of the Japanese anchovy cones is determined by three visual pigments; furthermore, the absorbance of most of the long and short cones results from a mixture of at least two pigments, similar to the absorbance of the lateral components of triple cones (Kondrashev et al., 2012). The spectral properties and ratio of the mixtures have not been investigated because of the absence of important data on the nature of the chromophore and opsins of the anchovies, which are needed for the correct approximation of the spectral data by visual pigment templates (Govardovskii et al., 2000). Toyama et al. (Toyama et al., 2008) state that the retina of *E. japonicus* contains only A1 pigments, but no experimental details were provided; thus, this question should be examined carefully. Information about the opsin proteins of the clupeid fishes, a fish family that includes anchovies, is scarce, and new data will significantly benefit the understanding of the evolution of visual pigments.

In this study, the nature of the chromophore bound to the opsins found in the retina of the Japanese anchovy was determined by high performance liquid chromatography (HPLC). Furthermore, the opsin genes were cloned and sequenced, and the absorption spectra of different types of cones were determined by microspectrophotometry (MSP). These data were used for the accurate approximation of the spectral data by known templates (Govardovskii et al., 2000), which allowed us to speculate on the properties of the visual pigments that provide color and polarization vision in the retina of anchovies.

MATERIALS AND METHODS

Fish

For the histological investigations and MSP and HPLC analyses, adult Japanese anchovy *Engraulis japonicus* Temminck & Schlegel 1846 were caught in July–August 2009–2012 in Vostok Bay (Peter the Great Bay, Sea of Japan) near the Marine Biological Station 'Vostok' (A. V. Zhirmunsky Institute of Marine Biology, Far East Branch, Russian Academy of Sciences, Vladivostok, Russia). The fish were caught at night with a net, placed in a thermos on ice and delivered to the laboratory within 30 min of being caught. The fish were immobilized in seawater with a high concentration of the anesthetic MS-222 (Sigma-Aldrich, St Louis, MO, USA) and decapitated. The eyes were enucleated and dissected in saline (0.9% NaCl solution in 0.06 mol l^{-1} phosphate buffer, pH 7.2) under a stereomicroscope.

For the molecular experiments, fish samples of the adult Japanese anchovy were obtained from the set-net fishery in May 2010 and August 2011 at Shima-Bay, Mie prefecture, Japan. Specimens were frozen whole immediately after collection. The fish were treated in accordance with the EU Directive of 2010/63/EU and with the approval of the Scientific Council of the Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences.

RNA extraction and cDNA synthesis

The retinas of *E. japonicus* were shaved off from the frozen eyeballs using a scalpel. Total RNA was isolated using a single-step guanidinium thiocyanate-phenol-chloroform extraction method, specifically TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Promega, Madison, WI, USA) with Poly-T primers designed by Takara Bio (Shiga, Otsu, Japan) (Table 1). The first-strand cDNA was then poly-G-tailed at the 5′-end by terminal deoxynucleotidyl transferase (Invitrogen) as described previously (Miyazaki et al., 2008). This single-strand cDNA was used as a template for the following polymerase chain reactions (PCRs).

Degenerate PCR, cloning and sequencing

To perform the screening for opsins in *E. japonicus*, we used a degenerate PCR primer set (HPO-Fw and HPO-Rv; Table 1) designed by Helvik et al. (Helvik et al., 2001) that was based on the conserved regions of the opsin sequences of several vertebrates. The PCRs were performed with the cycles recommended in Helvik et al. (Helvik et al., 2001). The expected size of the PCR product was 735 bp; this PCR product was excised from the agarose gel after electrophoresis, gel-purified and subcloned into the pGEM-T Easy vector (Promega). After the transformation of the vector into *Escherichia coli* cells, multiple clones were obtained and sequenced on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). A BLAST analysis on the DNA Data Bank of Japan server (http://www.ddbj.nig.ac.jp) was used to confirm which opsin class the nucleotide sequences belonged to.

Green and red opsin cDNA isolations

Our MSP results (Kondrashev et al., 2012) showed that *E. japonicus* possesses cone visual pigments with λ_{max} values of 474–513 nm; these values can be produced by opsin genes belonging to the green (RH2) and red (LWS) classes of opsins. The degenerate PCR above, however, yielded a single rod opsin class gene (RH1). To isolate green and red opsins in *E. japonicus*, we designed specific primers (Table 1) that exhibit high homology in the forward and reverse directions based on known teleost LWS and RH2 opsin sequences.

Rapid amplification of cDNA ends (RACE) PCR was used to generate full-length cDNAs of the green and red opsins. 3′-RACE was performed with a T-amp primer, and 5′-RACE was carried out with a Poly-C primer (Table 1); the PCR program consisted of an initial denaturation step of 94°C for 5 min followed by a 30-reaction cycle (94°C for 30 s, 57°C for 50 s and 72°C for 90 s). In both RACE reactions, a second round of PCR was performed using 0.5 μl of the first-round PCR product and the same PCR program described above. The T-amp primer was used for the second round of 3′-RACE, and the second-round 5′-RACE was performed with the C-amp primer (Table 1). The 3′-RACE reaction produced products with the expected sizes for both the green and red opsins; however, the 5′-RACE yielded only the green opsin. Thus, we designed two new reverse primers for the red opsin amplification (RED-Rv1 and RED-Rv2; Table 1) specific to the *E. japonicus* sequence derived from the 3′-RACE product; we then carried out the 5′-RACE reaction again. In addition, during the 5′-RACE amplification of the green

Table 1. Primers used in the present study

Application	Primer name	Primer sequence
RT-PCR	Poly-T	$CTGATCTAGAGGTACCGGATCCT_{17}$
Degenerate PCR	HPO-Fw	AAGAAGYTCMGTCMACCTCTYAAYT
	HPO-Rv	GTTCATGAAGACRTAGATDAYAGGGTTRTA
5′-RACE	GRN-Rv1	GGTGTAGTAGTCTGGTCCAGCAGGAG
	GRN-Rv2	CCTCAGGGATGTACCTTGACCAGCC
	RED-Rv1	ACACAGTAGGCCAGGATCGTGACAACCACC
	RED-Rv2	TATAGCAGAGGATGATGATAGCGAGGGG
	RHOD-Rv	ATGGGCTTGCAGACGACCAACCACCTC
	Poly C-amp	$GCGCATGCAGTACTAAGCTTC_{17}$
	C-amp	GCGCATGCAGTACTAAGCTT
3′-RACE	GRN-Fw1	TGGTCACTGGTGGTTCTTGCTGTCG
	GRN-Fw2	ACATTGTTGTGTGCAAGCCTATGGG
	GRN-a-Fw1	AGCAAATGTGTGGATCTACAGGTTG
	GRN-a-Faw2	TGCTTATGTGGATTTACTGTCACTTTC
	RED-Fw1	TGTGGTCCCTGACTGTCATCTCTTG
	RED-Fw2	GAAACGTCAAGTTTGATGCCAAATGG
	RHOD-Fw	TGCTCGTGCGGAATCGACTACTAC
	T-amp	CTGATCTAGAGGTACCGGATCC
Southern blotting probe	CLPGRN-FwQ	AACTTYTACATCCCCATGTCYAACARG
	CLPGRN-RvQ	AGGGTGTAGTARTCAGGTCCACAGGAG
	RED-RvA	GGGTTGTATATGGTGGCGCTCTTG

opsin gene, another distinct amino acid sequence of green opsin-like cDNA was obtained; thus, we designed gene-specific 3′-RACE primers (GRN-a-Fw1 and GRN-a-Fw2; Table 1) and conducted additional PCRs. 3′- and 5′-RACE reactions for rhodopsin were also carried out with gene-specific primers that were designed using the fragment sequence determined by the degenerate PCRs. The amplified products were subcloned and sequenced as above. The nucleotide sequences were aligned and translated into amino acid sequences. These amino acid sequences were identified as belonging to the red, green and rod opsin classes.

Southern blotting

To screen for duplications of the red and green opsin genes in the *E. japonicus* genome, we performed a Southern blot analysis on *E. japonicus* genomic DNA. The genomic DNA was extracted from *E. japonicus* muscles using a QuickGene DNA Tissue Kit S (Fujifilm, Tokyo, Japan) according to the manufacturer's instructions. Approximately 1 μg of genomic DNA was used per lane; the genomic DNA was digested overnight with restriction enzymes, electrophoresed on a 1% agarose gel, transferred to a positively charged nylon membrane (Roche Applied Science, Penzberg, Germany) and immobilized by UV cross-linking. For the red opsin probe preparation, 428 bp of the coding region was generated using the RED-Fw2 and RED-RvA primer set and the initial PCR product amplified with the RED-Fw1 and T-amp primer set (Table 1). A 498 bp cDNA fragment was amplified with a degenerate primer set (CLPGRN-FwQ and CLPGRN-RvQ) to produce the probe for green opsin detection. The cDNA fragments were labeled using the PCR DIG Probe Synthesis Kit (Roche Applied Science) and then hybridized to the membrane-bound DNA in hybridization buffer at 60°C overnight. The membranes were washed twice in low-stringency buffer [2× saline-sodium citrate (SSC) buffer, 0.1% sodium dodecyl sulphate (SDS)] at 25°C for 5 min, washed twice in high-stringency wash buffer (0.5× SSC, 0.1% SDS) at 65°C for 15 min, incubated in blocking reagent, and then conjugated with the anti-fluorescein antibody using alkaline phosphatase. The membranes were then incubated in CDP-Star, a chemiluminescent substrate (Roche Applied Science), for 5 min at room temperature. The immunoreactivity was detected by exposure to X-ray film.

Phylogenetic analysis

Complete nucleotide sequences of fish red and green opsins and rod opsin were retrieved from the GenBank. A phylogenetic analysis was performed with the MEGA5 computer program (Tamura et al., 2011). Multiple sequence alignments of the deduced amino acids were carried out using ClustalW (www.clustal.org) and were further refined manually. The nucleotide sequences were aligned according to the amino acid alignment. The evolutionary distance values were estimated using the Kimura two-parameter method (Kimura, 1980). A phylogenetic tree was constructed by applying the neighbor-joining (NJ) method (Saitou and Nei, 1987). A bootstrap analysis with 1000 replicates was performed using the Seqboot program (University of Washington, Seattle, WA, USA).

HPLC analysis

To investigate the nature of the chromophore from the anchovy visual pigments, we used the method described by Suzuki and Makino-Tasaka (Suzuki and Makino-Tasaka, 1983) with some modifications in consideration of techniques published elsewhere (Groenendljk et al., 1980; Hasegawa et al., 2002; Hasegawa, 2005). This method has been developed for the retinal and 3-dehydroretinal chromophores to be extracted as oximes and analyzed by HPLC.

The retinas from one to two fish were isolated in a Petri dish with saline and transferred to an opaque Eppendorf tube. The samples were quick-frozen with liquid nitrogen and stored at −80°C for later use (<3 weeks). The retinas were homogenized using an ultrasonic homogenizer three times for 5 s at 4°C in 1 ml phosphate buffer ($0.1\,mol\,l^{-1}$, pH 6.8). A 100 μl amount of homogenate was mixed (IKA MS 3 Basic Shaker, Wilmington, DE, USA) with 200 μl of $1.92\,mol\,l^{-1}$ hydroxylamine sulfate (MP Biomedicals, Solon, OH, USA; buffered to pH 6.2 with sodium bicarbonate) and 700 μl of methanol to form retinaloxime and 3-dehydroretinaloxime. After the mixture was kept for 15 min on ice, 600 μl of dichloromethane and 200 μl of water were added, and the mixture was shaken vigorously for 10 min. After centrifugation at 7500 ***g*** for 5 min, the organic (lower) layer was carefully collected with a pipette and transferred to another tube. Dichloromethane was again added to the aqueous residue, and the extraction procedure was repeated. The dichloromethane extracts of the two extractions were combined and

evaporated under a vacuum using a filter pump. The oximes were dissolved in 50 µl of *n*-hexane and subjected to HPLC analysis.

The HPLC system consisted of a pump and multisolvent delivery system (LC-20 Prominence, Shimadzu, Kyoto, Japan), a photodiode array detector with thermostatic flow cell (Shimadzu SPD-M20A) and a 5 µm YMC-Pack Silica 2.1×250 mm column (YMC Europe, Dinslaken, Germany). The isocratic mobile phase was 7% diethylether in *n*-hexane containing 0.075% ethanol (v/v) with a flow rate of 0.4 ml min^{-1}. A 10 µl sample of each extract was injected into the column. The absorbances at 360 and 400 nm were monitored with an SPD-M20A detector in full scale at 0.01 OD, and each peak area was determined by integration with the Shimadzu LC Solution 1.24 SP1 software.

A commercial *all-trans*-retinal (Sigma-Aldrich) and retinal extracts from wild goldfish *Carassius gibelio* and carp *Cyprinus carpio* from the aquaculture pond were used as standards. Standards were created using the same method described previously (Provencio et al., 1992). The peaks of the oximes were identified by the retention time of standard solutions using the same Shimadzu HPLC equipment and close working regime described above (Hasegawa et al., 2002; Hasegawa, 2005; Hasegawa et al., 2008). The relative quantity of oximes was evaluated by the amplitude or square of the chromatographic peaks at 360 and 400 nm.

MSP

The methods and additional protocols of the MSP research of retinal photoreceptors are described in detail elsewhere (Bowmaker et al., 1991; Bowmaker et al., 1994; Govardovskii et al., 2000; Govardovskii and Zueva, 2000; Kondrashev, 2008; Kondrashev and Gnyubkina, 2011).

After enucleation of the eye, the cornea, lens and vitreous humor were removed, and the retina was separated from the eyecup and placed in chilled saline. All operations and visual observations were conducted in infrared light (LED array) using a WAT-902B video camera (Watec Co., Tsuruoka, Japan) mounted on a stereomicroscope and a video monitor screened by a dark red acrylic glass filter. A small piece of the retina that did not include the pigment epithelium was torn up into tiny fragments using sharp needles on a glass slide in several drops of saline. Then, a drop of methylcellulose (4000 cP, Sigma-Aldrich) solution was added to one to two drops of the saline containing suspended photoreceptors to increase the viscosity of the solution and prevent cell movement. The mixed solution containing the photoreceptors was placed between two cover glasses and then sealed with silicone Vaseline.

The absorbance spectra of visual pigments from the outer segments of the isolated cones were measured with a Govardovskii single-beam microspectrophotometer (Govardovskii and Zueva, 1988; Govardovskii et al., 2000). This same instrument was used in earlier MSP studies and is based on a system containing a grating monochromator and a microscope equipped with a quartz-mirror condenser (Bowmaker et al., 1994; Kondrashev, 2010; Kondrashev and Gnyubkina, 2011). To avoid the potential bleaching of the pigments, only one measurement was performed in the range of 350–750 nm; the readings were taken every 1 nm starting from 750 nm at a scanning speed of 20 nm s^{-1}. The outgoing measuring beam from the monochromator varied in dimension from 2×10 to 1.5×3 µm. With a proper selection of the intensity of the measuring beam, the level of bleaching was thus solely dependent upon the total exposure. With our instrument settings, in-scan bleaching was estimated at ≤5% based on two subsequent scans of the same cell. As for the possible shift in the λ_{max} value, we relied on the MacNichol (MacNichol, 1986) and Govardovskii et al. (Govardovskii et al., 2000) fitting of a template to the long-wave slope of the absorbance curve, which is minimally affected by the accumulation of bleaching products.

Fragments of 20 retinae from 16 different fish specimens were used for MSP. We measured the spectral sensitivity of 96 long, 46 short and 50 triple cones. The data were imported into a personal computer *via* an AC/DC converter, saved as text files and subjected to further processing. The results obtained for 160 cells met the main acceptance criteria (Bowmaker et al., 1991) and were selected for further analysis.

Differential bleaching

To determine the properties of the supposed pigment mixtures, MSP experiments using differential bleaching (Douglas and Partridge, 1997) were performed. After recording the initial curve, the outer segment of the cone was bleached for 40–60 s using a monochromator beam at 610 nm, namely a wavelength, which is in the range of absorbance for a presumptive long-wavelength-sensitive component of the pigment mixture; however, this wavelength is out of the range of the spectral absorbance for the presumptive short-wavelength-sensitive component. After bleaching, a second recording from the same spot of the outer segment was performed, and the difference between the two data sets resulted in the absorbance of the long-wave pigment.

MSP data processing

To estimate the value of the spectral peak, the experimental data for every cell were smoothed by a single algorithm using TableCurve 2D software (Systat Software, Chicago, IL, USA). The raw data were then summarized, averaged and processed using MSP-PROC software, which was generously provided by V. I. Govardovskii (Institute of Evolutionary Physiology and Biochemistry, St Petersburg, Russia). This program's main functions were outlined in previous studies (Bowmaker et al., 1994; Govardovskii et al., 2000); it generates templates for A1 and A2 visual pigments at a given λ_{max} value and fits the experimental data to the templates for the visual pigments.

RESULTS

Screening for opsin class genes

A degenerate PCR approach was used to screen for opsin class genes in *E. japonicus*. Thirty clones were sequenced and all corresponded to the rod opsin gene. By aligning these fragment sequences, 680-base rod opsin gene fragments encoding 226 amino acid residues were obtained.

Nucleotide and amino acid sequences of the opsin genes

To generate red and green opsin genes, we performed standard RACE reactions with gene-specific primers and obtained two cDNAs belonging to the green opsin class and one cDNA corresponding to the red opsin class. The RACE reactions for rod opsin yielded the 5′- and 3′-end sequences of this gene; these fragments were then aligned with the 680-base rod opsin gene fragments determined by the degenerate PCR experiment described above. The full coding lengths of both green opsins (EJ-RH2-1, EJ-RH2-2) were 1035 bases, which predicts a 345 amino acid residue protein. The red opsin cDNA (EJ-LWS) was 1002 bases long and encoded a 334 amino acid protein, while the rod opsin cDNA (EJ-RH1) was 1056 bases long and encoded a 352 amino acid protein (Fig. 1; GenBank accession nos AB731899, AB731900, AB731901 and AB731902, respectively). The deduced amino acid sequences of EJ-RH2-1, EJ-RH2-2, EJ-LWS and EJ-RH1 showed 67–73%,

75–84%, 82–85% and 81–85% identity, respectively, to corresponding opsins in goldfish (*Carassius auratus*), salmon (*Salmo salar*), tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*), halibut (*Hippoglossus hippoglossus*) and fugu (*Takifugu rubripes*).

Southern blotting of the cone opsins

Southern blot analyses were used to determine whether duplications of the LWS and RH2 genes had occurred in the genomic DNA. The LWS probe showed a single band in the lane in which genomic DNA was digested with *Eco*RI at approximately 4.0 kb (Fig. 2A2); however, a clear band and a weak band were observed at ~8.0 and 2.5 kb, respectively, in the lane in which was *Bam*HI digested (Fig. 2A1). These results indicate that at least one copy of the LWS gene is present in the genomic DNA of *E. japonicus*. As for the analysis of the RH2 gene, EJ-RH2-1 and EJ-RH2-2 are 79% identical in the coding region, and it was expected that both of the RH2 genes would be detected when the 498-base cDNA fragment of EJ-RH2-1 was used as the probe. As expected, the probe detected two bands in the lane that was digested by *Hin*dIII and *Sac*I, which strongly suggests that *E. japonicus* has more than two copies of the RH2 gene (Fig. 2B).

HPLC

The results of the chromatography are presented in Fig. 3. The chromatogram for the anchovy (Fig. 3A) does not show any traces of 3-dehydroretinal or its isomeres and contains only one main peak corresponding to the retention time of the *all-trans*-retinal oxime standard (Fig. 3B). In contrast, the chromatograms of the carp and goldfish contained an additional main peak at a slightly longer retention time (carp) or at the peak (goldfish) corresponding to the *all-trans*-3-dehydroretinal oxime (Fig. 3C,D). Moreover, these chromatographs exhibited a fourth peak of smaller amplitude, indicating the presence of the *anti*-3-dehydroretinal oxime. The chromatogram of the carp at 400 nm showed sharp increases in peaks 2 and 3 relative to anti-peaks 1 and 4, respectively (Fig. 3D).

It is well known that the visual pigments of the cyprinid fish contain exclusively (goldfish) or predominantly (carp) porphyropsin based on a 3-dehydroretinal oxime (Bowmaker, 2008; Toyama et al., 2008), which is characterized by maximums of absorbance spectra that are shifted to longer wavelengths, as well as longer retention times, in comparison with the products of retinal (Groenendljk et al., 1980; Suzuki and Makino-Tasaka, 1983; Kondrashev, 2008). These data, in addition to the absence of a characteristic peak 2 on the anchovy chromatogram (Fig. 3A), convincingly show that anchovy visual pigments are based only on retinal.

MSP

Different types of cones could be easily recognized under the light microscope. The morphology and ultrastructure of photoreceptors of the adult Japanese anchovy and other representatives of the genus *Engraulis* were detailed in several publications (Awaiwanont et al., 2001; Kondrashev et al., 2012); therefore, we only provide a short description here of our observations to make the results of the MSP more transparent.

The cones in the retina of the *E. japonicus* are arranged regularly in parallel rows (Fig. 4A). In the dorsal and ventral regions of the retina, the rows consist exclusively of triple cones, but in the central, nasal and temporal regions the rows are composed of alternating long and short cones. Under the light microscope, the long cones look similar to the long cones seen in ordinary vertebrates, but the short cones are unusual because of their short, bi-lobed outer

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EJ-RH2-1  M N G T E G E N F Y I P M S N K T G V V R S P Y E Y P Q Y Y L A N V W I Y R L Q A L Y M F F L I C A G L P I N V L T L A  60
EJ-RH2-2  M N G T E G S N F Y I P M S N R T G L V R S P F E Y P R Y Y L A P P W Q F Y L L A F Y M F C L I C F G F P I N G L T L A  60
EJ-LWS                  M F T Y T N A N N T R D P F E G P N Y H I A P R W V Y N I S T L W M F F V V I A S V F T N G L V L V  50
EJ-RH1    M N G T E G P F F Y I P M S N A T G V V R S P Y E Y P Q Y Y L V A P W G F A C L A A Y M F F L I L V G F P V N F L T L Y  60

                                                                                    ▼     ▼
EJ-RH2-1  V T A M H K K L R Q P L N F I L V N L A V A G T I M C L C G F T V T F I T A L C G Y F V F G P M G C A I E G F S A T L G 120
EJ-RH2-2  V T A L H K K L R Q P L N F I L V N L A V A G M I M V L F G F T I T I T S A L N G Y F V F G A M G C A I E G F M A T L G 120
EJ-LWS    A T A K F K K L R H P L N W I L V N L A I A D L G E T V L A S T I S V I N Q F F G Y F I L G H P M C V F E G Y T V S T C 110
EJ-RH1    V T I E H K K L R S P L N Y I L L N L A V A N L F M V I G G F T T T M W T S L N G Y F V F G R M G C N I E G F F A T L G 120

EJ-RH2-1  G Q V A L W S L V V L A V E R Y M V V C K P M G N I K F G T P A A T V G V L F T W V M A F S C A A P P L F G W S R Y M P 180
EJ-RH2-2  G Q V A L W S L V V L A V E R Y I V V C K P M G S F K F G T A H A G A G V A F T W V M A M S C A A P P L F G W S R Y I P 180
EJ-LWS    G I A A L W S L T V I S W E R W V V V C K P F G N V K F D A K W A T G G I V F S W V W A A V W C A P P V F G W S R Y W P 170
EJ-RH1    G E I A L W S L V V L S I E R W L V V C K P I S S F R F T E T H A I G G V A F S W I M A A A C A V P P L V G W S R Y I P 180

                ▼
EJ-RH2-1  E G L Q T S C G P D Y Y T L N P V Y D N E N Y V I Y M F T F H F C V P V C T I F F T Y G C L V L T V K A A A A Q Q Q D S 240
EJ-RH2-2  E G M Q C S C G P D Y Y T L S P E F N N E S Y V I Y M F T C H F C A P V F I I F F T Y G S L V L T V K A A A A Q Q Q D S 240
EJ-LWS    H G L K T S C G P D V F S G S D D P G V K S Y M I V L M V T C C F L P L A I I I L C Y I A V W M A I R A V A A Q Q K D S 230
EJ-RH1    E G M Q C S C G I D Y Y T R A E G F N N E S F V I Y M F V V H F M C P F F I I T F C Y G N L V C A V K A A A A A Q Q E S 240

                                                                                    ▼
EJ-RH2-1  A S T Q K A E K E V T R M C V L M V L G F I V A W T P Y A S M A A W I F F N R G A A F S A V Q M A V P A F F S K T S A V 300
EJ-RH2-2  E S T Q K A E R E V T R M C V L M V L G F L V A W T P Y A S F A A W I F F N K G A A F S A Q S M A I P A F F S K S S A L 300
EJ-LWS    E S T Q K A E K E V S R M V V V T I L A Y C V C W G P Y T V F A C F A A A N P G Y A F H P L A A A M P A Y F A K S A T I 290
EJ-RH1    E T T Q R A E R E V T R M V V I M F I A F L V C W V P Y A S V A W F I F C N Q G S E F G P V F M T M P A F F A K S S A I 300

EJ-RH2-1  F N P V I Y I G L N Q Q F P W M H D A D S Y G I T P E D E T S V S Q S K T E V S S V A P A                             345
EJ-RH2-2  F N P I I Y I G M N K Q F R G C M M Q T V F G K T P E D E T S V S T S K T E V S S V G P A                             345
EJ-LWS    Y N P I I Y V F M N R Q F R S C I M Q L F G K A G D D A S E V S T S K T E V S S V S P S                                 334
EJ-RH1    Y N P L I Y V C M N K Q F R H C M I T T L C C G K N P F E E E E E G A S T T A S K T E A S S V S S V S P A             352
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Fig. 1. Amino acid alignment of *Engraulis japonicus* opsin sequences. Arrowheads indicate functionally important residues for the Schiff-base linkage to the chromophore (lysine, K), the Schiff-base counter ion (glutamate, E) and stabilizing disulfide bond (cysteine, C).

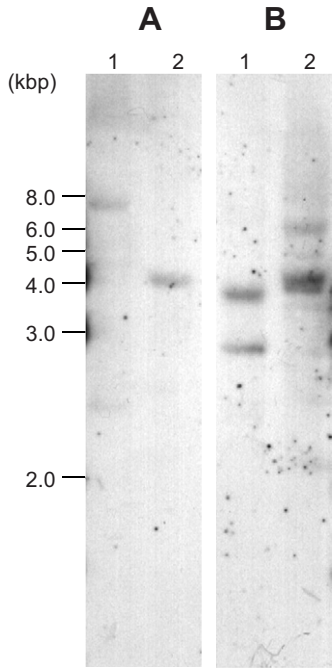

Fig. 2. Southern hybridization of the *E. japonica* genomic DNA to the EJ-LWS (A) and EJ-RH2-1 (B) probes. The genomic DNA in the A1, A2, B1 and B2 tracks was digested with *Bam*HI, *Eco*RI, *Hind*II and *Sac*I, respectively. The sizes of markers are indicated on the left.

segment. When isolated from the retina, the long and short cones are always coupled in so called 'polycones' (Fineran and Nicol, 1998), which are small units consisting of three to 10 cells that often have melanin- and guanine-containing pigment cells inserted between the cone outer segments (Fig. 4B).

The cones from different retinal zones exhibit different morphologies. The long and short cones isolated from the ventro-temporal zone, presumably from the area temporalis, are more slender than the cones from the central retina. In the central and nasal retina, the ellipsoids of all the cones are positioned at almost the same level (Fig. 4C). In contrast, in the ventro-temporal zone, the short cones are displaced vitreally relative to the long cones (Fig. 4B).

The triple cones are composed of two large lateral components with one smaller central component between them (Fig. 4D). The ellipsoid of the central component is thinner and the outer segment of the central component is significantly shorter than similar elements of the lateral components. During retinal preparation, these components were separated as a single triple unit.

The absorbance curves for the long and short cones obtained from the ventro-temporal retinal zone were identical and were best fitted by the template for the rhodopsin pigment at λ_{max}=492 nm (Kondrashev et al., 2012). The λ_{max} values of the long and short cones isolated outside the ventro-temporal retina and of the lateral members of the triple cones were ~502 nm. It was hypothesized (Kondrashev et al., 2012) that the cones contain a mixture of at least two visual pigments as the experimental data did not correspond to the template for a single pigment. Our HPLC data indicate the sole presence of the retinal chromophore, and our molecular experiments demonstrate the presence of three different cone opsins. Thus, a superposition of two rhodopsin templates with different maximums was combined to fit normalized data for the pigment mixture. This matching was made by the superposition of the rhodopsin templates generated using MSC-PROC software (Govardovskii et al., 2000), MS Excel and SigmaPlot v9.01. The best fit of the recorded and calculated data was obtained at a given maximum of the smoothed spectrum. It was found that the long and short cones as well as the lateral components of the triple cones contain a mixture of two rhodopsins with λ_{max} values at 492 and 512 nm (Fig. 5A,C,D). The content ratio of these pigments is close to 50:50 over the area of the retina except for the ventro-temporal zone; however, this ratio may vary from 30:70 to 60:40 in different individuals and retinal areas (Fig. 5A,D).

We performed experiments using differential bleaching to support the determined properties of the supposed pigment mixture. Both experiments revealed spectral components that best fit with the rhodopsin templates at the long wave slopes of the curves and corresponded with the maximums derived from the calculated superposition of the retinal-based templates (A1_492 and A1_512 nm), specifically A1_494 and A1_513 nm for the long cones, 489 and 513 nm for the short cones and A1_496 and A1_516 nm for the lateral components of the triple cones (Fig. 5B–D).

The absorbance curve of the central member of the triple cones exhibited a λ_{max} value of 474 nm and showed a good fit with the rhodopsin template (Fig. 5E). The orientation of cones in the MSP preparation is always a matter of consideration, and the components of the triple cone are densely packed (Fig. 4D); therefore, special attention was given to certain cases during the recording when the beam of the microspectrophotometer touched the border of the adjacent lateral component, which exhibits a much longer wave absorbance with a λ_{max} value ~502 nm. The result was a distortion and minute long-wave (4–6 nm) shift of the spectral curve. In the experiments with differential bleaching, this long-wave spectral 'contamination' was isolated, and the data did not fit the rhodopsin template, which runs far below these data points in the range of 540–600 nm (Fig. 5E).

DISCUSSION

Opsin types and visual pigment correspondence

We found two RH2, one LWS and one RH1 opsin in the retina of *E. japonicus*. Although we performed trials of (RACE) PCR using the SWS2 gene-specific primer sets, which have provided data for many fish species, no product encoding an SWS2 class gene from *E. japonicus* was obtained. In our experience, a primer is often inapplicable for isolating genes from some fish even when the fish belongs to the same order or family as other fish for which the primer was successful. It is possible that the primers that we used for SWS2 isolation were unsuitable for the sequence of the opsin gene in *E. japonicus*.

Visual pigments of the A1 type were revealed in the rods (Kondrashev et al., 2012) and three types of cones with λ_{max} values of 502, 474, 492 and 512 nm; they correspond to the RH1, both sub-classes of RH2 (RH2-1 and RH2-2) and LWS opsin genes, respectively.

Numerous RH1 genes of Clupeiformes are found in GenBank; however, there are only two entries of partial RH2 genes of *Clupea harengus* for cone opsins. A homology search on the BLAST server indicated the highest score for *C. harengus* for EJ-RH2-1 and EJ-RH2-2 and the highest score for *E. encrasicolus* and *Sardina pilchardus* for EJ-RH1.

In the Southern hybridization analysis (Fig. 2), the EJ-RH2-1 probe detected double bands in the genomic DNA. This result corresponds with our MSP results and the molecular cloning of opsin cDNA; it also strongly suggests that the RH2 gene has been duplicated in *E. japonicus*. The blotting analysis with the EJ-LWS

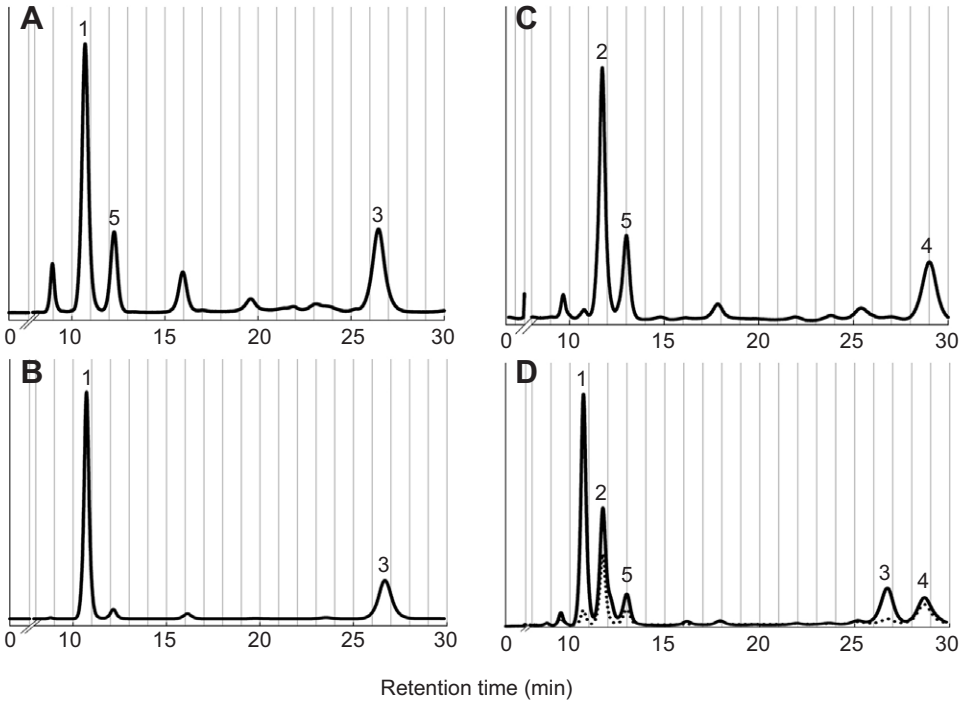

Fig. 3. HPLC analysis of the retina of *E. japonicus* (A), *Carassius gibelio* (C) and *Cyprinus carpio* (D). (B) Chromatogram of a standard *all-trans*-retinal oxime. Peaks: 1, *all-trans*-retinal oxime; 2, *all-trans*-3-dehydroretinal oxime; 3, *anti-trans*-retinal oxime; 4, *anti-trans*-3-dehydroretinal oxime; 5, unknown. Solid curve, absorption at 360 nm; dotted curve, absorption at 400 nm.

probe produced two bands, one weak and one clear. The weakly hybridized signal is frequently observed when hybridization and washing are carried out under low-stringency conditions. Although there are many fish species that have duplicate LWS genes, our current results suggest that *E. japonicus* has only one gene of LWS.

The full-length amino acid sequence of the *E. japonicus* LWS gene was determined (Fig. 1) to be 334 amino acids long, 22–23 residues shorter than the LWS gene of other fish species. When we conducted a homology search for a region of 33 nucleotides upstream from the start codon, no gene corresponding to the opsin class was found in GenBank. We carried out PCRs using a forward primer designed for a conserved coding region in the 5′-end of the LWS gene of teleosts (ATGGCAGAGCAGTGGGGAGATG) coupled with our reverse primers; no product was amplified with a cDNA or genomic DNA template. We expect the 334 amino acid sequence to fully encode the LWS gene of *E. japonicus*, although

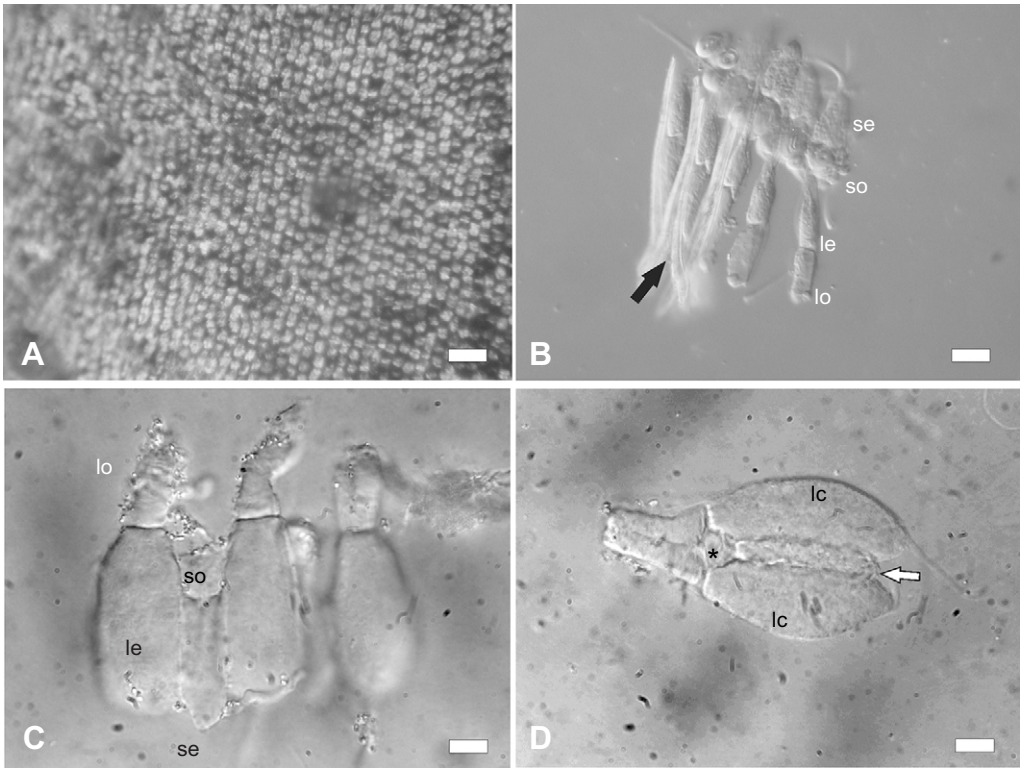

Fig. 4. Light microscopy photographs of cones of *E. japonicus*. (A) Retinal flatmount cleaned from the retinal epithelium; (B–D) isolated cones, Nomarski optics. (A) The rows of cones in the ventro-temporal retina. (B) Lateral view of a fragment of a polycone row from the ventro-temporal retina. se, short cone ellipsoids; so, short cone outer segments; le, long cone ellipsoids; lo, long cone outer segments; arrow, processes of pigment cells with guanine platelets. (C) Lateral view of part of a polycone from the central retina. For designations see B. (D) Isolated triple cone from the dorsal retina. lc, lateral component; arrow, central component; asterisk, outer segment of the central component. Scale bars: (A) 20 μm, (B) 10 μm, (C,D) 5 μm.

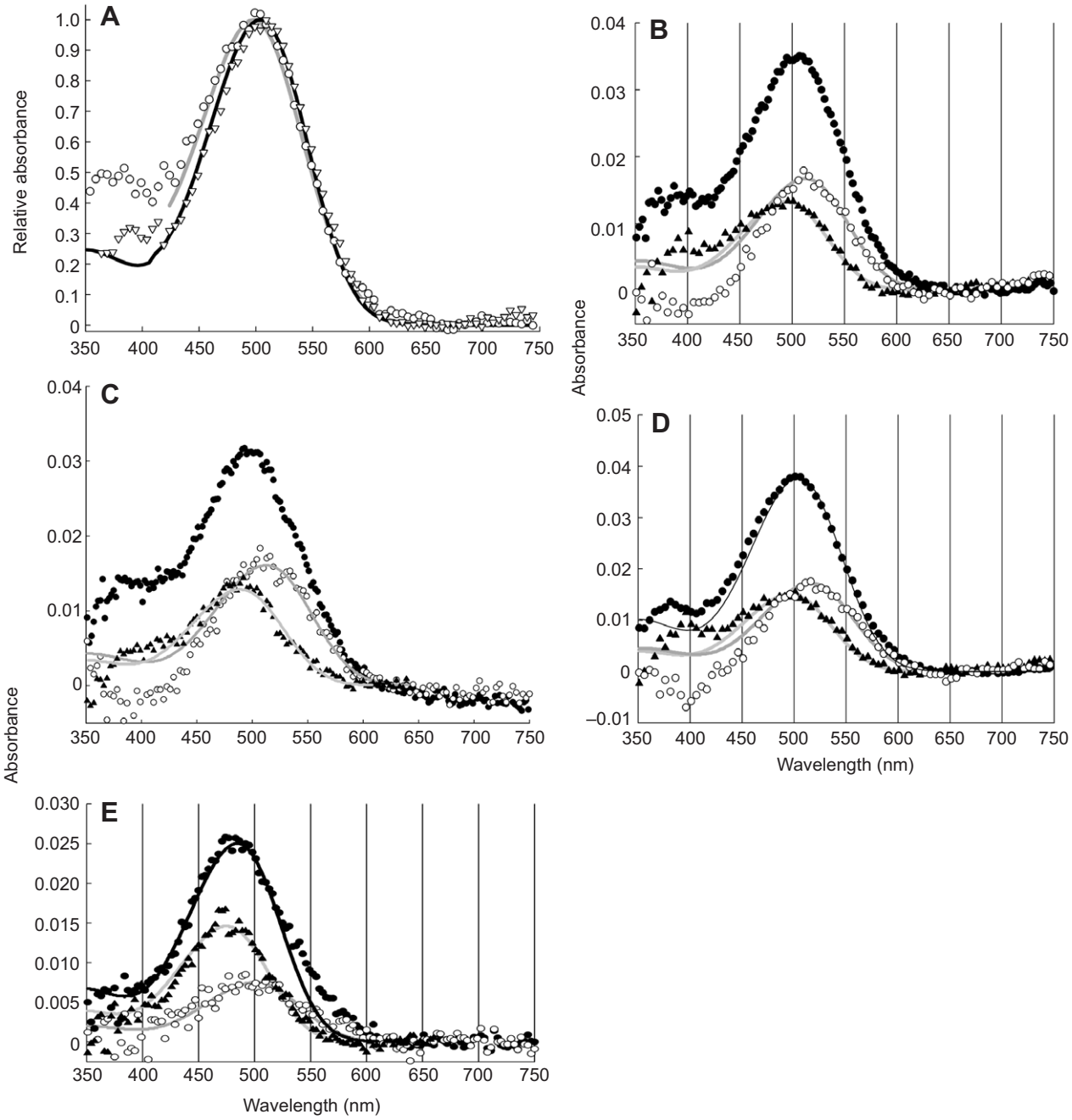

Fig. 5. Absorbance spectra of the photoreceptors of *E. japonicus.* The spectra are the means of records obtained from single cone outer segments. Symbols are the recorded and calculated data, solid lines are the best fit of the rhodopsin pigments single template curves (B–E) and a superposition of two rhodopsin templates (λ_{max}=492 and 512 nm) (A,D). (A) Variation in the absorbance of the long cones from different regions of the nasal retina. Triangles and circles indicate the A1_492+A1_512 pigment mixture at the ratios of 40:60 (λ_{max}=503 nm, *n*=10 records) and 60:40 (λ_{max}=500 nm, *n*=12), respectively. (B–E) Differential bleaching of different cone types. Filled circles, first recording; triangles, recording after bleaching; open circles, difference between the first and second spectral components. Solid curves are the rhodopsin templates for two A1 visual pigments. (B) Long cones (*n*=16, λ_{max}=503 nm; λ_{max} of spectral components=494 and 513 nm). (C) Short cones (*n*=12, λ_{max}=502 nm; λ_{max} of spectral components=490 and 513 nm). (D) Lateral components of the triple cones; solid line, fit by the combination of two A1 pigment templates (*n*=10, λ_{max}=504 nm; λ_{max} of spectral components=496 and 516 nm). (E) Central component of the triple cones (*n*=14). Solid line, template for A1_480 pigment. Triangles correspond to the true spectral absorbance of this component fitted by the A1 template at λ_{max}=474 nm, open circles correspond to data records of the difference spectrum (λ_{max}=503 nm); filled circles correspond to first data records.

we still need to investigate the LWS genomic sequence. Nevertheless, there is no doubt that a functional LWS gene is present in the retina of *E. japonicus*.

Takenaka and Yokoyama (Takenaka and Yokoyama, 2007) proposed that the RH2 λ_{max} values currently known are generated by eight key amino acid sites (49, 52, 83, 97, 122, 164, 207 and 292). These eight sites are occupied by CLGTQAMA in EJ-RH2-1. In goldfish RH2b (L11866), which has an A1-based λ_{max} of 505 nm (Johnson et al., 1993), these key sites were arranged as CLGTEAMA. The effect of amino acid change of Q122E is known

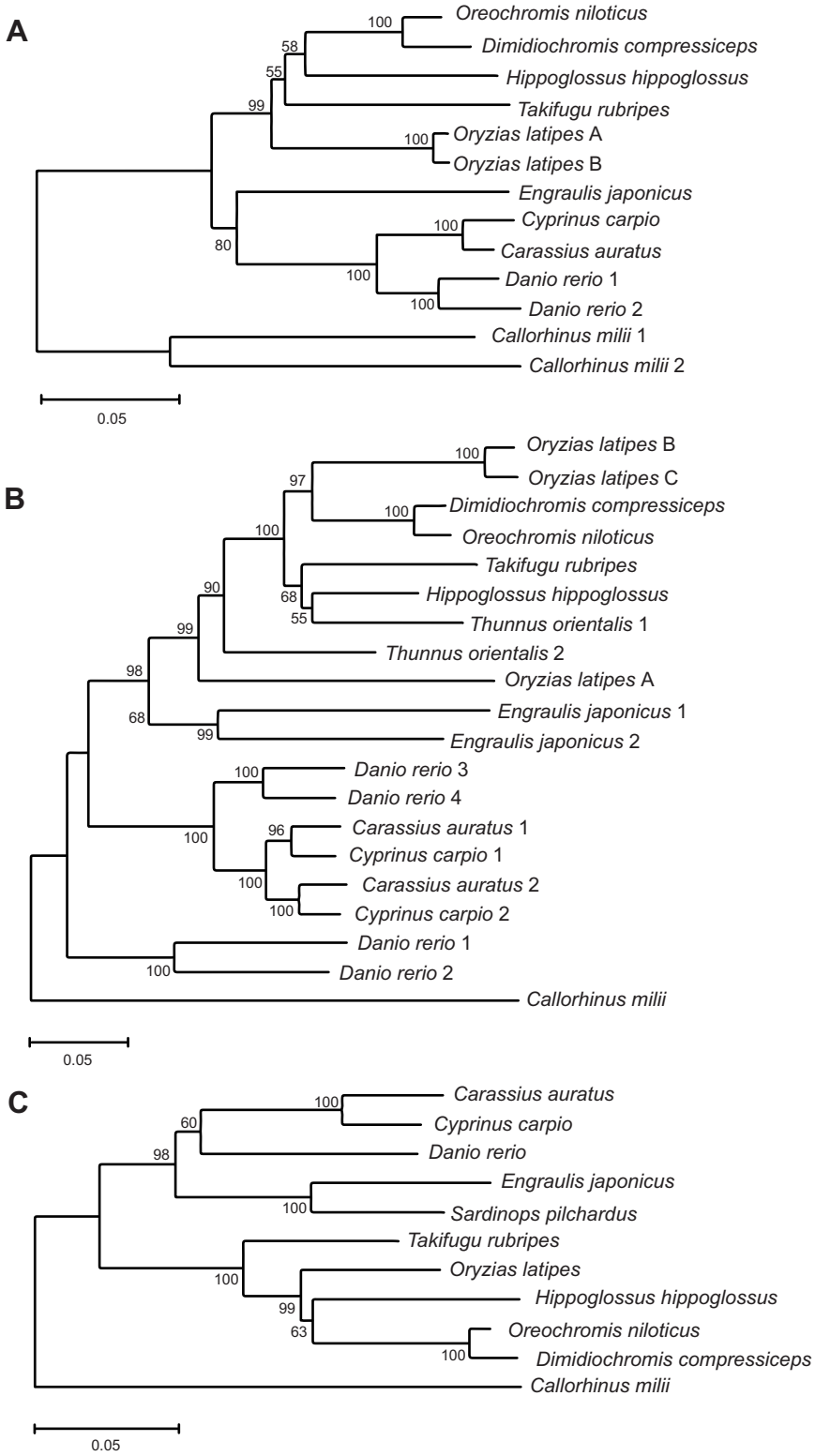

Fig. 6. Neighbor joining tree of LWS (A), RH2 (B) and RH1 (C) opsin genes based on their nucleotide sequences. *Callorhinchus milii* LWS1, LWS2, RH2 and RH1 (gene accession nos EF565165, EF565166, EF565168 and EF565167, respectively) were used as the outgroup for LWS, RH2 and RH1 trees, respectively. The bootstrap probabilities are given for each node. Scale bars indicate five substitutions per 100 sites. The nucleotide sequences of the following fish opsin genes were obtained from GenBank: *Carassius auratus* LWS (L11867), RH2-1 (L11865), RH2-2 (L11866), RH1 (L11863); *Cyprinus carpio* LWS (AB055656), RH2-1 (AB110602), RH2-2 (AB110603), RH1 (Z71999); *Danio rerio* LWS-1 (AB087803), LWS-2 (AB087804), RH2-1 (AB087805), RH2-2 (AB087806), RH2-3 (AB087807), RH2-4 (AB087808), RH1 (AB087811); *Oryzias latipes* LWS-A (AB223051), LWS-B (AB223052), RH2-A (AB223053), RH2-B (AB223054), RH2-C (AB223055), RH1 (AB180742); *Takifugu rubripes* LWS (AY598942), RH2 (AF226989), RH1 (AF201471); *Hippoglossus hippoglossus* LWS (AF316498), RH2 (AF156263), RH1 (AF156265); *Oreochromis niloticus* LWS (AF247128), RH2 (AF247124), RH1 (AY775108); *Dimidiochromis compressiceps* LWS (AF247131), RH2 (AF247130), RH1 (AY775059); *Thunnus orientalis* RH2-1 (AB290451), RH2-2 (AB290452); *Sardina pilchardus* RH1 (Y18677).

to cause a 13–16 nm red-shift (Takenaka and Yokoyama, 2007), so EJ-RH2-1 was expected to have a peak absorbance of 489–492 nm. In contrast, EJ-RH2-2 has CFGSQAMA at the critical eight sites. The amino acid composition of tilapia *Oreochromis niloticus* RH2b (DQ235681) is IFGSQAMS, and this pigment is known to have an A1-based λ_{max} value of 472 nm (Spady et al., 2006). Two amino acid changes, I49C and S292A, are found in EJ-RH2-2 when compared with the tilapia RH2b. Chinen et al. (Chinen et al., 2005) reported that mutation of I49C causes a 4 nm blue-shift, and Takenaka and Yokoyama (Takenaka and Yokoyama, 2007) have found that a 7 nm red-shift is caused by amino acid replacement of S292A. Therefore, it was predicted that EJ-RH2-2 would have a λ_{max} value of 475 nm. Thus, it can be concluded that the central component of the triple cones contain the EJ-RH2-2 gene, and the lateral components of the triple cones co-express EJ-RH2-1 and EJ-LWS genes.

With respect to the molecular mechanisms of spectral tuning in the LWS pigments, the five amino acid key sites (180, 197, 277, 285 and 308) are well known to tune the λ_{max} value of the LWS pigment class. In EJ-LWS, these sites were occupied by AHYTA residues and the predicted λ_{max} was 553 nm, longer than our measurement. Davies et al. (Davies et al., 2009; Davies et al., 2012) reported that the λ_{max} value of elephant shark *Callorhinchus milii* LWS1 (EF565165) expressed in an *in vitro* experiment was not consistent with the 'five-site' rule, because of inactivation of the chloride ion positive charge of H197 caused by amino acid substitution of A308S. The EJ-LWS has amino acid residues H and A at sites 197 and 308 and our present knowledge could not explain the molecular mechanisms involved in the blue shift. The co-expression of RH2-1 and LWS genes in the same cell may involve other interactions in the tuning of the peak sensitivity of the visual pigment.

Despite good correspondence of many experimental data with the predicted λ_{max}, caution is necessary when applying these calculations, as recent molecular studies on a variety of species provide clear evidence of the inconsistence of the 'site' rules in some cases. For example, according to our data, the Pacific saury *Cololabis saira*, which has the same sites with AHYTA residues (AB761214) as *E. japonicus*, possesses an A1-based visual pigment with a λ_{max} of 565 nm in double cones (Kondrashev and Gnyubkina, 2011) in comparison to the predicted λ_{max} of 553 nm. More examples of 'discrepancies' in other vertebrate classes can be found in the literature (e.g. Yokoyama, 2008).

The spectral absorbancies of the photoreceptors of the Japanese anchovy are similar to those of the European anchovy (Kondrashev et al., 2012), and thus both species may have the same complement of visual opsins. It is worth noting that in both species a retinal region rich in polycones specialized for polarization vision appears to be monochromatic (hence color blind). Other retinal regions containing triple cones obviously lack polarization sensitivity, but have color discrimination properties owing to the different spectral absorbances of three outer segments. Such segregation of polarization and color sensitivity between several specialized regions of the retina in anchovies is analogous to that found in insects in many aspects (Labhart and Meyer, 1999; Kamermans and Hawryshyn, 2011).

It was reported that the cones of the bay anchovy exhibit longer wave sensitivity at a λ_{max} of ~540 nm (Novales Flamarique and Hárosi, 2002). Judging from the value of the spectrum half-bandwidth, the authors concluded that the cone pigments were A1-based; however, according to our estimates, the cones fit an A2 template rather well with a λ_{max} value of 543 nm (Kondrashev et al., 2012). It is important to note that the λ_{max} difference between an A1/A2 pigment pair for the same opsin is determined by the Whitmore and Bowmaker (Whitmore and Bowmaker, 1989) or the Hárosi (Hárosi, 1994) equations, and it is the A1_512/A2_543 pigments that satisfy such a relationship with good accuracy. Thus, there is a real possibility that the bay anchovy has the same LWS opsin as both species of *Engraulis* that were previously studied. To provide more evidence of the properties of the LWS opsin in *E. japonicus*, we need to investigate the peak absorbance of recombinant visual pigments using A1 and A2 chromophores. However, there is no doubt that the LWS opsin gene of *E. japonicus* produces the shortest LWS pigment in teleosts.

The presence of two different visual pigments in the anchovy that utilize retinal as a chromophore in the same outer cone segments is quite rare for vertebrates. Prior to this, the expression of 'unpaired' visual pigments in the same photoreceptor was proposed for the guppy (Archer and Lythgoe, 1990) and was found in the guinea pig and some other mammals (Parry and Bowmaker, 2002; Lukáts et al., 2005).

Gene expression in different cone types

Considering that the distribution map of different cones across the retina is not uniform (Koch et al., 2010; Novales Flamarique, 2011; Zueva and Govardovskii, 1991) and given our MSP results, a pattern for the gene expression in cones can be outlined. In the central component of the triple cones the short-wave subclass of the RH2 gene is expressed; furthermore, in both lateral components of the triple cones a combination of the long-wave subclass of RH2 and the LWS genes are expressed. The same combination of RH2 subclass and LWS genes is characteristic for the long and short cones in the lateral retina, which 'reduces' to the long-wave subclass of RH2 in the ventro-temporal retinal zone. The presence of only one type of RH2 pigment in this area may be regarded as a special case of the pigment mixture variability, as the ratio of RH2-1 to RH2-2 pigment varied in different anchovy specimens (Fig. 5). This was obviously due to the different levels of the gene's expression as a result of reasons other than seasonal exposure, because all of the MSP experiments were performed in a limited period during the summer.

The results of the immunochemical labeling of the photoreceptors of closely related species, specifically the northern anchovy *Engraulis mordax* by RH1 and LWS antibodies (Novales Flamarique, 2011), are in accordance with our conclusions about the opsin content in the lateral components of the triple and long cones, as well as the opsin difference between the central component of triple cones and that of the long and lateral cones. The somewhat confusing labeling results (i.e. the different response of the long and short cones to the set of applied antibodies) could be explained by the fact that the anti-RH2 opsin antibodies were not used and that no assumption of the existing pigment mixtures was made. The RH1 antibody that was used may have a high level of non-specific binding to the two expressed RH2 opsins, or it may not bind at all to one or either of them. In addition, the level of expression of the RH2 opsins in different retinal zones of *E. mordax* may vary, as was shown here in *E. japonicus*.

Phylogenetic positions of *E. japonicus* opsins

The overall structure of the phylogenetic trees for LWS, RH2 and RH1 contained a branch that separated into two at the root of the tree (Fig. 6). We obtained the same tree topology when using maximum likelihood estimation of evolutionary distances. The fishes analyzed were divided into two clades, Cypriniformes and other fishes (i.e. Beloniformes, Perciformes, Pleuronectiformes and Tetraodontiformes). The *E. japonicus* opsins clustered with the Cypriniformes opsins in the LWS tree and the RH1 tree, whereas the *E. japonicus* opsins clustered with the other fishes' opsins in the RH2 tree. Phylogeny of a given opsin gene does not often follow the presumed phylogenetic tree of the families and/or genera (Yokoyama and Yokoyama, 1990; Sugawara et al., 2002). We believe that the clustering difference among the present trees is presumably due to convergent evolution of opsin genes, as suggested by the evolution of RH1 in cichlids (Sugawara et al., 2002). In the RH2 tree, two RH2 genes of *E. japonicus* were separated from other fishes at the node and formed a cluster with 99% bootstrap support. The RH1 gene of *E. japonicus* was clustered with *S. pilchardus* with 100% bootstrapping probability (Fig. 6C). The current NJ trees suggest that the opsin genes of *E. japonicus* evolved in the Clupeiformes lineage after divergence from the ancestral gene.

Again, long branches of two RH2 genes of *E. japonicus* strongly indicated that the amino acids of these genes have been changed until recently after the duplication, and it may mean that a rapid adaptive evolution to the light environment of RH2 genes has occurred. There are few data available on the cone opsin sequences of clupeoid fish. In addition, the molecular evolutionary path of the opsin genes is often different from the evolution of the species. More knowledge of the opsin sequences of the clupeoids is needed to better understand the expression and regulation of the opsin genes in *E. japonicus*. An *in situ* hybridization analysis is also required to elucidate the expression and localization of the RH2-1, RH2-2 and LWS genes in the retina; however, our current molecular results indicate that the RH2 and LWS class opsins are dominantly expressed in the retina of this species.

LIST OF SYMBOLS AND ABBREVIATIONS

LWS	long-wavelength-sensitive or red
NJ	neighbor joining
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RH1	rod opsin or rhodopsin
RH2	RH2-like or green
SDS	sodium dodecyl sulfate
SSC	saline-sodium citrate buffer
SWS2	short-wavelength-sensitive type 2 or blue
λ_{max}	wavelength of maximal absorption

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for their critical comments.

FUNDING

This work was supported by grants to S.K. from the Russian Foundation for Basic Research (project no. 10-04-00082) and the Far East Branch of the Russian Academy of Sciences (project nos12-I-P7-03 and 12-III-A-06-091). N.L. was partially supported by the grant from the Russian Government (no. 11.G34.31.0010). T.M. was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (no. 23570113) and the Mie University Center of Excellence Program A.

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