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Gene 371 (2006) 268–278
GENE
SECTION
EVOLUTIONARY GENOMICS
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Functional characterization of visual opsin repertoire in Medaka (*Oryzias latipes*)

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Received 9 September 2005; received in revised form 16 November 2005; accepted 8 December 2005
Available online 7 February 2006
Received by N. Saitou

Abstract

A variety of visual pigment repertoires present in fish species is believed due to the great variation under the water of light environment. A complete set of visual opsin genes has been isolated and characterized for absorption spectra and expression in the retina only in zebrafish. Medaka (*Oryzias latipes*) is a fish species phylogenetically distant from zebrafish and has served as an important vertebrate model system in molecular and developmental genetics. We previously isolated a medaka rod opsin gene (*RH1*). In the present study we isolated all the cone opsin genes of medaka by genome screening of a lambda-phage and bacterial artificial chromosome (BAC) libraries. The medaka genome contains two red, *LWS-A* and *LWS-B*, three green, *RH2-A*, *RH2-B* and *RH2-C*, and two blue, *SWS2-A* and *SWS2-B*, subtype opsin genes as well as a single-copy of the ultraviolet, *SWS1*, opsin gene. Previously only one gene was believed present for each opsin type as reported in a cDNA-based study. These subtype opsin genes are closely linked and must be the products of local gene duplications but not of a genome-wide duplication. Peak absorption spectra (λ_{max}) of the reconstituted photopigments with 11-*cis* retinal varied greatly among the three green opsins, 452 nm for *RH2-A*, 516 nm for *RH2-B* and 492 nm for *RH2-C*, and between the two blue opsins, 439 nm for *SWS2-A* and 405 nm for *SWS2-B*. Zebrafish also has multiple opsin subtypes, but phylogenetic analysis revealed that medaka and zebrafish gained the subtype opsins independently. The lambda and BAC DNA clones isolated in this study could be useful for investigating the regulatory mechanisms and evolutionary diversity of fish opsin genes.

Keywords: Medaka; Opsin; Visual pigment; Gene duplication; Absorption spectra

1. Introduction

Vertebrate visual opsins are classified into five types present prior to vertebrate radiation: rod opsin or rhodopsin RH1; RH1-like, or green, cone opsin RH2; short wavelength-sensitive type 1, or UV-blue, cone opsin SWS1; short wavelength-sensitive type 2, or blue, cone opsin SWS2; and middle to long wavelength-sensitive, or red–green, cone opsin M/LWS (Yokoyama, 2000). Many fish are known to possess two or more opsin subtypes in these five types. Among tetrapods, only primate M/LWS type opsins are known to contain subtypes as exemplified by human red and green opsins. The frequent observation of various opsin subtypes in fish presumably reflects their evolutionary adaptation to diverse aquatic light environments (Levine and MacNichol, 1982). Therefore, fish are excellent subjects for studying adaptive radiation of visual systems.

To understand the visual capability of an animal, it is necessary to identify the compliment of visual opsins present in the animal, clarify their absorption spectra and establish their spatial and temporal expression patterns in the retina. Most studies of fish opsin genes have utilized a cDNA-basis, and have rarely intended to excavate all of the opsin repertoires from a single species and to reveal the positional relationships among subtype opsin genes in the genome. Zebrafish is the only fish

Abbreviations: aa, amino acid(s); BAC, bacterial artificial chromosome; bp, base pair(s); cDNA, DNA complementary to RNA; kb, kilobase(s); λ_{max}, wavelength of maximal absorbance; M/LWS, middle to long wavelength-sensitive cone opsin; MSP, microspectrophotometry; nt, nucleotide(s); PCR, polymerase chain reaction; RH1, rod opsin or rhodopsin; RH2, RH1-like cone opsin; RT, reverse transcription; SWS1, short wavelength-sensitive type 1 cone opsin; SWS2, short wavelength-sensitive type 2 cone opsin; UV, ultraviolet.

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doi:10.1016/j.gene.2005.12.005

species whose visual opsins are fully characterized by gene screening from a genomic library, photopigment reconstitution and in situ hybridization in the retina (Chinen et al., 2003; Takechi and Kawamura, 2005). Zebrafish was found to possess two and four tandemly arranged M/LWS and RH2 opsin genes, respectively, in the genome, in addition to single copies of SWS1, SWS2 and RH1 opsin genes. The subtype opsins possess different absorption spectra and expression patterns. However, knowledge is incomplete about the opsin subtypes phylogenetic and ecologic distribution among fish species and their individual spatial and temporal expression in the retina.

Medaka (*Oryzias latipes*) is a small freshwater fish native to Asia and belongs to the Acanthopterygii superorder which diverged 115–200 million years ago from the Ostariophysi superorder which includes the zebrafish (Nelson, 1994; Furutani-Seiki and Wittbrodt, 2004). Medaka has a century of history as a subject of genetic study in Japan. It is relatively easy to maintain medaka and to apply transgenic methodologies to them in a small laboratory. Many inbred strains have been established for medaka and, as in zebrafish, many mutants are available and being employed for genome projects (Naruse et al., 2004). Given the large evolutionary distance from zebrafish and the ability to undertake functional studies, medaka is an excellent subject for studying the evolution of the opsin repertoire and regulation of their expression.

Hisatomi et al. (1997) reported five cDNA clones representing the five types of medaka opsins. However, it is not known whether subtypes of the five types of medaka opsins exist. The objectives of this paper are to establish the compliment of visual opsin genes present in the medaka genome, examine the absorption spectra of the photopigments reconstituted from the opsin genes, and estimate the relative expression of the opsin genes in the retina. On the basis of our cloning results, we discuss the importance of local gene duplication in opsin subtypes formation, the evolution of opsin repertoires in fish, opsin spectral tuning mechanisms and future utilization of the isolated clones in expression and evolutionary analyses of opsin genes.

2. Materials and methods

2.1. Genomic library screening

A lambda phage-based genomic library of a medaka (HNI strain) was previously constructed with an average insert size of 16 kb (Kawamura et al., 2005). For probe preparation, cDNA clones encoding full coding regions of M/LWS, RH2, SWS2, and SWS1 opsin genes were isolated from a medaka ocular RNA by reverse-transcription (RT) polymerase chain reaction (PCR) using the oligonucleotide primers designed on the basis of the published nt sequences of the corresponding medaka opsin cDNAs: *KFH-R* (M/LWS; GenBank accession number AB001604), *KFH-G* (RH2; AB001603), *KFH-B* (SWS2; AB001602), and *KFH-V* (SWS1; AB001605) (Hisatomi et al., 1997). Labeling, hybridization, and washing conditions were as previously described (Kawamura et al., 2005). Obtained clones were restriction-mapped and the restriction fragments hybridized to the screening probes were subcloned into the pBluescript II (SK-) plasmid vector (Stratagene, La Jolla, CA). All clones were sequenced in both strands using ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan).

We isolated two complete M/LWS type genes (*LWS-A* and *LWS-B*) which were juxtaposed in a tail-to-head manner, two partial SWS2 type genes (*SWS2-A* and *SWS2-B*) which were separated in different lambda clones, one complete RH2 type gene (*RH2-A*), and one complete SWS1 type gene (*SWS1*) from the lambda library. In a medaka genome database, which is run by the National Institute of Genetics (NIG) and the University of Tokyo (UT) (http://dolphin.lab.nig.ac.jp/medaka/; database version 200506), the two SWS2 and the two M/LWS genes were found to be arrayed in a tail-to-head manner in one contiguous sequence (scaffold 3). *LWS-A* appears to be misassembled in the database, because its exons 4–6 are placed upstream of its exons 1–3. In the database, we also found three RH2-like sequences which are arrayed in a tail-to-head manner in a scaffold 298. The DNA sequence of the head-most sequence corresponded to that of *RH2-A*. We designated the second and third ones *RH2-B* and *RH2-C*, respectively. In the database, an extra copy of a portion of exon 4 of *RH2-B*, accompanied by a large gap, was found in intron 4, which is possibly due to an assemblage error. The *SWS1* was found in a single copy in a scaffold 1278.

To isolate a genomic region encompassing the two SWS2 and the two M/LWS genes and a region encompassing the three RH2 genes, we screened a bacteria artificial chromosome (BAC) genomic library of a medaka (HNI strain) using the same probes used in the lambda library. The library consists of ~96,000 clones with an average insert size of 160 kb, covering 20 times the medaka genome (Kondo et al., 2002). Dot-blot membranes of the library were hybridized with the mixed probes using AlkPhos Direct Labelling and Detection System with CDP-*Star* (Amersham, Piscataway, NJ) according to the manufacturer's protocol. Positive clones were examined by PCR for each gene. Two clones, 82B21 and 84I18, were found to contain SWS2 and M/LWS genes, and one clone, 33O23, was found to contain RH2 genes.

To facilitate cloning and sequencing of the opsin genes and their surrounding regions from the BAC clones, clones 82B21 and 33O23 were subjected to subcloning into a lambda phage vector (Figs. 1A and B). The BAC clones were partially digested with *Sau*3AI and 12–20 kb DNA fragments were ligated to *Bam*HI-digested EMBL3 lambda phage vector. We picked up 200 plaques randomly from both 82B21 and 33O23 and sequenced both edges (designated λ-ends) of their insert DNA using an EMBL3 short-arm specific primer 5′-ataacgagtggatctgggtcgaccggtc-3′ and a long-arm specific primer 5′-gtcgacctgcaggtcaacggatc-3′ by ABI PRISM 3100-Avant Genetic Analyzer. The λ-end sequences from the BAC 82B21 were aligned to the scaffold 3 containing the SWS2 and M/LWS genes and those from the BAC 33O23 were aligned to the scaffold 298 containing the RH2 genes (Fig. 1). The lambda

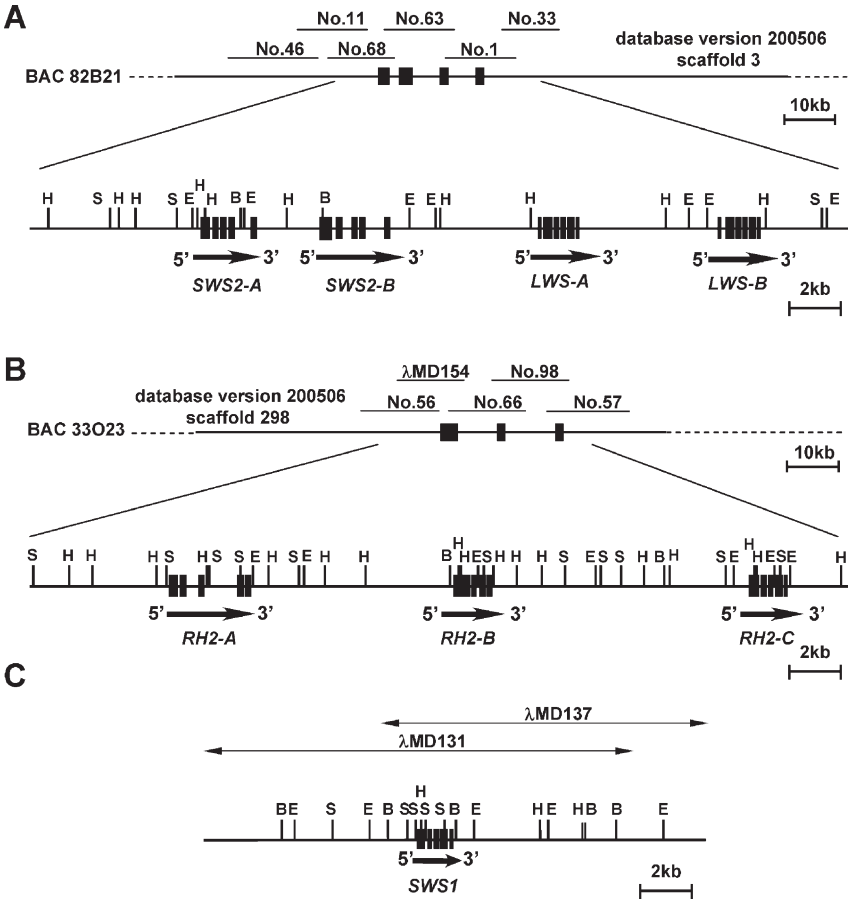

Fig. 1. A physical map of the genomic regions encompassing *SWS2-A*, *SWS2-B*, *LWS-A* and *LWS-B* (A), *RH2-A*, *RH2-B* and *RH2-C* (B) and *SWS1* (C) of HNI medaka. (A) The scaffold 3 (indicated with a solid line) is covered by the genomic region cloned in BAC 82B21. Positions and identification numbers of lambda clones isolated from the BAC clone are aligned to the scaffold 3 as indicated on top. *SWS2-A*, *SWS2-B*, *LWS-A* and *LWS-B* are depicted as solid boxes. A detailed restriction map of the genomic region encompassing the four genes is given in an expanded view, where exons are indicated as solid boxes and transcriptional orientations are depicted above the gene names. (B) The scaffold 298 is likewise contained in BAC 33O23. Lambda clones isolated from the BAC clone are indicated with clone numbers. λMD154 is from a lambda phage whole genome library. Locations and transcriptional orientations of *RH2-A*, *RH2-B* and *RH2-C* are indicated as in (A). (C) The exon–intron organization and transcriptional orientation of *SWS1* are indicated in the restriction map. The two overlapping clones (λMD131 and λMD137) are from the lambda genomic library. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I.

clones located in these scaffolds were subjected to restriction mapping and sequencing as described above.

2.2. Visual pigment reconstitution

Using the total RNA prepared from the eyes of an adult medaka (HNI strain), the first-strand cDNA was synthesized by using a poly (dT) primer (5′-aagcagtggtaacaacgcagagtact(30) vn-3′ (v: a, g, or c; n: a, c, g, or t; t(30), 30 succession of t)). Opsin genes were PCR-amplified using primer pairs (external primers in Table 1) which are designed immediately upstream of their start codons and immediately downstream of their stop codons. The PCR products were cloned into the pBluescript II (SK-) plasmids and sequenced. The DNA sequences were confirmed in duplicate RT-PCRs. The entire coding regions of the cDNAs were further amplified from these pBluescript-cDNA clones by primer pairs (pMT5 primers in Table 1) designed in 5′- and 3′-edges of the coding regions with necessary sequences for cloning and translation purposes (see Kawamura and Yokoyama, 1998). PCR products were cloned into pMT5 expression vector which contains the last 15 aa of the bovine rod opsin necessary for immunoaffinity purification by 1D4 monoclonal antibody (Kawamura and Yokoyama, 1998). The nt 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), the cells were incubated with 5 μM 11-*cis* retinal (Storm Eye Institute, Medical University of South Carolina, Charleston, SC) and solubilized with 1% dodesyl maltoside (Anatrace, Maumee, OH), and produced pigments were purified using the immobilized 1D4 monoclonal antibody (Cell Culture Center, Minneapolis, MN) as described in Kawamura and Yokoyama (1998). UV–visible absorption spectra of the visual pigments were recorded from 250 to 650 or 750 nm at 0.5 nm intervals using an U3010 dual beam spectrometer (Hitachi, Tokyo, Japan) at 20 °C for at least five times in dark and for at least five more times after 3 min of light exposure as described in Kawamura and Yokoyama (1998).

Table 1
List of oligonucleotide PCR primers used for photopigment reconstitution and real-time RT-PCR

Opsin		Primers
LWS-A	(External forward)	AGAGCCTCCTTGTGTGATCAG
	(External reverse)	TACATTCCCTCTCTGAAGTTA
	(pMT5 forward	TCCTTGGAATT*CCACC***ATG**GCAGAGGAGTGGGGAAA
	(pMT5 reverse)	AAAGAGGTCGACGCAGGAGCCACAGAGGAGAC
	(Real-time forward)	ATGAAGACACAACAAGAGGCTCTGC
	(Real-time reverse)	TTATGCAGGAGCCACAGAGGA
LWS-B	(External forward)	AGAGCCTCCTTGTGCGATCAA
	(External reverse)	TGCATTCTTTTTATGTGTAAT
	(pMT5 forward)	TCCTTGGAATT*CCACC***ATG**GCAGAGCAGTGGGGAAA
	(pMT5 reverse)	Same with LWS-A
	(Real-time forward)	Same with LWS-A
	(Real-time reverse)	Same with LWS-A
RH2-A	(External forward)	CTAATCCAGATCCTAACTTGCAAAG
	(External reverse)	CAGTCCAGAGACGTCCGGCGTGGTCT
	(pMT5 forward)	ATCCTAGAATT*CCACC***ATG**GAGAACGGCACAGAGGG
	(pMT5 reverse)	CCGGCGGTCGACGCAGCAGTAGAGACTTCTGT
	(Real-time forward)	ATGGAGAACGGCACAGAGGG
	(Real-time reverse)	GCAGTCCAGAGACGTCCGGCGTGGTCT
RH2-B	(External forward)	CAAGAGAGTCCAAGGGAGCATCAAAA
	(External reverse)	GTCATGAACTTTGCCAAAGCTTTGGTTTTGGATTT
	(pMT5 forward)	CAAGGGGAATT*CCACC***ATG**GGTTGGGAGCCTAATGGCACT
	(pMT5 reverse)	TGTGGTGTCGACGCTGCAGTTGAGACTTCTGTCTTG
	(Real-time forward)	CTCCGCTTCAAGAGAGTCCAAGGGAGCATC
	(Real-time reverse)	GCAATCACTGAAAGTGTCCACAAAAGCGAC
RH2-C	(External forward)	ACCAAGCAAGCAGACATGGGCTGGGAT
	(External reverse)	TAAGTCTGACACTTTCATTTAAAAGATCC
	(pMT5 forward)	TACCAAGAATT*CCACC***ATG**GGCTGGGATGGAGGAGA
	(pMT5 reverse)	ACATATGGTGTCGACGCTGCAGTTGAGACTTCTGTCTTGCTT
	(Real-time forward)	GAAGCTCCAGAAGACCAAGCAAGCAGAC
	(Real-time reverse)	GGATCTTTTAAATGAAAGTGTCAGACTTA
SWS2-A	(External forward)	CCAAAAAATCTTATTATTTGTTACAGTCAAGC
	(External reverse)	CCGCAGAGGAGTTATCACTAAGGGT
	(pMT5 forward)	AAAAAAGAATT*CCACC***ATG**AGGTTCATCAGTGGTGG
	(pMT5 reverse)	CTAAGGGTCGACGCTGGTCCGACTTTAGAGAC
	(Real-time forward)	ATGAGGTTCATCAGTGGTGGGGAGCTG
	(Real-time reverse)	CACTGATGTTGAGTCCTCCTCTTCTC
SWS2-B	(External forward)	GTCTCTTTTATCTGAGACAAAAG
	(External reverse)	GCATGAGTCAAAAATAAACAAGAAT
	(pMT5 forward)	TATCTGGAATT*CCACC***ATG**AGGGGAAATCGTGTTGT
	(pMT5 reverse)	ACAAGAGTCGACGAAGGGCCGACTTTTGAGAC
	(Real-time forward)	ATGAGGGGAAATCGTGTTGTGGAGTTT
	(Real-time reverse)	TGACTGGTTGAGGACTCTTCATCTTCGT
SWS1	(External forward)	GCGGTTCAGAGCTCAGCTTCACG
	(External reverse)	GGTTTGTTGCTTTTTGTCCCAAACC
	(pMT5 forward)	AGAGCTGAATT*CCACC***ATG**GGAAAATACTTCTACCT
	(pMT5 reverse)	CCCAAAGTCGACGAGGCCGTGGACACCTCCGT
	(Real-time forward)	ATGGGAAAATACTTCTACCTGTATGAGAACATCTCC
	(Real-time reverse)	TTAAGAGGCCGTGGACACCTCCGTCTTGGAGGACACC
RH1	(External forward)	GATAGCAACCGCAAGCCGCAACC
	(External reverse)	GAAACCTTTGCTTGATGTTGCTT
	(pMT5 forward)	ACCGCAGAATT*CCACC***ATG**AATGGCACAGAGGGACCA
	(pMT5 reverse)	TGTTGCGTCGACGCAGGGGACACAGAGCTTGA
	(Real-time forward)	ATGAATGGCACAGAGGGACC
	(Real-time reverse)	TTATGCAGGGGACACAGAGCT

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

2.3. Quantitative real-time RT-PCR

By RT-PCR using gene-specific primer pairs (real-time PCR primers in Table 1), cDNA was prepared for each opsin gene. Since *LWS-A* and *LWS-B* were highly similar in not only coding but also their immediate flanking regions and since the two genes encoded photopigments with indistinguishable absorption spectra (see Results section), we did not design gene-specific primers distinguishing the two M/LWS type genes. These cDNAs were cloned into pBluescript II (SK-) plasmids.

The pBluescript-cDNA clones were prepared for a series of concentrations diluted over three orders of magnitude. Real-time PCR was carried out for the standard templates with the real-time PCR primer pairs using the Smart cycler system (Cepheid, Sunnyvale, CA), where the amount of the PCR product was monitored through progression of PCR cycles by the fluorescence intensity of SYBR Green I (Takara Bio, Tokyo, Japan) intercalated in the double-stranded DNA. We thus obtained standard regression lines correlating initial DNA concentrations and the threshold PCR cycles as in Chinen et al. (2003). For all opsin genes, values of the correlation coefficient were between −0.99 and −1.0. Retina RNA was extracted from five 2 years old adult HNI medaka after 3 h of light exposure, all of which had been raised under the 14 h day/10 h dark cycle. Prior to RT reaction, samples were treated with DNase I, and PCR products were confirmed by electrophoresis to have the expected sizes with no introns, excluding a possibility of contamination from residual genomic DNA. Expression level of the opsin genes in the retina was evaluated for each fish by real-time RT-PCR using the real-time PCR primers and by the standard regression lines, with compensation of differences in RT efficiencies among the genes, as in Chinen et al. (2003). Relative RT efficiencies and expression levels among genes were given as those of *RH1* as 1.

2.4. Phylogenetic analysis

The following nt sequences of the fish M/LWS, RH2 and SWS2 type opsin genes were retrieved from the GenBank database: zebrafish (*Danio rerio*) M/LWS (accession No. AB087803 for *LWS-1* and AB087804 for *LWS-2*), RH2 (AB087805 for *RH2-1*, AB087806 for *RH2-2*, AB087807 for *RH2-3* and AB087808 for *RH2-4*) and SWS2 (AB087809); goldfish (*Carassius auratus*) M/LWS (L11867), RH2 (L11865 for *GFgr-1* and L11866 for *GFgr-2*) and SWS2 (L11864); carp (*Cyprinus carpio*) M/LWS (AB055656), RH2 (AB110602 for *gr-1* and AB110603 for *gr-2*) and SWS2 (AB113668); Mexican cavefish (*Astyanax fasciatus*) M/LWS (M90075 for R007, M38624 for *G101*, and M60945 for *G103*), RH2 (S75255) and SWS2 (AF134766); cichlid (*Metriaclima zebra*) M/LWS (AF247126), RH2 (DQ088651 for *RH2Aα*, AF247122 for *RH2Aβ* and DQ088652 for *RH2B*) and SWS2 (AF247114 for *SWS2A* and AF317118 for *SWS2B*); tilapia (*Oreochromis niloticus*) M/LWS (AF247128), RH2 (AF247124) and SWS2 (AF247120 for *SWS2A* and AF247116 for *SWS2B*); halibut (*Hippoglossus hippoglossus*) M/LWS (AF316498), RH2 (AF156263) and SWS2 (AF316497); cod icefish (*Dissostichus mawsoni*) RH2 (AY771352); Antarctic dragonfish (*Gymnodraco acuticeps*) RH2 (AY771355); Britain cottoid (*Cottus gobio*) SWS2 (AJ430489); Baikal cottoid (*Batrachocottus nicolskii*) SWS2 (AJ430474); mullet (*Mullus surmuletus*) RH2 (Y18680); sandgoby (*Pomatoschistus minutus*) RH2 (Y18679); bluefin killifish (*Lucania goodei*) M/LWS (AY296740 for *LWSA*, AY296741 for *LWSB*), RH2 (AY296739) and SWS2 (AY296737 for *SWS2A*, AY296736 for *SWS2B*); fugu (*Takifugu rubripes*) M/LWS (AY598942), RH2 (AF226989) and SWS2 (AY598947); *Tetraodon nigroviridis* M/LWS (AY598943), RH2 (AY598944) and SWS2 (AY598948); Atlantic salmon (*Salmo salar*) M/LWS (AY214131), RH2 (AY214132) and SWS2 (AY214134); rainbow trout (*Oncorhynchus mykiss*) M/LWS (AF425073), RH2 (AF425076) and SWS2 (AF425075); ayu (*Plecoglossus altivelis*) M/LWS (AB098702) and RH2 (AB098703 for *RH2-1*, AB098704 for *RH2-2*). As outgroup references for the fish genes, the following genes were used: pigeon (*Columba livia*) M/LWS (AF149248), RH2 (AF149233) and SWS2 (AH 007799); American chameleon (*Anolis carolinensis*) M/LWS (U08131), RH2 (AF134189) and SWS2 (AF133907).

Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 3.0 (Kumar et al., 2004). Alignment of the deduced aa sequences was carried out using CLUSTAL W and refined visually. Their nt sequences were aligned in accordance with the protein alignments. The transition/transversion (s/v) ratios for the fish M/LWS, RH2 and SWS2 type opsin genes were calculated to be 1.26, 1.05 and 1.15, respectively, according to Kimura's two-parameter model. The percentage of synonymous nt differences among the fish M/LWS genes, among fish RH2 genes and among fish SWS2 genes were calculated on the basis of the s/v ratio using the modified Nei–Gojobori method (Nei and Kumar, 2000). Synonymous nt differences among the fish genes were all below saturation level in all three opsin types (2.4–65.4% (average 46.8%) in M/LWS, 4.9–65.8% (average 50.9%) in RH2, and 3.6–67.5% (average 53.1%) in SWS2) and were considered to retain phylogenetic information. When aa sequences were used for tree construction, bootstrap values were generally low. Because of these reasons, we used nt sequences of complete coding regions for tree construction. The number of nt substitutions per site (*d*) for two sequences was estimated by the Tamura and Kumar (2002) model with gap

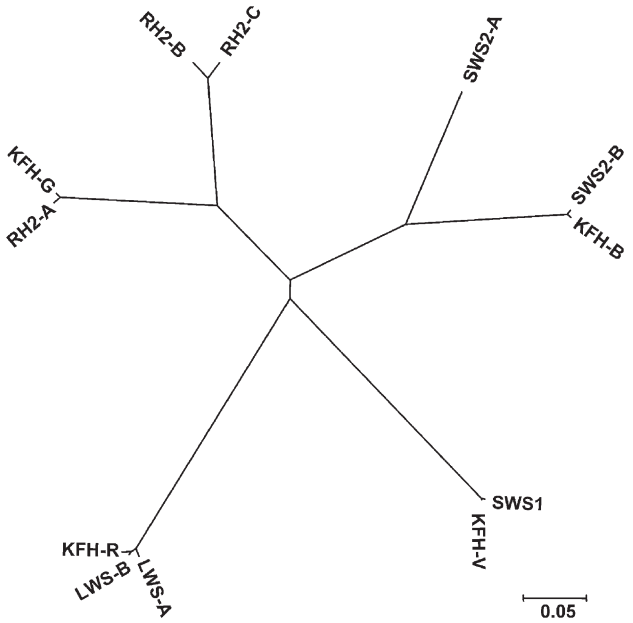

Fig. 2. The sequence relatedness among the medaka cone opsin genes isolated by Hisatomi et al. (1997), *KFH-R*, *KFH-G*, *KFH-B* and *KFH-V*, and in this study, *LWS-A*, *LWS-B*, *RH2-A*, *RH2-B*, *RH2-C*, *SWS2-A*, *SWS2-B* and *SWS1*. Scale bar indicates 5% nt difference.

sites excluded in a pairwise fashion. The phylogenetic tree was reconstructed by applying the neighbor-joining method (Saitou and Nei, 1987) to the *d* values. The reliability of the tree topology was evaluated by bootstrap analysis with 1000 replications.

3. Results

3.1. Identification of medaka cone opsin genes

The physical map of the cone opsin genes in the medaka genome, revealed by screening lambda-phage and BAC libraries of medaka genome and also by querying the NIG-UT medaka genome database version 200506 (http://dolphin.lab.nig.ac.jp/medaka/), is shown in Fig. 1. Two M/LWS genes (*LWS-A* and *LWS-B*) were identified (Fig. 1A). As in zebrafish (Chinen et al., 2003), the two genes were closely arrayed in tandem, with an intergenic region of ~5.5 kb, and were located ~6 kb downstream of SWS2 type genes. Contrary to zebrafish which only has a single SWS2 gene, medaka had two closely spaced genes, *SWS2-A* and *SWS2-B*, with an intergenic region of ~2.5 kb (Fig. 1A). Medaka was found to possess three closely spaced RH2 genes, *RH2-A*, *RH2-B* and *RH2-C*; zebrafish has four such genes (Chinen et al., 2003). Intergenic

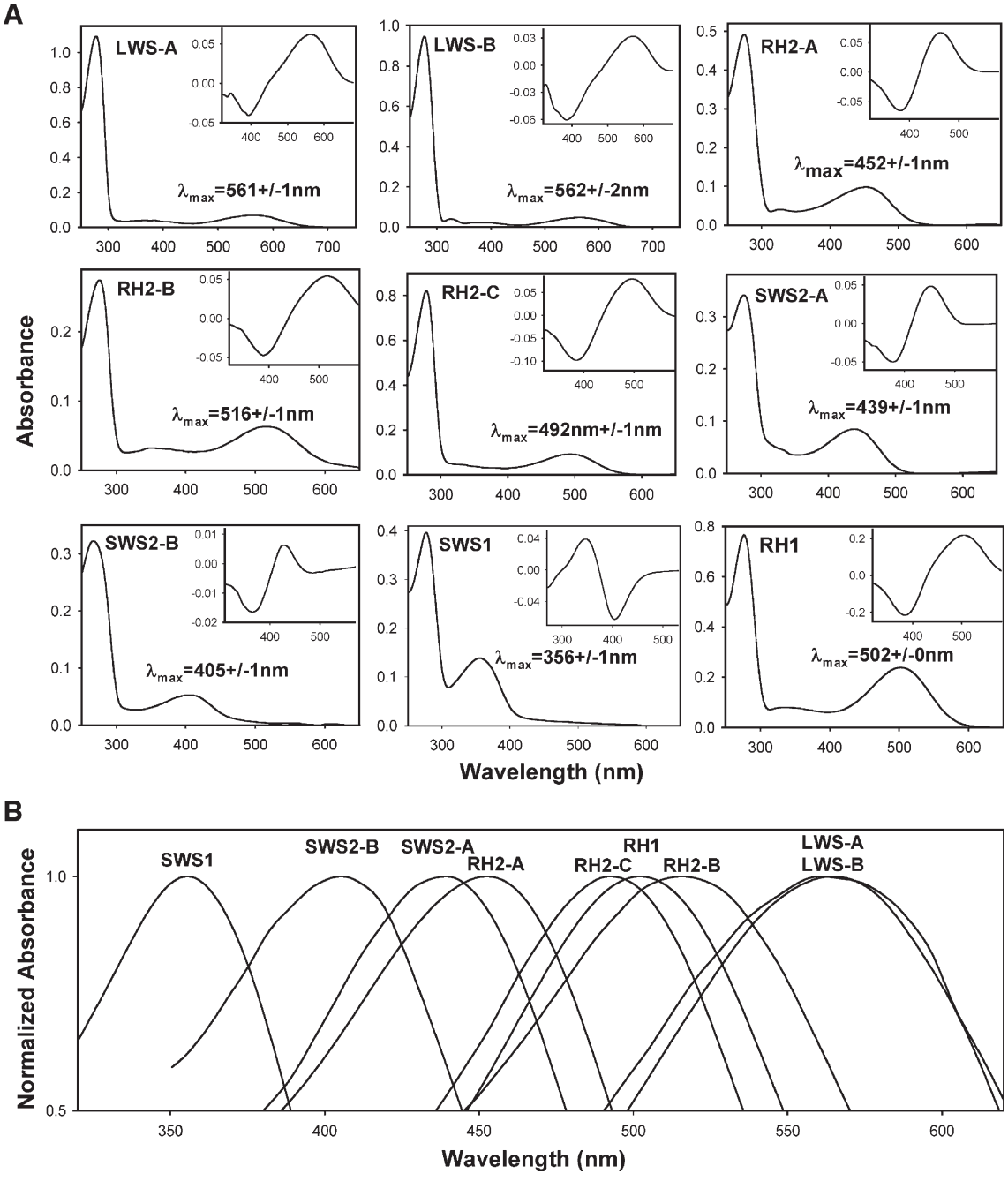

Fig. 3. Absorption spectra of the reconstituted visual pigments of medaka measured in the dark. (A) λ_{max} values are given with standard errors. Insets: the dark–light difference spectra. (B) A composite of normalized absorption spectra of the reconstituted photopigments by setting their peak height at λ_{max} to be 1.

regions between *RH2-A* and *RH2-B* and between *RH2-B* and *RH2-C* were ~8 kb and ~10 kb, respectively (Fig. 1B). A single SWS1 type gene, *SWS1*, was found in the screening as in zebrafish (Fig. 1C). No other cone opsin-like genes were detected by genomic Southern hybridization or in the genome database (data not shown), indicating that the cloned genes represent a complete set of the medaka cone opsin genes. Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under accession nos. AB223051, AB223052, AB223053, AB223054, AB223055, AB223056, AB223057 and AB223058.

Exon–intron organizations of these genes were consistent with those of their orthologous genes found in other vertebrates, there were five exons in RH2, SWS1 and SWS2 types and six exons in M/LWS type (Yokoyama, 2000). Splice junction signals (GT/AG) were conserved in all introns and nonsense mutations were not found in the coding regions. Functionally important residues were conserved, such as a lysine for the Schiff-base linkage to the chromophore, a glutamate residue for the Schiff-base counter ion, cysteine residues for the disulfide bond, and multiple serines and threonines in the C-terminal region for the targets of opsin kinase.

Fig. 2 shows a neighbor-joining dendrogram (Saitou and Nei, 1987) representing the percentage nt differences among the coding regions of medaka cone opsin genes identified in this genomic study and in a previous cDNA study (Hisatomi et al., 1997) where one gene was identified for each of the four cone opsin types (*KFH-R* (M/LWS), *KFH-G* (RH2), *KFH-B* (SWS2) and *KFH-V* (SWS1)). *LWS-A* and *LWS-B* were highly similar with 98.8% identity, and *KFH-R* was slightly more similar to *LWS-B* with 99.0% than to *LWS-A* with 98.0% identity. RH2 genes can be classified into the *RH2-A* and *RH2-B*/*RH2-C* groups with 74.3–75.5% identity between the groups and 96.8% identity between *RH2-B* and *RH2-C*, *KFH-G* was most similar to *RH2-A* with 98.9% identity, and its similarity to *RH2-B* and *RH2-C* was 74.7% and 75.8%, respectively. The similarity between *SWS2-A* and *SWS2-B* was 75.3% and *KFH-B* was more similar to *SWS2-B* with 99.2% identity than to *SWS2-A* with 75.0% identity. *KFH-V* was most similar to *SWS1* with 99.6% identity. These results suggest that *KFH-R*, *KFH-G*, *KFH-B* and *KFH-V* represent alleles of *LWS-B*, *RH2-A*, *SWS2-B* and *SWS1*, respectively. Consequently, *RH2-B*, *RH2-C*, and *SWS2-A* are the new genes identified in this study.

3.2. Spectral sensitivity of reconstructed visual pigments

We isolated opsin cDNAs corresponding to the genes isolated from the genome by RT-PCR using external primer pairs shown in Table 1, including the rod opsin gene *RH1* which was previously isolated (Kawamura et al., 2005). The cDNA sequences perfectly matched the genomic sequences. We reconstituted photopigments for all the opsin genes with 11-*cis* retinal as a chromophore. The pigments absorption spectra measured in the dark had a prominent absorption peak in addition to a protein absorbance at 280 nm as shown in Fig. 3A. The λ_{max} values were taken directly from the dark spectra (see Fig. 3A). When the reconstituted pigments were exposed to light, a new absorption peak appeared at 380 nm represented as a negative peak in the dark–light difference spectra indicated in the insets of Fig. 3A. This indicates that 11-*cis* retinal in the pigments was isomerized by light and all-*trans* retinal was released. These results demonstrate that the reconstituted pigments were in fact photo-reactive. The ultraviolet sensitivity of SWS1 pigment was verified by acid denaturation as in Chinen et al. (2003).

While the two M/LWS pigments showed similar λ_{max} values at about 560 nm, those of the three RH2 and the two SWS2 pigments differed considerably between subtypes. There was a

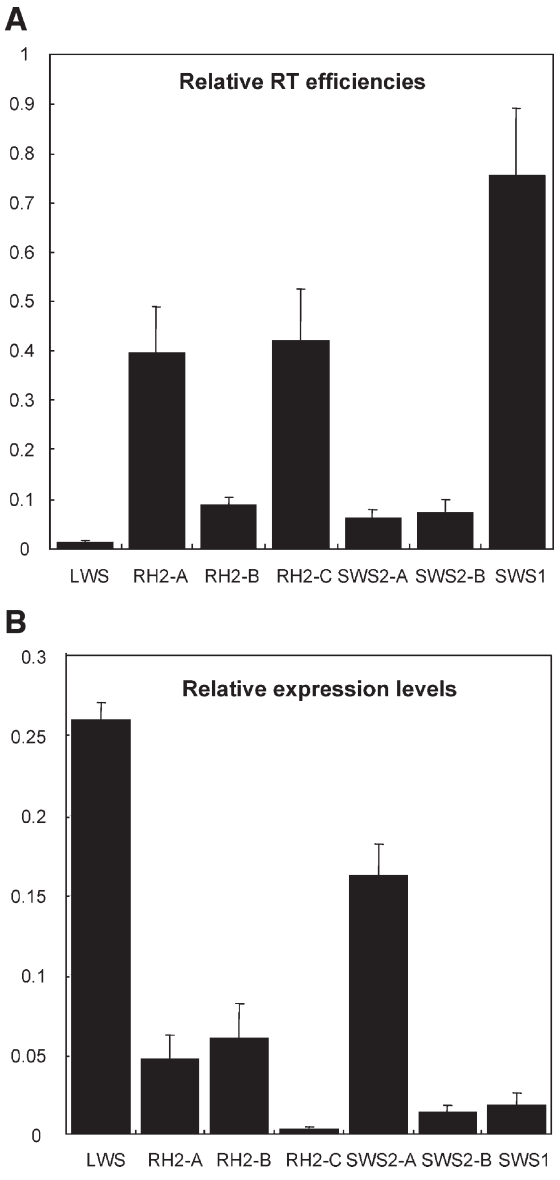

Fig. 4. Real-time RT-PCR analysis of medaka opsin expression in the retina. (A) Relative efficiencies of the reverse transcription among the medaka opsin genes. Efficiencies are given as that of *RH1* as 1. *LWS* (*LWS-A*+*LWS-B*), 0.014±0.003; *RH2-A*, 0.40±0.09; *RH2-B*, 0.088±0.016; *RH2-C*, 0.42±0.11; *SWS2-A*, 0.063±0.017; *SWS2-B*, 0.075±0.025; *SWS1*, 0.76±0.14. (B) Relative expression levels among the medaka opsin genes. Expression levels are given as that of *RH1* as 1. *LWS* (*LWS-A*+*LWS-B*), 0.26±0.01; *RH2-A*, 0.047±0.015; *RH2-B*, 0.060±0.022; *RH2-C*, 0.0029±0.0020; *SWS2-A*, 0.16±0.02; *SWS2-B*, 0.013±0.005; *SWS1*, 0.018±0.008. Error bars are 1 S.D.

24–64 nm difference between the three RH2 pigments and a 34 nm difference between the two SWS2 pigments (Fig. 3B). It was also noted that the λ_{max} value of 452 nm for *RH2-A* is the shortest one of vertebrate RH2 pigments thus far reported, which are typically in the range of 470–510 nm (Yokoyama, 2000). The 405 nm λ_{max} of *SWS2-B* is also the shortest one among those of vertebrate SWS2 pigments thus far reported, which are typically in the range of 420–460 nm (Yokoyama, 2000).

3.3. Relative expression levels of medaka opsin genes

To evaluate the relative expression levels among the several types and subtypes of the opsin genes, real-time RT-PCR was performed using retina RNA from adult medaka. The two M/LWS subtypes were highly similar in their DNA sequence and in λ_{max}, therefore, the expression levels of the two subtypes were evaluated collectively. As in a zebrafish study (Chinen et al., 2003), the relative RT efficiency differed considerably among opsin genes (Fig. 4A), emphasizing the necessity to measure this efficiency for evaluation of the relative expression levels among the genes.

The five fish tested showed a similar profile pattern of relative expression and representative data are shown in Fig. 4B. In medaka retina, expression of M/LWS type was most abundant, in contrast to the zebrafish case where it is expressed at a very low level (Chinen et al., 2003). Expression of RH2 and SWS2 types in medaka retina was relatively abundant as in zebrafish. Expression of *RH2-C* was significantly lower than the other two RH2 subtypes. Expression level of *SWS2-A* was much higher than that of *SWS2-B*. Although circadian, seasonal and developmental variations of the relative expression profile remain to be evaluated, the result revealed a large difference in the expression level among the types and subtypes of the opsin genes in medaka retina.

4. Discussion

We have shown that medaka cone opsin genes consist of two M/LWS (red) type (*LWS-A* and *LWS-B*), three RH2 (green) type (*RH2-A*, *RH2-B* and *RH2-C*), two SWS2 (blue) type (*SWS2-A* and *SWS2-B*) and one SWS1 (ultraviolet) type (*SWS1*) in the genome (Fig. 1). Photopigment reconstruction with 11-*cis* retinal revealed that there was a considerable spectral variance among the three RH2 subtypes with 24–64 nm λ_{max} differences and between the two SWS2 subtypes with 34 nm λ_{max} difference (Fig. 3). Employing a real-time RT-PCR assay, we showed that the expression level in the retina also differed between or among the subtypes (Fig. 4B). This study, together with our previous study on the medaka rod opsin gene (*RH1*) (Kawamura et al., 2005), provides information about the second complete set of fish visual opsin genes in addition to our study of zebrafish opsin genes (Chinen et al., 2003).

4.1. Local gene duplication

In both medaka and zebrafish, subtype opsin genes are closely linked and are clearly the products of local gene duplications but not the genome-wide duplication which occurred before the teleost radiation (Amores et al., 1998). It appears that genome duplication has little influence on the generation of visual opsin repertoires in fish species. For coordinated expression of the subtype opsin genes in the retina, regulatory regions and transcription factors associated with the genes need to be under interrelated control mechanisms. Such regulation would be more feasible if the genes are in close proximity. Advantage of the tandem organization in opsin genes is seen in the human red and green opsin genes. These are expressed in a mutually exclusive manner by the interaction of a locus control region located upstream of the red opsin gene and the promoter region of red or green opsin gene (Smallwood et al., 2002). Though expression patterns of opsin genes in medaka retina remain to be elucidated, our study on zebrafish M/LWS and RH2 opsin genes revealed that these subtype genes are indeed differentially expressed in the retina both spatially and temporally (Takechi and Kawamura, 2005). The BAC and lambda clones isolated in this study could be useful for the examination of the regulatory mechanism of coordinated expression of subtype opsin genes and to test the advantage of local gene duplications on differentiation of the opsin genes.

4.2. Phylogenetic implications

Given a large evolutionary distance of 115–200 million years between medaka and zebrafish belonging respectively to the different superorders Acanthopterygii and Ostariophysi (Nelson, 1994; Furutani-Seiki and Wittbrodt, 2004), information of complete gene organization of opsins in the two species illustrates the evolution of the visual opsin repertoire in fish.

4.2.1. M/LWS

Although medaka and zebrafish are similar in both having tandemly duplicated M/LWS opsin genes, phylogenetic analysis revealed that the gene duplication occurred independently in medaka and zebrafish (Fig. 5A). The two subtypes of medaka M/LWS opsins are spectrally undifferentiated and abundantly expressed but those of zebrafish are spectrally distinct and expressed only at a low level (Chinen et al., 2003). M/LWS subtypes have also been found in Mexican cavefish (Yokoyama and Yokoyama, 1990) and bluefin killifish, however, their chromosomal positions between subtypes are unknown. The possibility that some of these duplications may have a common origin should be considered and that subsequent concerted evolution by gene conversions might have masked any trace of ancient duplications. However, DNA sequences of the opsin subtype of medaka and zebrafish can be aligned only in the coding regions and it is difficult to detect duplication units in the genomic region. Future elucidation of the regulatory regions of M/LWS genes in the zebrafish and medaka might reveal the origin of the duplication event.

There is ancient gene duplication at a common ancestor of all fish shown in Fig. 5A which leads to the cavefish *R007* and *G101*/*G103*. *R007* and *G101*/*G103* have considerable differences in spectral sensitivity with λ_{max} at 558 nm in R007 and at 530 nm in G101/G103 (Yokoyama and Radlwimmer, 2001).

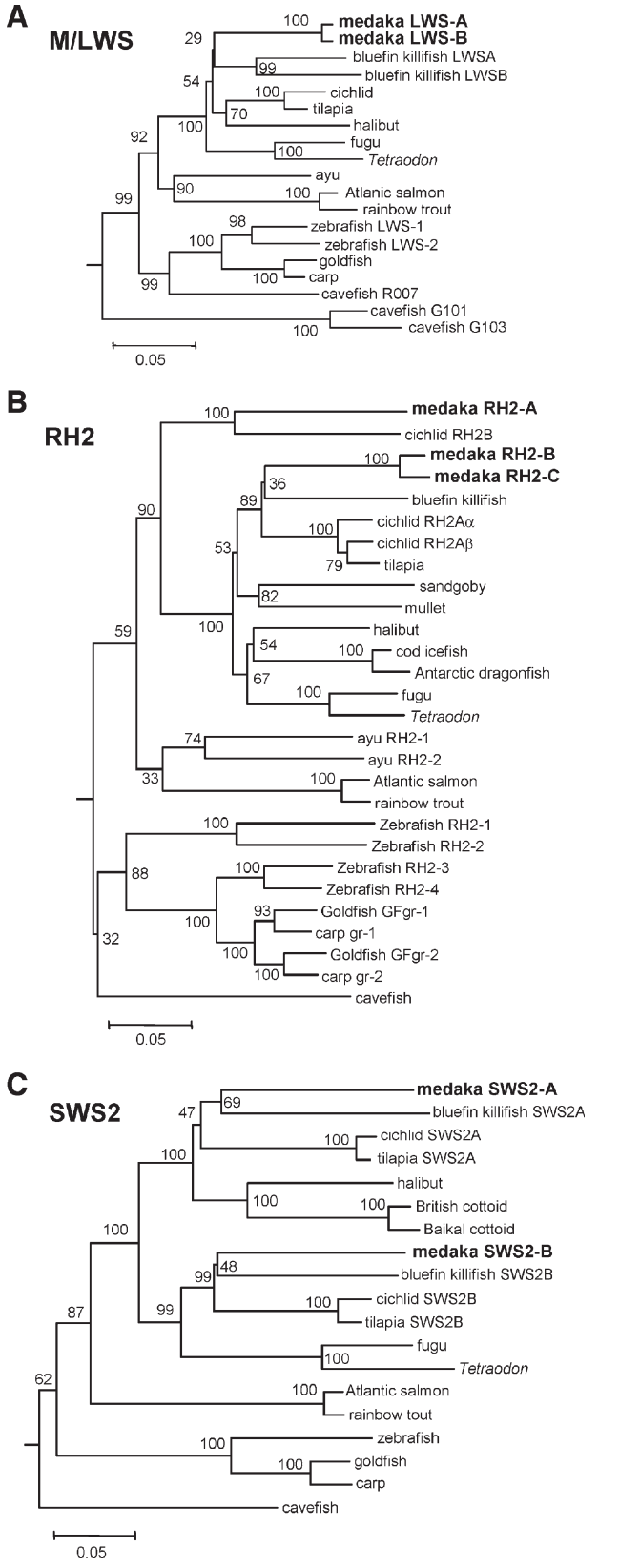

Fig. 5. Phylogenetic trees of the fish M/LWS (A), RH2 (B) and SWS2 (C) opsin genes based on their nt sequences. The medaka genes are highlighted with boldface letters. The bootstrap probabilities are given to each node. Phylogenetic root was given by using orthologous nt sequences of pigeon and American chameleon as outgroups in each tree. Scale bar: five nt substitutions per 100 sites.

Orthologous genes of cavefish *G101*/*G103* have not been found in other fish species including medaka and zebrafish. Although the genes must have been lost at least in medaka and zebrafish genomes, future examination of the orthologous gene in various fish taxa would clarify evolutionary diversity of the fish visual system.

4.2.2. RH2

A phylogenetic tree of fish RH2 opsin genes is shown in Fig. 5B which demonstrates that subtypes in zebrafish and medaka were formed by independent gene duplication events despite the similarity in their genomic organizations. The first gene duplication leading to *RH2-A* and *RH2-B*/*RH2-C* occurred in a common ancestor of the superorder Acanthopterygii which includes medaka, killifish, cichlid, tilapia, mullet, sandgoby, halibut and pufferfish (fugu and *Tetraodon*), and the second duplication leading to *RH2-B* and *RH2-C* occurred in medaka. An orthologous gene of the medaka *RH2-A* has been detected only in cichlids as a functional gene (*RH2B* in Parry et al., 2005) and in fugu and *Tetraodon* as a pseudogene (Neafsey and Hartl, 2005). In contrast, functional genes orthologous to the medaka *RH2-B*/*RH2-C* have been reported in many species. It is not clear whether orthologous genes to the medaka *RH2-A* have been lost or pseudogenized in many Acanthopterygii species as in pufferfish or were missed in previous studies. In the gene clade orthologous to the medaka *RH2-B*/*RH2-C*, there may be another gene duplication at a common ancestor of cichlid and tilapia, giving rise to their *RH2Aα* and *RH2Aβ* subtypes (Fig. 5) (Parry et al., 2005). The cichlids *RH2B*, *RH2Aα* and *RH2Aβ* subtypes are spectrally different as in medaka, with *RH2B* being shorter-wave sensitive with λ_{max} at ~485 nm and *RH2Aα* and *RH2Aβ* being longer-wave sensitive with λ_{max} at ~530 nm and 505–520 nm, respectively (Parry et al., 2005). *RH2-B*/*RH2-C* duplication in medaka and *RH2Aα*/*RH2Aβ* duplication in cichlids appear to be independent events in the phylogenetic tree (Fig. 5). However, the possibility cannot be ruled out that these duplications are of a common origin and subsequent gene conversion could have eliminated any trace of long divergence. Comparative studies of RH2 opsin genes in genomic arrangement, spectral tuning mechanisms and regulatory mechanisms of expression among various Acanthopterygii species should clarify the evolutionary process of functional diversification among RH2 opsin repertoires.

4.2.3. SWS2

Fig. 5C shows that a gene duplication leading to the medaka *SWS2-A* with λ_{max} at 439 nm and *SWS2-B* at 405 nm occurred in the common ancestor of Acanthopterygii. Bluefin killifish has two spectral classes of cone cells in the blue-violet range with λ_{max} at ~455 nm and at ~405 nm (Fuller et al., 2003) which may correspond to their *SWS2A* and *SWS2B* expressing cells, respectively (Fuller et al., 2004). In cichlids, λ_{max} values of *SWS2A* and *SWS2B* photopigments with 11-*cis* retinal have been measure by microspectrophotometry (MSP) and in vitro reconstitution to be at 452–456 nm and at 415–423 nm,

respectively (Parry et al., 2005). These observations suggest that the origin of spectral differentiation between medaka *SWS2-A* and *SWS2-B* is also ancient.

Pufferfish have a single SWS2 type opsin gene orthologous to the medaka *SWS2-B* but their *SWS2-A* ortholog is not found in the genome database (Neafsey and Hartl, 2005) and must have been lost as in *RH2-A* orthologs during pufferfish evolution. In contrast to pufferfish, cottoid fish and halibut have only a *SWS2-A* ortholog, and the orthologous gene to the medaka *SWS2-B* might have been lost or simply missed. In the phylogenetic tree of SWS2 genes, cavefish is located outside all the other fish (Fig. 5C) although it belongs to a superorder Ostariophysi with zebrafish, goldfish and carp (Nelson, 1994). This may suggest an additional gene duplication of SWS2 gene in a common ancestor of teleosts, but this is more likely to be a statistical artifact since the bootstrap support for the clade is not high (62%).

4.3. Spectral tuning

The λ_{max} values of *RH2-A* of 452 nm and *SWS2-B* of 405 nm of medaka are the shortest reported of vertebrate RH2 and SWS2 pigments, respectively, creating a large spectral separation between the subtypes of 64 nm in RH2 and 34 nm in SWS2 types. The aa sites located in the close vicinity to the retinal in the model of bovine rod opsin, i.e., the retinal-surrounding sites (Palczewski et al., 2000; Menon et al., 2001; Takahashi and Ebrey, 2003), are expected to exert direct interaction with the retinal. Therefore, these aa sites are so-called "key sites" which are the first candidates to be examined for variation associated with spectral differences among opsins (Chinen et al., 2005a,b).

When focusing on the retinal-surrounding sites, aa at sites 122, 292 and 295 (position numbers follow those of bovine rod opsin) are varied among the three RH2 subtypes of medaka: Gln at site 122, designated Gln122, occurs in *RH2-A* and *RH2-C* compared to Glu122 in *RH2-B*; Ser292 occurs in *RH2-A* compared to Ala292 in *RH2-B* and *RH2-C*; Ser295 occurs in *RH2-A* compared to Ala295 in *RH2-B* and *RH2-C*. It has been reported that aa substitution from Glu to Gln at site 122 (Glu122Gln) results in a −15 nm spectral shift in RH2 opsins (Yokoyama et al., 1999; Chinen et al., 2005a) and that a Ala292Ser substitution results in a −27 nm shift in M/LWS opsins (Yokoyama and Radlwimmer, 2001). Therefore, these substitutions may account for some of the spectral differences among the three RH2 subtypes in medaka.

Six aa sites of the two SWS2 subtypes of medaka differ in the retinal-surrounding region: Phe43 compared to Leu43; Ala94 compared to Cys94; Thr118 compared to Ala118; Ala164 compared to Gly164; Trp265 compared to Tyr265; and Leu294 compared to Val294 in *SWS2-A* and *SWS2-B*, respectively. Among these a Thr118Ala substitution was reported to result in a −5 nm spectral shift in the SWS2 opsin of cottoid fish (Cowing et al., 2002). A Ser94Ala substitution causes a −14 nm shift in the newt SWS2 opsin (Takahashi and Ebrey, 2003) but same substitution results only in a −3 nm shift in goldfish SWS2 (Chinen et al., 2005b). A Ser164Ala substitution causes a −7 nm shift in M/LWS opsins (Yokoyama and Radlwimmer, 2001). In addition to the retinal-surrounding sites, a Lue116Thr substitution was reported to cause a −6 nm shift in goldfish SWS2 (Chinen et al., 2005b) and there is a Ala116Thr difference between medaka *SWS2-A* and *SWS2-B*. These aa substitutions may contribute to the spectral difference between the two SWS2 opsins of medaka.

It should be noted that a large portion of the spectral differences could be attributed to the collective effects of multiple aa replacements which are located outside the retinal-surrounding region and have individually minor and indirect spectral effects, as in zebrafish RH2 and SWS2 opsins (Chinen et al., 2005a,b). Such collective effects could be additive (Chinen et al., 2005b) but are often synergistic (Shi and Yokoyama, 2003) or even regressive (Chinen et al., 2005a), making predictions uncertain of λ_{max} values from only aa variations at key sites. These issues can only be resolved through experimental verification using site-directed mutagenesis. The mutagenesis experiments for medaka RH2 and SWS2 opsins are targeting aa substitutions inferred to have occurred between the current and the reconstructed ancestral aa sequences as in Chinen et al. (2005a,b).

4.4. Toward intra-genus comparison

Up to 20 congeneric species of medaka are widely distributed in various climatic regions of Asia, many of these are available as laboratory stocks (Takehana et al., 2005). The Japanese medaka studied here is a freshwater surface swimmer which can utilize a wide range of sunshine spectrum. Their rich repertoire of visual opsins covering the broad spectrum should be correlated with its visual ecology. Little information has accumulated thus far on the visual behaviors, the light environments and the visual system of medaka. MSP and electro-physiological data on visual cells have not been reported for medaka. Previously, data on spectral sensitivity of medaka was only from whole-retina extracts showing a λ_{max} at ~510 nm (Hasegawa, 1998). Fish visual pigments consist of an opsin and a chromophore, either 11-*cis* retinal, Vitamin A1 aldehyde, or 11-*cis* 3,4-dehydroretinal, Vitamin A2 aldehyde. The A1/A2 chromophore ratio in medaka is reported to be A1-dominated, but having a broad range with the A1 percentage varying from 40–90% according to season (Hasegawa and Miyaguchi, 1997). Combined with an identical opsin, the A2 chromophore shows a λ_{max} at a longer wavelength than that of the A1 chromophore, and this spectral difference is larger when combined to a longer-wavelength sensitive opsin (Whitmore and Bowmaker, 1989). Therefore, seasonal variation of A1/A2 ratio would further expand the repertoire of visual pigments in medaka.

Using gene clones and PCR primer sequences of this study, it is now possible to compare opsin repertoires and the spatial and the temporal expression profile of opsin subtypes in the retina among the closely related medaka species. These studies, together with a detailed comparative study of A1/A2 chromophore ratio among these species at different temperatures, representing their seasonal variations, will increase our understanding of the biological significance of the visual pigment repertoires which medaka species have developed.

Acknowledgements

We greatly appreciate Drs. Shuichi Asakawa and Nobuyoshi Shimizu (Keio University School of Medicine) for the medaka BAC library. The whole genome sequence data has been provided freely by the National Institute of Genetics and the University of Tokyo for use in this publication only. This study was supported by Grants-in-Aid for Scientific Research (B) (12440243) and Exploratory Research (15657005) from Japan Society of the Promotion of Science to S.K. The manuscript was proofread by BioMed Proofreading Service.

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