Gene Duplication and Spectral Diversification of Cone Visual Pigments of Zebrafish

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ABSTRACT

Zebrafish is becoming a powerful animal model for the study of vision but the genomic organization and variation of its visual opsins have not been fully characterized. We show here that zebrafish has two red (*LWS-1* and *LWS-2*), four green (*RH2-1*, *RH2-2*, *RH2-3*, and *RH2-4*), and single blue (*SWS2*) and ultraviolet (*SWS1*) opsin genes in the genome, among which *LWS-2*, *RH2-2*, and *RH2-3* are novel. *SWS2*, *LWS-1*, and *LWS-2* are located in tandem and *RH2-1*, *RH2-2*, *RH2-3*, and *RH2-4* form another tandem gene cluster. The peak absorption spectra (λmax) of the reconstituted photopigments from the opsin cDNAs differed markedly among them: 558 nm (*LWS-1*), 548 nm (*LWS-2*), 467 nm (*RH2-1*), 476 nm (*RH2-2*), 488 nm (*RH2-3*), 505 nm (*RH2-4*), 355 nm (*SWS1*), 416 nm (*SWS2*), and 501 nm (*RH1*, rod opsin). The quantitative RT-PCR revealed a considerable difference among the opsin genes in the expression level in the retina. The expression of the two red opsin genes and of three green opsin genes, *RH2-1*, *RH2-3*, and *RH2-4*, is significantly lower than that of *RH2-2*, *SWS1*, and *SWS2*. These findings must contribute to our comprehensive understanding of visual capabilities of zebrafish and the evolution of the fish visual system and should become a basis of further studies on expression and developmental regulation of the opsin genes.

VISUAL pigments are photoreceptive molecules that occur in rod and cone photoreceptor cells in the retina and characterize the vision of an animal. They consist of a protein moiety, opsin, and a chromophore, either 11-*cis* retinal or 11-*cis* 3,4-dehydroretinal in vertebrates. Vertebrate opsins have been classified into five phylogenetic groups: rod opsin or the rhodopsin group (RH1), ultraviolet-blue or the short-wave-sensitive-1 cone-opsin group (SWS1), blue or the short-wave-sensitive-2 cone-opsin group (SWS2), green or the rod-opsin-like cone-opsin group (RH2), and red-green or the long-to-middle-wave-sensitive cone-opsin group (LWS/MWS; Yokoyama 2000).

Zebrafish (*Danio rerio*) is an excellent animal model featuring high fecundity, rapid oviparous development, embryonic transparency, and mutant screening feasibility (Detrich *et al.* 1999). Zebrafish plays a pivotal role in our understanding of molecular mechanisms of the vertebrate visual system (Malicki 2000) and the basic architecture of its retina has been well investigated. Zebrafish has rods and four morphologically distinct classes of cones in the retina: the long (or principal) and short (or accessory) members of double cones, long-single cones, and short-single cones (Branchek and Bremiller 1984). In a horizontal plane of the photoreceptor layer, the four types of cones are highly organized in a regular mosaic fashion (Robinson *et al.* 1993). The spectral characteristics of the cone and rod cells are investigated with microspectrophotometry (MSP; Nawrocki *et al.* 1985; Robinson *et al.* 1993; Cameron 2002) (Table 1). Zebrafish has all five groups of the opsins and produces them all in the retina: RH1 in rods, LWS/MWS (red) in the long members of double cones, RH2 (green) in the short members of double cones, SWS2 (blue) in long-single cones, and SWS1 (ultraviolet) in short-single cones (Raymond *et al.* 1993; Vihtelic *et al.* 1999). The cDNAs of all five groups of the opsins are cloned from the zebrafish retina including two subtypes of RH2 opsins (Vihtelic *et al.* 1999). However, it has remained unknown whether the two RH2 pigments show distinct absorption spectra and whether zebrafish has yet additional visual pigments.

We previously isolated a rod opsin gene (*RH1*) from the zebrafish genome (Hamaoka *et al.* 2002). In this study we aimed to examine the zebrafish genome for the cone opsin genes, determine the absorption spectra of all visual pigments, and compare the expression level among opsin genes in the retina to establish the molecular basis of visual capabilities of zebrafish.

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under accession nos. AB087803–10.

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MATERIALS AND METHODS

Genomic library screening: A zebrafish genomic library was constructed previously from a strain (AB) of zebrafish using

TABLE 1

The λmax values (nanometers) of the zebrafish visual pigments measured by *in vitro* reconstitution and MSP methods

Pigment	*In vitro*	Cell type	MSP
LWS-1	557.7 ± 3.3		
LWS-2	548.3 ± 0.5		
		LD cone	564 ± 6[a], 570[b], 556 ± 6[c] (540 ± 8)[d]
RH2-1	466.5 ± 1.5		
RH2-2	475.7 ± 0.5		
RH2-3	488.0 ± 0.0		
RH2-4	504.9 ± 0.7		
		SD cone	473 ± 5[a], 480[b], 478 ± 9[c]
SWS1	354.6 ± 0.5	SS cone	362 ± 3[b]
SWS2	416.0 ± 1.0	LS cone	407 ± 2[a], 415[b], 417 ± 5[c]
RH1	500.6 ± 0.5	Rod	502 ± 4[a], 501 ± 5[c]

[a] Measured by Cameron (2002).
[b] Measured by Robinson *et al.* (1993).
[c] Measured by Nawrocki *et al.* (1985).
[d] Measured by Nawrocki *et al.* (1985) for larvae (6–8 dpf).

the λ phage vector EMBL3 (Hamaoka *et al.* 2002). For the probe preparation, the cDNAs encoding full coding regions of the LWS/MWS, RH2, SWS2, and SWS1 opsin genes were isolated from the zebrafish ocular RNA by reverse-transcription (RT) polymerase chain reaction (PCR) using the oligonucleotide primers designed on the basis of the published nucleotide sequences of corresponding zebrafish opsin cDNAs, zfred (LWS/MWS), zfgr1 (RH2), zfgr2 (RH2), zfblue (SWS2), and zfuv (SWS1; Vihtelic *et al.* 1999).

The cDNA probes were labeled with [α-^{32}P]dCTP using the random primer method. Plaque hybridization was carried out at 65° in the solution consisting of 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 5 μg/ml *Escherichia coli* DNA. The hybridized membranes were washed in 1× SSC/0.1% SDS at 65° four times (20 min each), which allows ~20% mismatch (Sambrook and Russel 2001). Three overlapping clones, λzf-B31, λzf-B26, and λzf-B29, encompassing a SWS2 gene (*SWS2*) and two LWS/MWS genes (*LWS-1* and *LWS-2*) were isolated (Figure 1). Seven overlapping clones, λzf-C6, λzf-C2, λzf-C7, λzf-C13, λzf-C1, λzf-C3, and λzf-C16, were isolated encompassing four RH2 genes (*RH2-1*, *RH2-2*, *RH2-3*, and *RH2-4*). Two overlapping clones, λzf-A34 and λzf-A7, encompassing a SWS1 gene (*SWS1*) were isolated. After restriction mapping of these clones, the restriction fragments hybridized to the screening probes were subcloned into the pBluescript II (SK−) plasmid vector (Stratagene, La Jolla, CA). Sequencing of these subclones was carried out for both strands, using a Thermo Sequenase cycle sequencing kit (Amersham, Piscataway, NJ) with dye-labeled primers and the LI-COR 4200L-1 automated DNA sequencer.

Genomic PCR cloning: A 1.9-kb genomic DNA containing entire *RH2-4* was isolated by the PCR method from the same DNA source used for the genomic library construction. The forward (5′-TGGATCTTTAGCAGGTAGAG-3′) and reverse (5′-TAC AGTACATTTCAACCAAAATA-3′) primers correspond to the 20 nucleotides (nt) immediately upstream of the start codon and the reverse complement sequence of 196–174 nt downstream of the stop codon of zfgr2 of Vihtelic *et al.* (1999), respectively. PCR was carried out at 95° for 5 min followed by 30 cycles of 95° for 30 sec, 55° for 30 sec, and 72° for 3.5 min. The resulting DNA fragment was cloned into pBluescript II (SK−) (designated as PCR-RH2-4, see Figure 1) and was subjected to DNA sequencing as described above. The DNA sequence was confirmed in duplicate PCRs.

Southern hybridization: The genomic DNA was extracted from a zebrafish (strain Tuebingen). Approximately 1 μg per lane of the zebrafish genomic DNA was digested with a restriction enzyme, electrophoresed on a 0.5% agarose gel, and transferred to a positively charged nylon membrane (Biodyne B, Pall) by using the VacuGene vacuum-blotting system (Pharmacia, Piscataway, NJ). For the probe preparation, the first 361 bp of the coding region of zfgr1 (*RH2-1*), corresponding to its exon 1, was PCR amplified from its full-length cDNA clone used in the library screening. Likewise, the exon 1 portions of zfgr2 (*RH2-4*; 361 bp), zfblue (*SWS1*; 340 bp), and zfuv (*SWS2*; 382 bp) were amplified from the corresponding cDNA clones. As for zfred (*LWS-1*), the first 393 bp of the coding region, corresponding to exons 1 and 2, were amplified from the cDNA clone. Exon 1 of *RH2-2* (361 bp) was amplified from a genomic DNA clone containing the gene. The probe labeling, hybridization, and washing were carried out in the same conditions as in the library screening.

Phylogenetic analysis: The deduced amino acid sequences of zebrafish opsins were aligned using CLUSTAL W (Thompson *et al.* 1994) and the alignment was refined visually (Figure 2). Their nucleotide sequences were aligned in accordance with the protein alignment. The percentages of nucleotide differences among the zebrafish opsin genes were calculated according to the alignment by excluding gap sites in pairwise fashion. The dendrogram of zebrafish opsin genes (Figure 3) was constructed from the nucleotide differences by using the neighbor-joining method (Saitou and Nei 1987).

The following nucleotide sequences of the fish LWS/MWS and RH2 genes were retrieved from the GenBank database: goldfish (*Carassius auratus*) LWS/MWS (accession no. L11867) and RH2 (L11865 for GFgr-1 and L11866 for GFgr-2; Johnson *et al.* 1993), carp (*Cyprinus carpio*) LWS/MWS (AB055656), Mexican cavefish (*Astyanax fasciatus*) LWS/MWS (M90075 for R007, M38619–24 for G101, and M60938–40 and M60943–5 for G103; Yokoyama and Yokoyama 1990) and RH2 (S75251–5; Register *et al.* 1994), cichlid (*Dimidiochromis compressiceps*) LWS/MWS (AF247125) and RH2 (AF247121; Carleton and Kocher 2001), tilapia (*Oreochromis niloticus*) LWS/MWS (AF247128) and RH2 (AF247124; Carleton and Kocher 2001), halibut (*Hippoglossus hippoglossus*) LWS/MWS (AF316498) and RH2 (AF156263; Helvik *et al.* 2001), medaka (*Oryzias latipes*) LWS/MWS (AB001604) and RH2 (AB001603; Hisatomi *et al.* 1997), fugu (*Takifugu rubripes*) RH2 (AF-226989), mullet (*Mullus surmuletus*) RH2 (Y18680), and sand goby (*Pomatoschistus minutus*) RH2 (Y18679). As outgroup references of the fish genes, the following genes were used: pigeon (*Columba livia*) LWS/MWS (AF149243–8) and RH2 (AF149232–3), chicken (*Gallus gallus*) LWS/MWS (M62903) and RH2 (M88178), zebra finch (*Taeniopygia guttata*) LWS/MWS (AF222333) and RH2 (AF222330), canary (*Serinus canaria*) LWS/MWS (AJ277925) and RH2 (AJ277924), American chameleon (*Anolis carolinensis*) LWS/MWS (U08131) and RH2 (AF134189-91), gecko (*Phelsuma madagascariensis*) LWS/MWS (AF074043) and RH2 (AF074044), salamander LWS/MWS (*Ambystoma tigrinum*; AF038947), and frog LWS/MWS (*Xenopus laevis*; U90895).

Alignments of the LWS/MWS genes and the RH2 genes were carried out by their deduced amino acid sequences using CLUSTAL W and were refined visually. Their nucleotide sequences were aligned in accordance with the protein alignments. Subsequent phylogenetic analyses were conducted using the MEGA2 program version 2.1 (Nei and Kumar 2000; Kumar *et al.* 2001). The transition/transversion ratios for the

fish LWS/MWS and RH2 opsin genes were calculated to be 1.08 and 0.95, respectively, and the percentage of synonymous nucleotide differences among the fish LWS/MWS genes and among fish RH2 genes were calculated using the modified Nei-Gojobori method (Ina 1995) by setting the transition/transversion ratio at 1.0 for both LWS/MWS and RH2 genes. The number of nucleotide substitutions per site (d) for two sequences was estimated by the Tamura and Nei (1993) method with gap sites excluded in pairwise fashion. The phylogenetic tree was reconstructed by applying the neighbor-joining method to the d values. The reliability of the tree topology was evaluated by bootstrap analysis with 1000 replications. For protein-tree construction, the number of amino acid substitutions per site was estimated by Poisson correction (Nei and Kumar 2000) and the phylogenetic tree was reconstructed by the neighbor-joining method with 1000 bootstrap replications.

Visual pigment reconstitution: Using the total RNA prepared from the eyes of an adult zebrafish (strain AB), which is a different individual from that used for construction of the genomic library, we synthesized the first-strand cDNA by using a poly(dT) primer {5′-AAGCAGTGGTAACAACGCAGAG TACT(30)*VN*-3′ [V = A, G, or C; N = A, C, G, or T; T(30), 30 succession of T]}. The sequences of forward and reverse primers (external primers) specific to *LWS-1*, *LWS-2*, *RH2-1*, *RH2-2*, *RH2-3*, *RH2-4*, *SWS1*, *SWS2*, and *RH1* were taken from immediately upstream of their start codons and immediately downstream of their stop codons, respectively (Table 2). PCR was carried out using the high-fidelity Pyrobest DNA polymerase (TaKaRa, Berkeley, CA) at 95° for 5 min followed by 35 cycles of 94° for 30 sec, 55° for 5–60 sec, and 72° for 1 min. The resulting DNA fragments were cloned into the pBluescript II (SK−) plasmids and were subjected to DNA sequencing as described above. The DNA sequences were confirmed in duplicate PCRs. The entire coding regions of the cDNAs were further amplified from these pBluescript-cDNA clones by the forward and reverse primers (internal primers) set in 5′- and 3′-edges of the coding regions, respectively (Table 2). The forward and reverse primers contain *Eco*RI and *Sal*I linkers, respectively, and the Kozak (1984) sequence was inserted between *Eco*RI and the initiation codon in the forward primers to promote translation as previously described (Kawamura and Yokoyama 1998). The amplified DNA fragments were cloned into the *Eco*RI/*Sal*I-digested pMT5 expression vector (Khorana *et al.* 1988), which contains the last 15 amino acids of the bovine rod opsin necessary for immunoaffinity purification by 1D4 monoclonal antibody (Molday and Mackenzie 1983). The nucleotide sequences of the pMT5-cDNA clones were confirmed to match those of the template pBluescript-cDNA clones.

The pMT5-cDNA clones were expressed in cultured COS-1 cells (RIKEN Cell Bank, Tsukuba, Japan), incubated with 11-*cis* retinal, and the resulting pigments were purified using the immobilized 1D4 (The Cell Culture Center, Minneapolis) by following the method described in Kawamura and Yokoyama (1998) with minor modifications. The UV-visible absorption spectra of the visual pigments were recorded at 20° at least five times for each pigment using the Hitachi U3010 dual-beam spectrometer.

Quantitation of mRNA expression level by real-time RT-PCR: Real-time PCR was carried out using the Smart Cycler system (Applied Cepheid), where the amount of the PCR product was monitored through progression of PCR cycles by the fluorescence intensity of SYBR Green I (Molecular Probes, Eugene, OR) intercalated in the double-stranded DNA. The cycle number where the secondary derived function of the fluorescence intensity gives the highest peak was defined as the threshold cycle that most effectively reflects the initial amount of the target DNA. For each of the nine opsin genes, *LWS-1*, *LWS-2*, *RH2-1*, *RH2-2*, *RH2-3*, *RH2-4*, *SWS1*, *SWS2*, and *RH1*, the pBluescript-cDNA clones isolated for visual pigment reconstitution were used for the standard templates. The cDNA clones of the known concentrations were prepared for a series of dilutions over three orders of magnitude. The real-time PCR was conducted for each opsin cDNA using the gene-specific primer pairs (the internal forward and the external reverse primers for *RH1* and the external primer pairs for the others; see Table 2) by 40 cycles of 95° for 30 sec, 55° for 30 sec, 72° for 60 sec, and 80° for 8 sec. The reaction contained 1.25 units of R-PCR Ex Taq polymerase (TaKaRa), 1× R-PCR buffer (TaKaRa), 3 mM of $MgCl_2$, 0.3 mM each of dNTP, 1:30,000 dilution of SYBR Green I (Molecular Probes), and 0.5 μM each of the forward and reverse primers in a total volume of 25 μl. The standard regression line was obtained for each opsin gene, which showed the negative linear correlation between logarithmic values of the initial DNA concentrations and the threshold cycles. For all opsin genes, values of the correlation coefficient were between −0.99 and −1.0.

To estimate the relative amount of mRNA among the nine opsin genes from the DNA amount evaluated by the real-time PCR, the difference of the RT efficiencies among the nine opsin genes was evaluated. The sense-strand RNA was transcribed *in vitro* from the pBluescript-cDNA clones of the nine opsin genes. The reaction was carried out using 1 μg of the linearized cDNA, T3 or T7 RNA polymerase (Epicentre, Madison, WI), 1× reaction buffer (Epicentre), 7.5 mM each NTPs, and 10 mM DTT in a total volume of 20 μl at 37° for 2 hr. The concentration of the transcribed RNA was determined by measuring the optical density at 260 nm. Then 120 ng of *LWS-2* and 60 ng each of the other eight opsin RNAs were mixed together and were reverse transcribed, using their gene-specific external reverse primers (Table 2) simultaneously in the same tube to set the reaction conditions identically among the nine genes. The reaction was carried out at 42° for 90 min in a total volume of 20 μl containing 5 units of ReverTra Ace reverse transcriptase (Toyobo), 1× ReverTra Ace buffer (Toyobo), 1 mM each of dNTP, and 0.1 μM each of the primers. After the reaction, 80 μl of water was added and the solution was incubated at 72° for 7 min. Two microliters from 100 μl of the RT solution was subjected to the real-time PCR. The real-time PCR was carried out in the conditions described above two to three times for each opsin gene. The initial DNA amount in the PCR solution was estimated using the standard regression line for each opsin gene. The ratio (R) of the estimated DNA amount to the original RNA amount was calculated for each opsin gene with standard deviations (r). Then ratio of R in pigment X (R_x) to R in *LWS-1* (R_{LWS1}) was calculated ($R_x/R_{LWS1} \equiv A_x$) and the standard deviation of A_x was given by $(R_x/R_{LWS1})\sqrt{(r_x/R_x)^2 + (r_{LWS1}/R_{LWS1})^2} \equiv a_x$. The A_x values were averaged for five RT reactions ($\Sigma_{i=1}^{5} A_{xi}/5 \equiv \overline{A_x}$) and the standard deviations of $\overline{A_x}$ values were given by $\sqrt{\Sigma_{i=1}^{5}(A_{xi} - \overline{A_x})^2/4} + \sqrt{\Sigma_{i=1}^{5} a_{xi}^2/5}$ ($\equiv \overline{a_x}$), where A_{xi} and a_{xi} stand for A_x and a_x values at the ith RT experiment, respectively. The $\overline{A_x} \pm \overline{a_x}$, values were defined as the relative RT efficiencies.

Three adult fish (1 year old, strain AB) were killed at 1.5 hr after onset of the light and three young adults (2 months old, strain AB) were killed at 7 hr after the light onset, all of which had been raised under 14-hr light/10-hr dark cycles. For each fish, the nine opsin mRNAs were reverse transcribed from the total ocular RNA (~1 μg), using the gene-specific reverse primers (the external reverse primers in Table 2) simultaneously in the same tube in the conditions described above. The real-time PCR was carried out three times for each opsin gene in the conditions described above. The initial DNA amount of pigment X (D_x) in the PCR solution was estimated from the standard regression line for each opsin gene. The D_x values were averaged for the three PCR reactions ($\overline{D_x}$) and

TABLE 2

List of the oligonucleotide PCR primers used for the visual pigment reconstitution

Opsin	Primers
LWS-1	(External forward) TATACAACAAACCCCAAAAA
	(External reverse) ACTCAGATCTGGTGCACAAC
	(Internal forward) CAACAAGAATT*CCACC***ATG**GCAGAGCATTGGGGAGA
	(Internal reverse) TGCACAGTCGACGCAGGAGCCACAGAAGACAC
LWS-2	(External forward) TTGATCTTCAGGGTTTCCAAA
	(External reverse) GAGCTATAAATCACGTAAGAT
	(Internal forward) CTTCAGGAATT*CCACC***ATG**GCAGAGTGGGCCAATGC
	(Internal reverse) same with *LWS-1*
RH2-1	(External forward) TAATTTAGGTTTCAGTAAAA
	(External reverse) GGAGCACTGAATAGGCAGAT
	(Internal forward) TTAGGTGAATT*CCACC***ATG**AACGGGACAGAAGGGAG
	(Internal reverse) AGGCAGGTCGACGCAGGAGACACAGAGGACAC
RH2-2	(External forward) CGTGCAACACCTCCAGCAGG
	(External reverse) AGCATAGTTGGCAATTAGTT
	(Internal forward) CAACACGAATT*CCACC***ATG**AACGGCACCGAAGGAAA
	(Internal reverse) AATTAGGTCGACGCTGGAGACACAGAGGACAC
RH2-3	(External forward) TGGATCACTAGCAGGCAGAG
	(External reverse) CACAAAGAAGTTCAAAAAGT
	(Internal forward) TCTTTAGAATT*CCACC***ATG**AACGGCACTGAAGGAAA
	(Internal reverse) TCAAAAGTCGACGCTGGAGATACAGAAGACAC
RH2-4	(External forward) TGGATCTTTAGCAGGTAGAG
	(External reverse) CTGTAAAGAAGTTCAAAAGT
	(Internal forward) same with *RH2-3*
	(Internal reverse) same with *RH2-3*
SWS1	(External forward) AGGCCTCCAACGGCACAACC
	(External reverse) TAAATGTGCTGCGGGAGGAT
	(Internal forward) CTCCAAGAATT*CCACC***ATG**GACGCGTGGGCCGTTCA
	(Internal reverse) GGGAGGGTCGACGCAGACACAGATGAGGTTTC
SWS2	(External forward) GGGCACCAATTACAAGCAAG
	(External reverse) AGGTTACATGAGAACTGTGT
	(Internal forward) ACCAATGAATT*CCACC***ATG**AAGCAACAACAGCAAAC
	(Internal reverse) AACTGTGTCGACTTCTCTGGCGCAACAGAGGA
RH1	(External forward) CGCCACATCCAACCGCAGCC
	(External reverse) AGAGTGTCTGGAAGGAGAGT
	(Internal forward) ACATCCGAATT*CCACC***ATG**AACGGTACAGAGGGACC
	(Internal reverse) AGGAGAGTCGACGCCGGAGACACGGAGCTGGA

*Eco*RI and *Sal*I linkers are indicated with single and double underlines, respectively, the Kozak sequence with italic letters, and the initiation codons with boldface letters.

standard deviation (d_x) was calculated. The relative RNA amount was given by dividing $\overline{D}_x$ by the relative RT efficiency ($\overline{D}_x/\overline{A}_x \equiv E_x$) with the standard deviations given as $(\overline{D}_x/\overline{A}_x)\sqrt{(d_x/\overline{D}_x)^2 + (\overline{a}_x/\overline{A}_x)^2} \equiv e_x$. Finally the E_x values were normalized to set that of *LWS-1* as one and those of the other opsin genes as the relative values to it as $E_x/E_{LWS1} \pm (E_x/E_{LWS1})\sqrt{(e_x/E_x)^2 + (e_{LWS1}/E_{LWS1})^2}$.

RESULTS

Isolation of genomic DNA clones of zebrafish cone opsin genes: From the genomic library of a zebrafish, we isolated two LWS/MWS opsin genes, *LWS-1* and *LWS-2*; four RH2 opsin genes, *RH2-1*, *RH2-2*, *RH2-3*, and *RH2-4*; and one each of SWS1 and SWS2 opsin genes, *SWS1* and *SWS2*, respectively (Figure 1). Among them *SWS2*, *LWS-1*, and *LWS-2* genes were found to be linked in tandem and *RH2-1*, *RH2-2*, *RH2-3*, and *RH2-4* genes were likewise located in tandem. The distance between *SWS2* and *LWS-1*, evaluated by nucleotide sequencing and restriction mapping as that between the stop codon of the former and the start codon of the latter, is ~2.5 kb. That between *LWS-1* and the *LWS-2* is ~1.8 kb. The distances between *RH2-1* and *RH2-2*, *RH2-2* and *RH2-3*, and *RH2-3* and *RH2-4* are ~2.8, ~2.6, and ~12 kb, respectively. The two LWS/MWS genes contain six putative exons and five introns whereas the others contain five exons and four introns (Figure 1). These exon-intron structures are highly conserved among the vertebrate visual opsin genes (Yokoyama 2000). In all genes splice junction signals (GT/AG) are conserved in all introns and there is no nonsense mutation in the coding regions. Functionally important residues are also con-

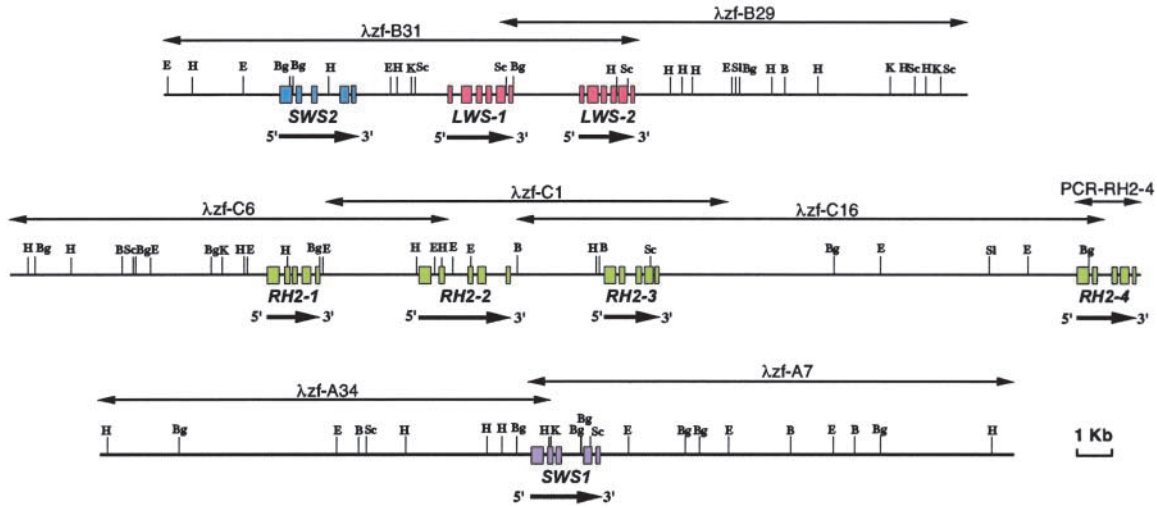

FIGURE 1.—The physical maps of the zebrafish cone opsin genes. The coding regions are indicated by solid boxes with red, green, blue, and violet colors for LWS/MWS, RH2, SWS2, and SWS1 genes, respectively. The gene names are indicated below the boxes with their transcriptional orientations. The genomic regions covered by the λphage and PCR clones are indicated above the physical maps. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Sc, *Sac*I; Sl, *Sal*I.

served, which include a lysine for the Schiff-base linkage to the chromophore in the seventh transmembrane domain (WANG *et al.* 1980), a glutamate residue for the Schiff-base counter ion in the third transmembrane domain (SAKMAR *et al.* 1989; ZHUKOVSKY and OPRIAN 1989), two cysteine residues for the disulfide bond in the first and second extracellular loops (KARNIK *et al.* 1988), and multiple serines and threonines in the C-terminal region for the targets of opsin kinase (OHGURO *et al.* 1994) (Figure 2).

Comparison of the genomic DNA clones to the previously reported cDNA clones: VIHTELIC *et al.* (1999) isolated one species each of cDNA encoding LWS/MWS (zfred), SWS1 (zfuv), and SWS2 (zfblue) opsins and two cDNA species encoding RH2 (zfgr1 and zfgr2) opsins. Figure 3 shows a dendrogram representing the percentage of nucleotide differences among the coding regions of the five cDNA and the eight genomic opsin genes. zfred is 100% identical to *LWS-1*. *LWS-2* is 7.0% different from *LWS-1* (and zfred) and has a 1-amino-acid deletion in the deduced N-terminal region (Figure 2). zfgr1 is most similar to *RH2-1* (99.6% identity) and secondary to *RH2-2* (85.0%) while zfgr2 is most similar to *RH2-4* (99.4%) and secondary to RH2-3 (92.6%; Figure 3). When dividing RH2 genes into two groups, zfgr1/*RH2-1*/*RH2-2* and *RH2-3*/*RH2-4*/zfgr2, the nucleotide identities between them range from 76.4 to 78.8%. zfblue and zfuv are 99.9 and 99.6% identical to *SWS2* and *SWS1*, respectively. These percentages of identities among the sequences strongly suggest that zfred, zfgr1, zfgr2, zfblue, and zfuv cDNAs of VIHTELIC *et al.* (1999) represent *LWS-1*, *RH2-1*, *RH2-4*, *SWS1*, and *SWS2* genes, respectively, in the genome and indicate that *LWS-2*, *RH2-2*, and *RH2-3* were newly identified in this study.

The number of different nucleotides in the coding region between zfgr1 and *RH2-1* is four, among which three are synonymous [T in the former and C in the latter at site 447 (denoted T447C), A459G, and T504A] and one is nonsynonymous [T864G resulting in Ile in the former and Met in the latter at amino acid residue 288 (denoted Ile288Met)]. Met at residue 288 of *RH2-1* (Figure 2) is highly conserved among vertebrate RH2 opsins including those of birds and reptiles. That between zfgr2 and *RH2-4* is six, among which five are synonymous (C72T, A108G, C514T, G540A, and G672A) and one is nonsynonymous (C526T resulting in Pro176Ser). In this case, Ser at residue 176 of *RH2-4* (Figure 2) is completely conserved among all vertebrate visual opsins examined so far except zfgr2. There is only one synonymous difference between zfblue and *SWS2* (G342A). The number of differences between zfuv and *SWS1* is four, all of which are nonsynonymous (C263T, C290T, T310G, and C875G, resulting in Ser88Phe, Ser97Phe, Ser104Ala, and Ser292Cys, respectively). Phe at residue 88 and Cys at residue 292 of *SWS1* (Figure 2) are completely conserved among all vertebrate SWS1 opsins examined to date except zfuv, while Phe at residue 97 and Ala at residue 104 of *SWS1* (Figure 2) are conserved among all fish SWS1 opsins but zfuv. These amino acids observed in *RH2-1*, *RH2-4*, and *SWS1* were also observed in the corresponding cDNA clones isolated from zebrafish eyes in this study (see below). These results led us to infer that these amino acid differences may be due to cloning artifacts in zfgr1, zfgr2, and zfuv.

Genomic Southern analysis of zebrafish opsin genes: To examine whether yet other related opsin genes are in the zebrafish genome, we performed Southern blot analysis for the genomic DNA. *LWS-1* and *LWS-2* are 93% identical in the coding region and both of the genes should be detected when the *LWS-1* (zfred) cDNA frag-

FIGURE 2.—Alignment of the deduced amino acid sequences of the zebrafish cone opsins. Gaps necessary to optimize the alignment are indicated by dashes. The seven transmembrane domains (HARGRAVE *et al.* 1983) are indicated by horizontal lines above the sequences. A Lys (K) residue for the Schiff-base linkage to the chromophore in the seventh transmembrane domain, a Glu (E) residue for the Schiff-base counter ion in the third transmembrane domain, and two Cys (C) residues for the disulfide bond in the first and second extracellular loops are highlighted with boldface letters. The spectral-tuning residues Ala (A) 177 of *LWS-1* and A 176 and Phe (F) 273 of *LWS-2* are indicated in red and E 122 of *RH2-4* is in green. The amino acid differences of *RH2-1* from zfgr1 [Met (M) 288], of *RH2-4* from zfgr2 [Ser (S) 176], and of *SWS1* from zfuv (F 88, F 97, A 104, and C 292) are highlighted with underlines.

ment is used as a probe. As expected, the probe detected two bands, sizes of which correspond to the cloned *LWS-1* and *LWS-2* (Figures 1 and 4). Likewise, the *RH2-4* (zfgr2) probe detected both *RH2-3* and *RH2-4* (92.8% identical in the coding region; Figures 1 and 4). Because of the lower similarity between *RH2-1* and *RH2-2* (85.3%), it was necessary to examine the two genes separately. The *RH2-1* and *RH2-2* probes, as well as the *SWS1* and *SWS2* probes, detected only one band, respectively, the size of which matches the corresponding gene cloned (Figures 1 and 4). When the genomic DNA was examined with other restriction enzymes, we observed only hybridization bands corresponding to the cloned genes (data not shown). The same hybridization pattern was observed when hybridization and washing were carried out under the low-stringency conditions, allowing ~30% mismatch (data not shown). These results using the Tuebingen strain were consistent with those using other strains (AB, WIK, and TL; data not shown) and strongly suggest that zebrafish has no cone opsin genes other than the cloned ones in the genome.

Phylogenetic positions of *LWS-1* and *LWS-2*: Figure 5A shows a phylogenetic tree of the fish LWS/MWS genes where the phylogenetic root was given by the pigeon LWS/MWS gene. For construction of the phylogenetic tree we used nucleotide sequences of the entire coding regions because synonymous nucleotide differences among the fish genes are below saturation level, ranging from 5.5 to 63.4% (47.5% on average), and were considered to retain phylogenetic information. The reconstructed tree supports the clustering of *LWS-1* and *LWS-2* with 100% bootstrap probability, strongly

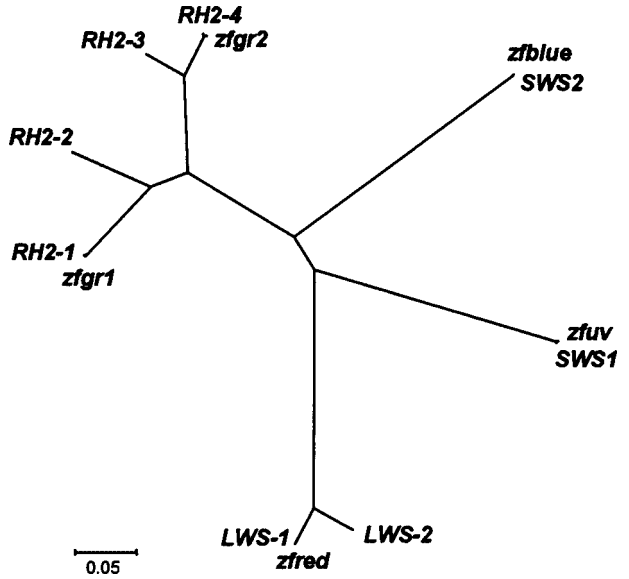

FIGURE 3.—The sequence relatedness among the zebrafish cone opsin genes isolated by VIHTELIC *et al.* (1999), zfred, zfgr1, zfgr2, zfblue, and zfuv; and in this study, *LWS-1*, *LWS-2*, *RH2-1*, *RH2-2*, *RH2-3*, *RH2-4*, *SWS1*, and *SWS2*. Scale bar indicates 5% nucleotide difference.

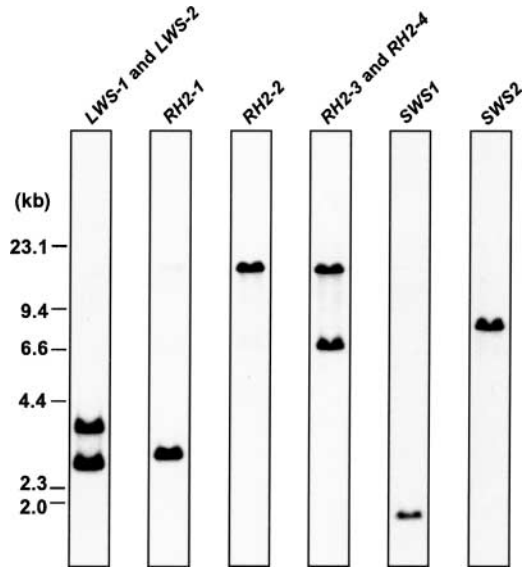

FIGURE 4.—Southern hybridization of the zebrafish genomic DNA to the *LWS-1*, *RH2-1*, *RH2-2*, *RH2-4*, *SWS1*, and *SWS2* probes. The exon-1 DNA fragments are used as the probes (however, exons 1 and 2 are used for *LWS-1*). The *LWS-1* and *LWS-2* and *RH2-3* and *RH2-4* tracks represent the hybridizations to the *LWS-1* and *RH2-4* probes, respectively. The genomic DNA in the *LWS-1* and *LWS-2* track is digested with *Sac*I while the DNA in the other tracks are with *Bgl*II. λ*Hin*dIII size standards are indicated in kilobases.

suggesting that the *LWS-1/LWS-2* gene duplication occurred in zebrafish lineage after its separation from the common ancestor of goldfish and carp (Figure 5A). A virtually identical tree was obtained when Jukes and Cantor's, Kimura's two-parameter, Tajima and Nei's, and Tamura's methods (NEI and KUMAR 2000) were used for estimating evolutionary distances (*d* values) and when amphibian, reptile, or other bird species was used as an outgroup reference (data not shown). When the protein sequences were used, topology of the tree was the same except that medaka was most closely related to cichlid but with low bootstrap probability (~50%; data not shown).

By the phylogenetic analysis of Mexican cavefish and goldfish opsin genes, REGISTER *et al.* (1994) noted that the gene duplication of Mexican cavefish LWS/MWS genes, which led to R007 and an ancestral gene of G101 and G103, predated the speciation leading to Mexican cavefish and goldfish. They suggested that goldfish has an additional gene orthologous to the cavefish G101 and G103 genes. The ancient duplication leading to the cavefish R007 and G101/G103 was also supported with 99% bootstrap probability in our phylogenetic tree (Figure 5A), where the duplication occurs even before the separation of the superorders Ostariophysi (including zebrafish, goldfish, and carp) and Acanthopterygii (including medaka, halibut, tilapia, and cichlid; NELSON 1994). This suggests the presence of orthologous genes to G101/G103 in all fishes belonging to the subdivision Euteleostei unless the genes have been lost. Overall nucleotide differences between G101 (or G103) and the other fish LWS/MWS genes examined in this study ranged from 22.2 to 26.0%. In the zebrafish genome we could detect only *LWS-1* and *LWS-2* for LWS/MWS group of genes even in the low-stringency hybridization conditions that allow ~30% mismatch. Thus we suppose that the orthologous gene to the cavefish G101/G103 was lost from the zebrafish genome.

Phylogenetic positions of the four RH2 genes of zebrafish: Figure 5B shows a phylogenetic tree of the fish RH2 genes where the phylogenetic root was given by the pigeon RH2 gene. Synonymous nucleotide differences among the fish genes are below saturation level, ranging from 8.3 to 65.1% (52.4% on average), and the nucleotide sequence of the entire coding region was used for reconstructing the phylogenetic tree. In the tree zebrafish *RH2-3* and *RH2-4* and goldfish GFgr-1 and GFgr-2 form separate clusters with high bootstrap supports (100 and 99%, respectively), strongly suggesting that these gene duplications occurred independently in zebrafish and goldfish lineages. A cluster consisting of *RH2-3*/*RH2-4* and GFgr-1/GFgr-2 is also highly reliable with 100% bootstrap probability. Zebrafish *RH2-1* and *RH2-2* form a cluster with 100% bootstrap support. The tree strongly suggests that gene duplication leading to an ancestral gene of zebrafish *RH2-1* and *RH2-2* and of *RH2-3* and *RH2-4* occurred before the speciation leading to goldfish and zebrafish and after the divergence between Cypriniformes (including goldfish and zebrafish) and Characiformes (including Mexican cavefish). This implies that goldfish has additional RH2 gene(s)

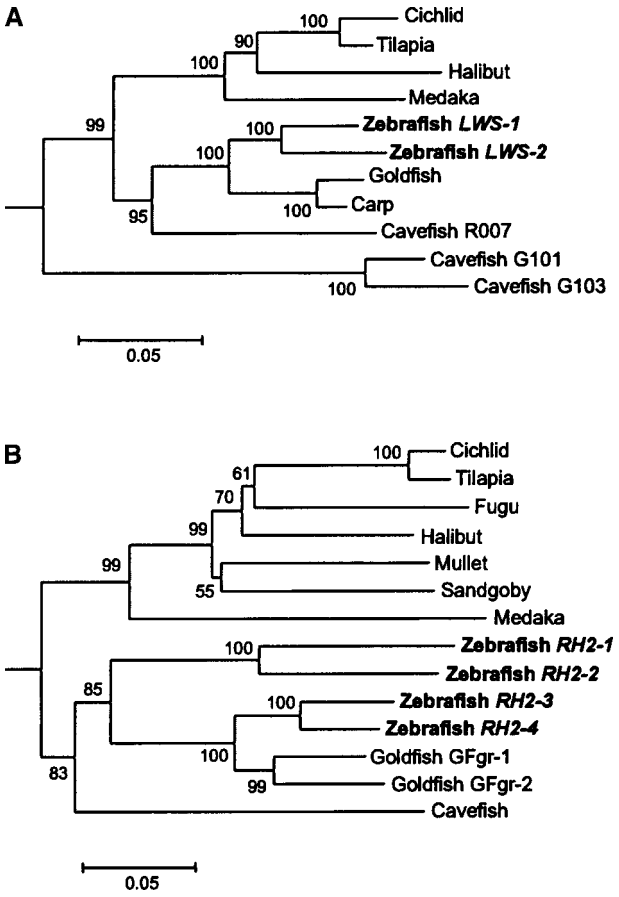

FIGURE 5.—Phylogenetic trees of the fish LWS/MWS (A) and RH2 (B) genes based on their nucleotide sequences. The zebrafish genes are highlighted with boldface letters. The bootstrap probabilities are given to each node. Scale bar, five nucleotide substitutions per 100 sites.

orthologous to zebrafish *RH2-1* and *RH2-2*. As in the case of LWS/MWS genes, we obtained the same tree topology when using other estimation methods of evolutionary distances. When using reptile genes as outgroups, the position of Mexican cavefish changed to outside of all the other fish genes (data not shown). However, the bootstrap support for the branch node was low (40–64%). When we used the protein sequences, the relationship among zebrafish, goldfish, and cavefish genes varied depending on outgroup genes chosen. In any of the protein tree topologies, bootstrap values of the clusterings were generally low (14–77%) for the genes except for the *RH2-1*/*RH2-2* clustering (100%).

Spectral properties of the reconstituted photopigments: The ocular RNA was extracted from a zebrafish that is different from that used for constructing the genomic library. The cDNA clones of *LWS-1*, *LWS-2*, *RH2-1*, *RH2-2*, *RH2-3*, *RH2-4*, *SWS1*, *SWS2*, and *RH1* were isolated by RT-PCR from the RNA using the gene-specific external primers (Table 2). The deduced amino acid sequences of the cDNAs were identical to those of the corresponding genomic clones in all genes except in *RH2-2* and *RH2-3*. The discrepancies between the genomic and the cDNA clones of *RH2-2* were at residue position 198 [Phe in the genomic clone and Tyr in the cDNA clone (denoted Phe/Tyr)] and at residue 332 (Glu/Asp). Those of *RH2-3* were at residue 166 (Ser/Ala) and at residue 173 (Val/Phe). These cDNA sequences were confirmed in independent RT-PCR experiments. It was noted that these sites were varied among the known RH2 opsins of vertebrates and there was no apparent association between the residues and the peak absorption spectra (λmax). Thus, the differences between the cDNA and genomic sequences were interpreted as naturally occurring neutral polymorphisms rather than as the cloning artifacts.

Absorption spectra of the reconstituted visual pigments are shown in Figure 6. The pigments show spectra with a prominent absorption peak in addition to a protein absorbance at 280 nm. The λmax values were directly measured from the dark spectra (Table 1). When the reconstituted pigments were bleached by light, new absorption peaks of 380 nm were achieved, showing that 11-*cis* retinal in the pigments was isomerized by light and all-*trans* retinal was released. Insets of Figure 6 show the dark-light difference spectra where the post-bleaching absorption maxima appear as a negative peak at around 380 nm. These demonstrate that the reconstituted pigments are in fact photosensitive. The λmax values measured from the difference spectra are 558.7 ± 1.3 nm (*LWS-1*), 551.2 ± 1.5 nm (*LWS-2*), 472.7 ± 1.6 nm (*RH2-1*), 479.7 ± 1.9 nm (*RH2-2*), 488.6 ± 0.5 nm (*RH2-3*), 508.1 ± 3.2 nm (*RH2-4*), 344.9 ± 2.1 nm (*SWS1*), 428.2 ± 0.4 nm (*SWS2*), and 502.1 ± 0.6 nm (*RH1*). These values are close to those from dark spectra, except for *SWS1*, *SWS2*, and *RH2-1*, where the peak positions are affected by a post-bleaching absorption curve in the subtraction because pre- and post-bleaching peaks are not sufficiently distant from each other.

When *SWS1* pigment was denatured by sulfuric acid to eliminate the opsin-induced spectral shift, the resulting dark spectrum had a peak absorbance at 440 nm (data not shown), which is identical to that of a protonated Schiff-base 11-*cis* retinal free in solution (KITO *et al.* 1968). When the acid was added to the pigment after the light exposure, the peak position did not shift from 380 nm, indicating that all-*trans* retinal had been dissociated from opsin before the acid was added. The 355-nm peak appeared only in the *SWS1* transfection experiment. These observations indicate that the 355-nm peak in the *SWS1* dark spectrum (Figure 6) is achieved by the reconstituted pigment itself but not by the residual free 11-*cis* retinal in the solution or by the residual 11-*cis* retinal, which formed random Schiff-base adducts with other proteins.

Relative expression levels among zebrafish opsin genes: To estimate relative expression levels among the nine visual opsin genes in the eye by the real-time RT-PCR, we first evaluated the relative RT efficiencies

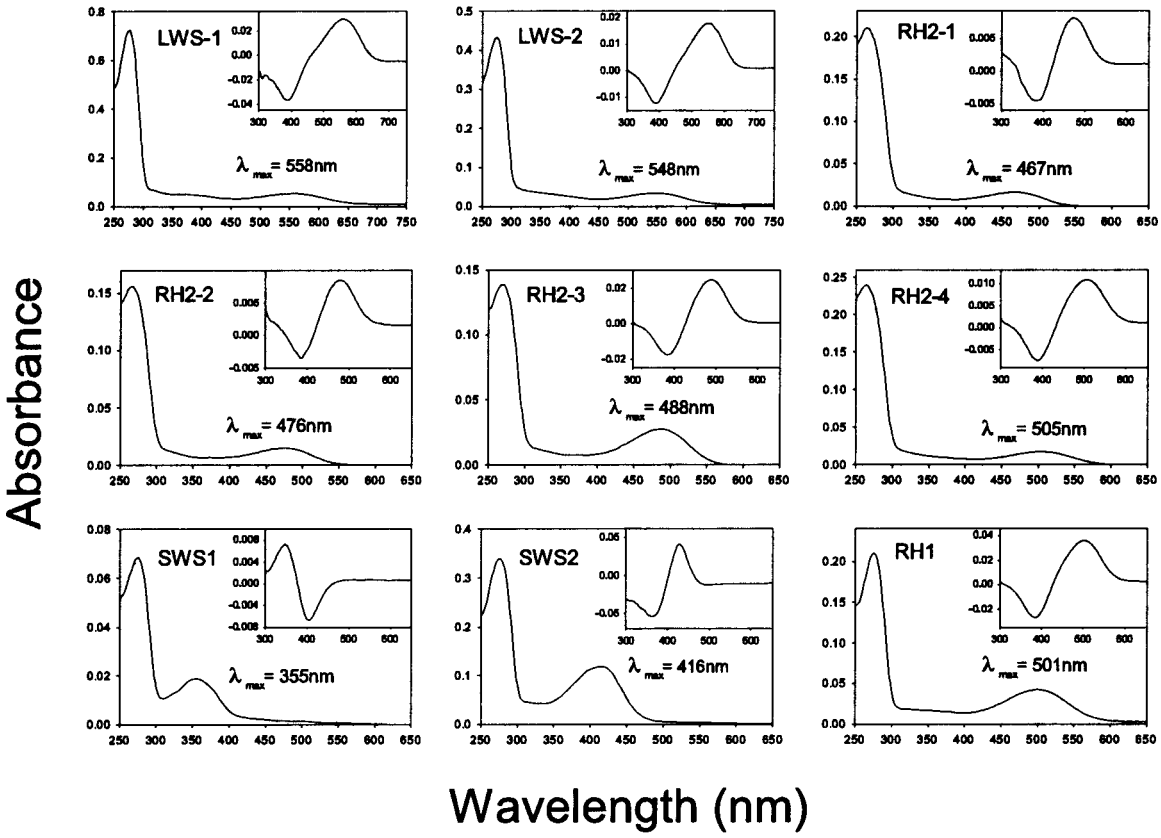

FIGURE 6.—Absorption spectra of the reconstituted visual pigments of zebrafish measured in the dark. Insets show the dark-light difference spectra.

among them. It was noted that the efficiencies differed markedly among the genes (Figure 7). We examined three adult fish (1 year old) killed 1.5 hr after onset of the light (denoted group A) and three young adults (2 months old) killed 7 hr after the light onset (group B), all of which had been raised under the 14-hr light/10-hr dark cycle. Since the expressional patterns were similar among the three fish within each group, the expression levels of one individual from each group (A and B) are shown in Figure 8, A and B, respectively. The expression levels of the rod opsin gene, *RH1*, were out of the scale and were not included in the figure. In both groups, expression levels of *RH2-2* and *SWS2* were significantly higher than those of the others, among which *SWS1* expression level was significantly higher than those of the rest. Notably, expression levels of the two LWS/MWS genes were considerably lower; that of *LWS-2* was even lower than that of *LWS-1*. Among the four RH2 genes, expression levels of *RH2-1*, *RH2-3*, and *RH2-4* were much lower than that of *RH2-2*. The difference between groups A and B appeared to be the lower relative expression levels of *RH2-2*, *SWS2*, and *RH1* in group B than in group A. It is not clear from the present data set whether the difference is due to the age of the fish or the circadian time when the measurement was carried out. However, irrespective of these differences, the relative expression levels among the opsin genes are common between the two groups.

DISCUSSION

Gene duplications of zebrafish opsins: It has been suggested that a genome-wide duplication occurred at the base of the teleost radiation (AMORES *et al.* 1998; MEYER and MALAGA-TRILLO 1999; POSTLETHWAIT *et al.* 2000). On top of it, further genome duplications are considered to have occurred in salmonids, goldfish, and carp (LARHAMMAR and RISINGER 1994). We showed here that zebrafish has two LWS/MWS, four RH2, and single SWS1 and SWS2 opsin genes in the genome (Figure 4). The duplications of the LWS/MWS and RH2 genes do not appear to be the result of the genome duplication since the two LWS/MWS genes, *LWS-1* and *LWS-2*, are arrayed in tandem and the four RH2 genes, *RH2-1*, *RH2-2*, *RH2-3*, and *RH2-4*, form another tandem gene cluster (Figure 1). These results suggest that all counterparts of the opsin gene pairs by the whole-genome duplication have been lost or become pseudogenes by mutations, as in the case of many other genes in the zebrafish genome (~80% of the gene pairs are considered to have lost their counterparts; POSTLETHWAIT *et al.* 2000).

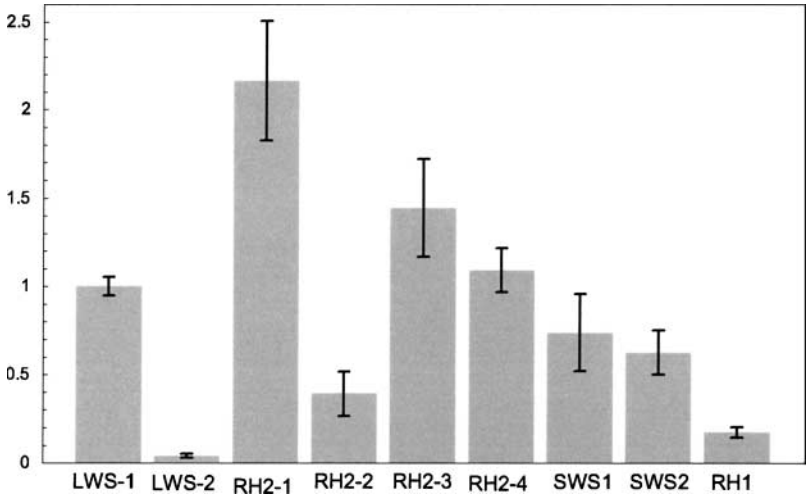

FIGURE 7.—The relative efficiencies of reverse transcription among the zebrafish opsin genes. The efficiencies are given as that of *LWS-1* as 1. *LWS-1*, 1.00 ± 0.05; *LWS-2*, $4.08 \times 10^{-2} \pm 1.05 \times 10^{-2}$; *RH2-1*, 2.17 ± 0.34; *RH2-2*, $3.92 \times 10^{-1} \pm 1.23 \times 10^{-1}$; *RH2-3*, 1.45 ± 0.28; *RH2-4*, 1.09 ± 0.12; *SWS1*, $7.39 \times 10^{-1} \pm 2.20 \times 10^{-1}$; *SWS2*, $6.26 \times 10^{-1} \pm 1.25 \times 10^{-1}$; *RH1*, $1.74 \times 10^{-1} \pm 0.29 \times 10^{-1}$.

Besides LWS/MWS genes of higher primates, gene duplications in the five groups of visual opsins have been documented only for fishes: LWS/MWS of Mexican cavefish (R007, G101, and G103; YOKOYAMA and YOKOYAMA 1990), RH2 of goldfish (GFgr-1 and GFgr-2; JOHNSON *et al.* 1993) and zebrafish (zfgr1 and zfgr2; VIHTELIC *et al.* 1999), SWS2 of cichlid (SWS-2A and SWS-2B; CARLETON and KOCHER 2001), and RH1 of eels (freshwater and deep-sea types; ARCHER *et al.* 1995; ZHANG *et al.* 2000). However, a thorough genomic survey of fish opsin genes has not been accomplished and positional relationships among these duplicated genes have been largely unknown. Our study on the zebrafish cone opsin genes together with our previous study on the rod opsin gene (*RH1*; HAMAOKA *et al.* 2002) provides the first complete set of information on the genomic organization of the visual opsin genes in fish.

The close linkage between SWS2 and LWS/MWS genes has been documented for Mexican cavefish (YOKOYAMA and YOKOYAMA 1993), cichlid (CARLETON and KOCHER 2001), and pigeon (KAWAMURA *et al.* 1999). While the distances between SWS2 and LWS/MWS genes are longer in the three species (~6 kb) than in zebrafish (2.5 kb), conservation of the linkage is suggestive of the associated expressional regulation between the genes by an analogy from the human LWS/MWS expression system where the red and green opsin genes are regulated by a common enhancer motif, the locus control region (SMALLWOOD *et al.* 2002). With the information on the positional relationship among *SWS2*, *LWS-1*, and

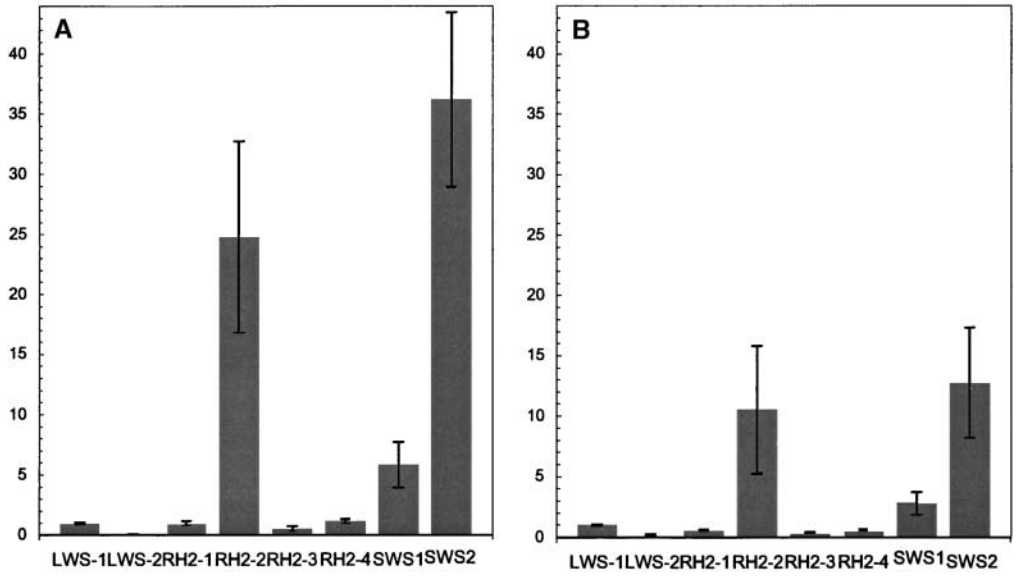

FIGURE 8.—The relative expression levels of the cone opsin genes in the zebrafish eye measured by the real-time RT-PCR for (A) a 1-year-old fish at 1.5 hr after the onset of the light and (B) a 2-month-old fish at 7 hr after it. The expression levels are given as that of *LWS-1* as 1. (A) *LWS-1*, 1.00 ± 0.07; *LWS-2*, 0.07 ± 0.03; *RH2-1*, 0.98 ± 0.16; *RH2-2*, 24.7 ± 8.0; *RH2-3*, 0.52 ± 0.23; *RH2-4*, 1.16 ± 0.18; *SWS1*, 5.8 ± 1.9; *SWS2*, 36.2 ± 7.3. (B) *LWS-1*, 1.00 ± 0.09; *LWS-2*, 0.15 ± 0.11; *RH2-1*, 0.53 ± 0.12; *RH2-2*, 10.5 ± 5.2; *RH2-3*, 0.34 ± 0.07; *RH2-4*, 0.52 ± 0.16; *SWS1*, 2.8 ± 0.9; *SWS2*, 12.7 ± 4.5. The relative expression levels of the rod opsin gene, *RH1*, are 739 ± 135 in A and 76 ± 23 in B. Error bars are 1 SD.

LWS-2 and among *RH2-1*, *RH2-2*, *RH2-3*, and *RH2-4* in zebrafish, the expressional regulation of these genes can be systematically explored by transgenesis using a proper reporter gene, such as green fluorescent protein (GFP), as has been demonstrated for the RH1 gene of zebrafish (Kennedy *et al.* 2001; Hamaoka *et al.* 2002).

Comparison of MSP and *in vitro* measurements of absorption spectra: MSP analyses of zebrafish retinal photoreceptor cells have shown that the long (LD) and short (SD) members of the double-cone cell are red (λmax of ~560 nm) and green sensitive (~480 nm), respectively, while the long-single- (LS) and short-single- (SS) cone cells are blue (~410 nm) and ultraviolet sensitive (~360 nm), respectively (Nawrocki *et al.* 1985; Robinson *et al.* 1993; Cameron 2002; Table 1). The λmax of the rod cell is measured to be ~500 nm (Nawrocki *et al.* 1985; Cameron 2002). Raymond *et al.* (1993) applied the goldfish opsin cRNA probes for *in situ* hybridization to the zebrafish retina. Vihtelic *et al.* (1999) used the antibodies against zebrafish opsins for immunohistochemical analysis. Consistent with the MSP results, both studies detected expression of LWS/MWS (red) opsin in one and RH2 (green) opsin in the other members of the double cones (most likely LD and SD cones, respectively, based on the MSP data) and that of SWS2 (blue), SWS1 (ultraviolet), and RH1 (rod) opsins in the LS cone, SS cone, and rod cells, respectively, in the zebrafish retina.

Because of the high level of the nucleotide sequence identities between *LWS-1* and *LWS-2* and among *RH2-1*, *RH2-2*, *RH2-3*, and *RH2-4*, the cRNA probes and the antibodies should be capable of detecting corresponding groups of the opsin transcripts and proteins, respectively, irrespective of their subtypical differences (Vihtelic *et al.* 1999). Since these hybridization and immunostaining signals are confined to these specific cell types in entire retina, all opsin transcripts detected in the ocular RNA in this study most likely originated only from them. The λmax values of *SWS1* (355 nm), *SWS2* (416 nm), and *RH1* (501 nm) pigments measured *in vitro* are reasonably close to those of the SS cone, LS cone, and rod cells (Table 1), respectively. The fact that the λmax value of SD cones (~480 nm) is closest to that of *RH2-2* pigment (476 nm) is consistent with our quantitative RT-PCR result that the majority of the RH2 transcript in the ocular RNA is from *RH2-2* (Figure 8). Likewise, the λmax of LD cones (~560 nm) is close to that of *LWS-1* pigment (558 nm), which dominates over *LWS-2* in the expression level (Figure 8).

The chromophore of the visual pigments of zebrafish is predominantly 11-*cis* retinal and not the 11-*cis* 3,4-dehydroretinal that shifts the λmax of the pigments to longer wavelength (Nawrocki *et al.* 1985). When zebrafish is housed in cold temperature (22–25°), mixed usage of the two types of chromophore is observed in rod cells but not in cone cells (Saszik and Bilotta 1999). The consistency between MSP and our *in vitro* measurements provides further support for the predominant usage of the retinal-based visual pigments in zebrafish.

It should be noted that Nawrocki *et al.* (1985) observed a remarkable difference in λmax values between developmental stages only for the LD cones, *i.e.*, ~540 nm in early larvae [6–8 days postfertilization (dpf)] and ~560 nm in late larvae (11–17 dpf) and adults (1–2 years old) [the cell type is denoted "SD" in the literature probably due to the misclassification of the isolated cone cells; see Raymond *et al.* (1993) and Robinson *et al.* (1993)]. Although 540 nm is somewhat shorter than the λmax of *LWS-2* pigment (548 nm), this could imply that *LWS-1* and *LWS-2* may be expressed at different developmental stages, with the latter in the early stages and the former in the later stages. Temporal control of the visual pigment production is a rather common feature in fish (Bowmaker 1995) and the differential usage of the duplicated opsin genes at different life stages has been demonstrated for the RH1 (rod) opsin genes of European eel (*Anguilla anguilla*) and Japanese eel (*A. japonica*; Archer *et al.* 1995; Zhang *et al.* 2000).

Blue shift of retinal sensitivity of zebrafish: The electroretinogram measured for adult zebrafish has showed lower sensitivity of the retina to the long-wavelength light (Hughes *et al.* 1998; Cameron 2002). The low level of LWS/MWS RNA (Figure 8) appears to be consistent with this tendency. It has been documented in other organisms that the expression level of opsins oscillates with a circadian rhythm (Pierce *et al.* 1993; Von Schantz *et al.* 1999). Although the expression level of each opsin gene may well oscillate with the circadian time in zebrafish as in some other retina-specific genes (Rajendran *et al.* 1996), the relative scantiness of the LWS/MWS RNA to those of the other pigment groups appears to be irrespective of the circadian time (Figure 8).

In zebrafish, as in other teleost fish, cone cells are arranged in a regular geometric array called a mosaic (Robinson *et al.* 1993). On the basis of the retinal mosaic configuration, the ratio of the cell numbers among LD, SD, LS, and SS cones can be estimated to be 2:2:1:1. If all cone cells express equal amounts of opsin RNA, the ratio of mRNA amount in the retina among LWS/MWS, RH2, SWS2, and SWS1 genes would be likewise 2:2:1:1. Our quantitative RT-PCR result deviated considerably from the expectation, especially in the LWS/MWS group, which is represented at very low level. This suggests that expression of the LWS/MWS opsin genes is downregulated in zebrafish.

It should be noted that the λmax of *SWS2* pigment (416 nm) is relatively short compared to those of many other SWS2 pigments characterized to date (~440 nm) and that the λmax of *RH2-2* pigment (476 nm), a representative RH2 in the zebrafish retina, is also short compared to many other RH2 pigments (~500 nm, except for some nocturnal or deep-sea organisms; see Yokoyama 2000). The low expressional level of the LWS/

MWS genes appears to be consistent with this blue shift of retinal sensitivity.

The amino acid residues responsible for the spectral tuning of LWS/MWS and SWS1 pigments have been well investigated with the site-directed mutagenesis and pigment-reconstitution methodologies (see Yokoyama 2000 for a review), but relatively few studies have been done on the RH2 and SWS2 pigments (Yokoyama *et al.* 1999; Cowing *et al.* 2002). We could assign no relevant amino acid changes from the literature to those in the zebrafish RH2 and SWS2 pigments to explain their spectral shifts, except for the change that could account for the "red shift" of *RH2-4* among the blue-shifted RH2 pigments (Gln to Glu at residue 122, Figure 2; Yokoyama *et al.* 1999). To investigate the molecular mechanisms accounting for the large spectral diversity among the four zebrafish RH2 pigments and the blue shift of zebrafish SWS2, it is of great importance to conduct site-directed mutagenesis for the reconstituted photopigments.

Interestingly, the λmax of *LWS-1* pigment may also be blue shifted according to the "five-sites rule" where the λmax values of the LWS/MWS pigments are largely determined by the five amino acid residues at positions 180, 197, 277, 285, and 308 (the residue numbers represented by those in the human red opsin; Yokoyama and Radlwimmer 1998, 1999, 2001). In typical "red" opsins, these sites are occupied by amino acids Ser, His, Tyr, Thr, and Ala, respectively. The amino acid changes from Ser to Ala at 180 (denoted Ser180Ala), His197Tyr, Tyr277Phe, Thr285Ala, and Ala308Ser shift the λmax values toward blue by 7, 28, 8, 15, and 27 nm, respectively (Yokoyama and Radlwimmer 2001). The zebrafish *LWS-1* and *LWS-2* pigments have the "green"-type amino acid, Ala, at residues 177 and 176, respectively (the sites corresponding to residue 180 of the human red opsin; Figure 2). Furthermore, the *LWS-2* pigment has an additional green-type amino acid, Phe, at residue 273 (the site corresponding to residue 277 of the human red opsin; Figure 2). About 10 nm difference of λmax between the two pigments likely results from it.

The short-wave-shifted character of λmax in zebrafish photoreceptors has been pointed out by Nawrocki *et al.* (1985) and is typical of the fish in "freshwater group I" (Levine and Macnichol 1979), which inhabit freshwater either in the shallow margins of lakes and rivers or near the surface of deeper waters. Zebrafish is indigenous to freshwater in the Indian subcontinent, especially in the Ganges River system. The blue shift presumably enhances the luminance contrast at relatively short wavelengths that dominate its ambient background, which could be of specific benefit in the detection of dark profiles, such as foods or predators, against the relatively bright background of the down-welling light (Cameron 2002).

Spectral and expressional variation among subtypes of LWS/MWS and RH2 opsin genes of zebrafish: We have seen the large spectral variation among subtypes of the LWS/MWS and RH2 pigments of zebrafish. Despite the remarkable differences, significance of the subtypical variation is not clear because only one type from each group, *LWS-1* and *RH2-2*, is dominated in expression level in these groups. In the pineal organ, the expression of both LWS/MWS and RH2 opsin genes has been documented for zebrafish (Mano *et al.* 1999; Forsell *et al.* 2001). To understand biological significance of the gene duplications and spectral diversity of these genes, it is of great importance to investigate (1) whether the subtypes are coexpressed in the same cone cells or not; (2) if not, whether they are distributed with some spatial pattern or in a random fashion in the retina; (3) whether the subtypes are expressed in temporally different ways as supposed for *LWS-1* and *LWS-2*; and (4) which subtypes are expressed in the pineal organ and possibly in other tissues. *In situ* hybridization using the 3′ untranslated regions of these opsin genes should reveal the spatio-temporal expression patterns of the LWS/MWS and RH2 subtypes of zebrafish.

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LITERATURE CITED

Amores, A., A. Force, Y. L. Yan, L. Joly, C. Amemiya *et al.*, 1998 Zebrafish hox clusters and vertebrate genome evolution. Science **282:** 1711–1714.

Archer, S., A. Hope and J. C. Partridge, 1995 The molecular basis for the green-blue sensitivity shift in the rod visual pigments of the European eel. Proc. R. Soc. Lond. Ser. B Biol. Sci. **262:** 289–295.

Bowmaker, J. K., 1995 The visual pigment of fish. Prog. Retinal Eye Res. **15:** 1–31.

Branchek, T., and R. Bremiller, 1984 The development of photoreceptors in the zebrafish, Brachydanio rerio. I. Structure. J. Comp. Neurol. **224:** 107–115.

Cameron, D. A., 2002 Mapping absorbance spectra, cone fractions, and neuronal mechanisms to photopic spectral sensitivity in the zebrafish. Vis. Neurosci. **19:** 365–372.

Carleton, K. L., and T. D. Kocher, 2001 Cone opsin genes of African cichlid fishes: tuning spectral sensitivity by differential gene expression. Mol. Biol. Evol. **18:** 1540–1550.

Cowing, J. A., S. Poopalasundaram, S. E. Wilkie, J. K. Bowmaker and D. M. Hunt, 2002 Spectral tuning and evolution of short wave-sensitive cone pigments in cottoid fish from Lake Baikal. Biochemistry **41:** 6019–6025.

Detrich III, H. W., M. Westerfield and L. I. Zon, 1999 *The Zebrafish: Genetics and Genomics.* Academic Press, San Diego.

Forsell, J., P. Ekstrom, I. N. Flamarique and B. Holmqvist, 2001 Expression of pineal ultraviolet- and green-like opsins in the pineal organ and retina of teleosts. J. Exp. Biol. **204:** 2517–2525.

Hamaoka, T., M. Takechi, A. Chinen, Y. Nishiwaki and S. Kawamura, 2002 Visualization of rod photoreceptor development using *GFP*-transgenic zebrafish. Genesis **34:** 215–220.

Hargrave, P. A., J. H. Mcdowell, D. R. Curtis, J. K. Wang, E. Juszczak *et al.*, 1983 The structure of bovine rhodopsin. Biophys. Struct. Mech. **9:** 235–244.

Helvik, J. V., O. Drivenes, T. H. Naess, A. Fjose and H. C. Seo, 2001 Molecular cloning and characterization of five opsin genes from the marine flatfish Atlantic halibut (Hippoglossus hippoglossus). Vis. Neurosci. **18:** 767–780.

Hisatomi, O., T. Satoh and F. Tokunaga, 1997 The primary structure and distribution of killifish visual pigments. Vision Res. **37:** 3089–3096.

Hughes, A., S. Saszik, J. Bilotta, P. J. Demarco, Jr. and W. F. Patterson, II, 1998 Cone contributions to the photopic spectral sensitivity of the zebrafish ERG. Vis. Neurosci. **15:** 1029–1037.

Ina, Y., 1995 New methods for estimating the numbers of synonymous and nonsynonymous substitutions. J. Mol. Evol. **40:** 190–226.

Johnson, R. L., K. B. Grant, T. C. Zankel, M. F. Boehm, S. L. Merbs *et al.*, 1993 Cloning and expression of goldfish opsin sequences. Biochemistry **32:** 208–214.

Karnik, S. S., T. P. Sakmar, H. B. Chen and H. G. Khorana, 1988 Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. Proc. Natl. Acad. Sci. USA **85:** 8459–8463.

Kawamura, S., and S. Yokoyama, 1998 Functional characterization of visual and nonvisual pigments of American chameleon (*Anolis carolinensis*). Vision Res. **38:** 37–44.

Kawamura, S., N. S. Blow and S. Yokoyama, 1999 Genetic analyses of visual pigments of the pigeon (*Columba livia*). Genetics **153:** 1839–1850.

Kennedy, B. N., T. S. Vihtelic, L. Checkley, K. T. Vaughan and D. R. Hyde, 2001 Isolation of a zebrafish rod opsin promoter to generate a transgenic zebrafish line expressing enhanced green fluorescent protein in rod photoreceptors. J. Biol. Chem. **276:** 14037–14043.

Khorana, H. G., B. E. Knox, E. Nasi, R. Swanson and D. A. Thompson, 1988 Expression of a bovine rhodopsin gene in *Xenopus* oocytes: demonstration of light-dependent ionic currents. Proc. Natl. Acad. Sci. USA **85:** 7917–7921.

Kito, Y., T. Suzuki, M. Azuma and Y. Sekoguti, 1968 Absorption spectrum of rhodopsin denatured with acid. Nature **218:** 955–957.

Kozak, M., 1984 Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res. **12:** 857–872.

Kumar, S., K. Tamura, I. B. Jacobsen and M. Nei, 2001 *MEGA2, Molecular Evolutionary Genetics Analysis.* Arizona State University, Tempe, AZ.

Larhammar, D., and C. Risinger, 1994 Molecular genetic aspects of tetraploidy in the common carp *Cyprinus carpio*. Mol. Phylogenet. Evol. **3:** 59–68.

Levine, J. S., and E. F. Macnichol, Jr., 1979 Visual pigments in teleost fishes: effects of habitat, microhabitat, and behavior on visual system evolution. Sens. Processes **3:** 95–131.

Malicki, J., 2000 Harnessing the power of forward genetics—analysis of neuronal diversity and patterning in the zebrafish retina. Trends Neurosci. **23:** 531–541.

Mano, H., D. Kojima and Y. Fukada, 1999 Exo-rhodopsin: a novel rhodopsin expressed in the zebrafish pineal gland. Brain Res. Mol. Brain Res. **73:** 110–118.

Meyer, A., and E. Malaga-Trillo, 1999 Vertebrate genomics: more fishy tales about Hox genes. Curr. Biol. **9:** R210–R213.

Molday, R. S., and D. Mackenzie, 1983 Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes. Biochemistry **22:** 653–660.

Nawrocki, L., R. Bremiller, G. Streisinger and M. Kaplan, 1985 Larval and adult visual pigments of the zebrafish, *Brachydanio rerio*. Vision Res. **25:** 1569–1576.

Nei, M., and S. Kumar, 2000 *Molecular Evolution and Phylogenetics.* Oxford University Press, New York.

Nelson, J. S., 1994 *Fishes of the World.* John Wiley & Sons, New York.

Ohguro, H., R. S. Johnson, L. H. Ericsson, K. A. Walsh and K. Palczewski, 1994 Control of rhodopsin multiple phosphorylation. Biochemistry **33:** 1023–1028.

Pierce, M. E., H. Sheshberadaran, Z. Zhang, L. E. Fox, M. L. Applebury *et al.*, 1993 Circadian regulation of iodopsin gene expression in embryonic photoreceptors in retinal cell culture. Neuron **10:** 579–584.

Postlethwait, J. H., I. G. Woods, P. Ngo-Hazelett, Y. L. Yan, P. D. Kelly *et al.*, 2000 Zebrafish comparative genomics and the origins of vertebrate chromosomes. Genome Res. **10:** 1890–1902.

Rajendran, R. R., E. E. Van Niel, D. L. Stenkamp, L. L. Cunningham, P. A. Raymond *et al.*, 1996 Zebrafish interphotoreceptor retinoid-binding protein: differential circadian expression among cone subtypes. J. Exp. Biol. **199** (12): 2775–2787.

Raymond, P. A., L. K. Barthel, M. E. Rounsifer, S. A. Sullivan and J. K. Knight, 1993 Expression of rod and cone visual pigments in goldfish and zebrafish: a rhodopsin-like gene is expressed in cones. Neuron **10:** 1161–1174.

Register, E. A., R. Yokoyama and S. Yokoyama, 1994 Multiple origins of the green-sensitive opsin genes in fish. J. Mol. Evol. **39:** 268–273.

Robinson, J., E. A. Schmitt, F. I. Harosi, R. J. Reece and J. E. Dowling, 1993 Zebrafish ultraviolet visual pigment: absorption spectrum, sequence, and localization. Proc. Natl. Acad. Sci. USA **90:** 6009–6012.

Saitou, N., and M. Nei, 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406–425.

Sakmar, T. P., R. R. Franke and H. G. Khorana, 1989 Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. Proc. Natl. Acad. Sci. USA **86:** 8309–8313.

Sambrook, J., and D. W. Russel, 2001 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Saszik, S., and J. Bilotta, 1999 The effects of temperature on the dark-adapted spectral sensitivity function of the adult zebrafish. Vision Res. **39:** 1051–1058.

Smallwood, P. M., Y. Wang and J. Nathans, 2002 Role of a locus control region in the mutually exclusive expression of human red and green cone pigment genes. Proc. Natl. Acad. Sci. USA **99:** 1008–1011.

Tamura, K., and M. Nei, 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. **10:** 512–526.

Thompson, J. D., D. G. Higgins and T. J. Gibson, 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

Vihtelic, T. S., C. J. Doro and D. R. Hyde, 1999 Cloning and characterization of six zebrafish photoreceptor opsin cDNAs and immunolocalization of their corresponding proteins. Vis. Neurosci. **16:** 571–585.

Von Schantz, M., R. J. Lucas and R. G. Foster, 1999 Circadian oscillation of photopigment transcript levels in the mouse retina. Mol. Brain Res. **72:** 108–114.

Wang, J. K., J. H. Mcdowell and P. A. Hargrave, 1980 Site of attachment of 11-*cis*-retinal in bovine rhodopsin. Biochemistry **19:** 5111–5117.

Yokoyama, R., and S. Yokoyama, 1990 Convergent evolution of the red- and green-like visual pigment genes in fish, *Astyanax fasciatus*, and human. Proc. Natl. Acad. Sci. USA **87:** 9315–9318.

Yokoyama, R., and S. Yokoyama, 1993 Molecular characterization of a blue visual pigment gene in the fish *Astyanax fasciatus*. FEBS Lett. **334:** 27–31.

Yokoyama, S., 2000 Molecular evolution of vertebrate visual pigments. Prog. Retin. Eye Res. **19:** 385–419.

Yokoyama, S., and F. B. Radlwimmer, 1998 The "five-sites" rule and the evolution of red and green color vision in mammals. Mol. Biol. Evol. **15:** 560–567.

Yokoyama, S., and F. B. Radlwimmer, 1999 The molecular genetics of red and green color vision in mammals. Genetics **153:** 919–932.

Yokoyama, S., and F. B. Radlwimmer, 2001 The molecular genetics and evolution of red and green color vision in vertebrates. Genetics **158:** 1697–1710.

Yokoyama, S., H. Zhang, F. B. Radlwimmer and N. S. Blow, 1999 Adaptive evolution of color vision of the Comoran coelacanth (*Latimeria chalumnae*). Proc. Natl. Acad. Sci. USA **96:** 6279–6284.

Zhang, H., K. Futami, N. Horie, A. Okamura, T. Utoh *et al.*, 2000 Molecular cloning of fresh water and deep-sea rod opsin genes from Japanese eel *Anguilla japonica* and expressional analyses during sexual maturation. FEBS Lett. **469:** 39–43.

Zhukovsky, E. A., and D. D. Oprian, 1989 Effect of carboxylic acid side chains on the absorption maximum of visual pigments. Science **246:** 928–930.

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