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A novel spectral tuning in the short wavelength-sensitive (SWS1 and SWS2) pigments of bluefin killifish (*Lucania goodei*)

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Abstract

The molecular bases of spectral tuning in the UV-, violet-, and blue-sensitive pigments are not well understood. Using the *in vitro* assay, here we show that the SWS1, SWS2-A, and SWS2-B pigments of bluefin killifish (*Lucania goodei*) have the wavelengths of maximal absorption (λ_{max}'s) of 354, 448, and 397 nm, respectively. The spectral difference between the SWS2-A and SWS2-B pigments is largest among those of all currently known pairs of SWS2 pigments within a species. The SWS1 pigment contains no amino acid replacement at the currently known 25 critical sites and seems to have inherited its UV-sensitivity directly from the vertebrate ancestor. Mutagenesis analyses show that the amino acid differences at sites 44, 46, 94, 97, 109, 116, 118, 265, and 292 of the SWS2-A and SWS2-B pigments explain 80% of their spectral difference. Moreover, the larger the individual effects of amino acid changes on the λ_{max}-shift are, the larger the synergistic effects tend to be generated, revealing a novel mechanism of spectral tuning of visual pigments.

Keywords

visual pigments; absorption spectra; mutagenesis; bluefin killifish

1. Introduction

The photosensitive molecules, visual pigments, consist of an opsin and a chromophore, either 11-*cis*-retinal or 11-*cis*-3, 4-dehydroretinal, and their major function can be characterized by the wavelength of maximal absorption (λ_{max}). In the early stage of vertebrate evolution, our ancestor already possessed five paralogous visual pigments: 1) rhodopsin (RH1); 2) RH1-like (RH2); 3) short wavelength-sensitive type 1 (SWS1); 4) SWS type 2 (SWS2); and 5) middle and long wavelength-sensitive (M/LWS) pigments (e. g., Yokoyama and Yokoyama, 1996; Yokoyama, 2000a; Ebrey and Koutalos, 2001). To adapt to their diverse ecological and physiological environments, organisms have modified the absorption spectra of their visual pigments during vertebrate evolution (e.g., Lythgoe, 1979; Yokoyama and Yokoyama, 1996). Using different experimental methods, currently known RH1, RH2, SWS1, SWS2, and M/LWS pigments are shown to haveλ_{max}'s of 480–520, 460–510, 360–440, 410–460, and 510–570 nm, respectively (e.g., Yokoyama, 2000a; Ebrey and Koutalos, 2001; Takahashi and Ebrey, 2003). These evolutionary characteristics and the availability of functional assays make

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visual pigments one of only handful molecules that allow us to conduct experimental analyses of adaptive evolution in vertebrates (Yokoyama and Yokoyama, 1996; Yokoyama, 1997; Yokoyama, 2000a; Carroll, 2006).

The bluefin killifish, *Lucania goodei*, live in freshwater in South Carolina, Georgia, and Florida. Using the microspectrophotometry (MSP), Fuller and her colleagues have identified cone pigments of bluefin killifish with λ_{max}'s of 538 nm (RH2 pigment), 359 nm (SWS1 pigment), 454 nm (SWS2-A pigment), 405 nm (SWS2-B pigment), and 572 nm (LWS pigment) (Fuller *et al*., 2003). Thus, in addition to the UV-sensitive SWS1 pigment, bluefin killifish uses blue-sensitive SWS2-A and violet-sensitive SWS2-B pigments. More recently, medaka (Matusmoto et al., 2006), tilapia (Spady et al., 2006), and cichlids (e. g., Parry et al., 2005) are also shown to have two types of SWS2 pigments. Among these, bluefin killifish has the largest spectral difference between SWS2-A and -B pigments and provides an opportunity to explore the molecular basis of spectral tuning in SWS2 pigments. Therefore, we constructed the bluefin killifish SWS1, SWS2-A, and SWS2-B pigments and studied the evolutionary patterns of their functional differentiation. The results show that the λ_{max}'s of the three pigments evaluated using the *in vitro* assay are consistently 5–8 nm shorter than the corresponding MSP estimates. Furthermore, the majority of the λ_{max} difference between the two SWS2 pigments can be explained by nine amino acid differences; in particular, those at sites 94, 97, 118, and 265 significantly modulate the λ_{max}'s of the SWS2 pigments individually as well as synergistically.

2. Materials and methods

2.1. In vitro assays of SWS1 and SWS2 pigments

Total retinal RNAs of bluefin killifish (*Lucania goodei*) are gift from Dr. R. C. Fuller at the University of Illinois at Urbana/Champaign. The full-length SWS1, SWS2-A, and SWS2-B opsin cDNAs were obtained by RT-PCR using primers based on the nucleotide sequences of the 5′ and 3′ end cDNA clones (SWS1: 5′–ATTGCAGAATTCCACCATGGGGAAACATTTTC-3′ (forward) and 5′–TCCCAAGTCGACGAAGCTGTGGACAC-3′ (reverse); SWS2-A: 5′–GCAGAAGAATTCCACCATGAGGTCCACCCGAGTCATAGAGC-3′ (forward) and 5′–CTGTTAGTCGACGCTGGGCCAACTTTGGAGA-3′ (reverse); and SWS2-B: 5′–AAACAAGAATTCCACCATGAAGATGAGGGTAAAC-3′ (forward) and 5′–GCCGAGAGTCGACGAGGGTCCAACTTTTGAG-3′ (reverse)). The opsin cDNAs of full length were subcloned into the *Eco*RI and *Sal*I restriction sites of the expression vector pMT5 (e.g., Yokoyama, 2000b). To rule out spurious mutations, these DNA fragments were sequenced by cycle sequencing reactions using the Sequitherm Excel II long-read kits (Epicentre Technologies, Madison, WI) with dye-labeled M13 forward and reverse primers. Reactions were run on a LI-COR (Lincoln, NE) 4200LD automated DNA sequencer.

These opsins were expressed in COS1 cells by transient transfection. The pigments were regenerated by incubating the opsins with 11-*cis*-retinal (Storm Eye Institute, Medical University of South Carolina) and were purified using immobilized 1D4 (The Culture Center, Minneapolis, MN) in buffer W1 (50 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 6.6), 140 mM NaCl, 3mM $MgCl_2$, 20% (w/v) glycerol and 0.1% dodecyl maltoside) (for more details, see Yokoyama, 2000b). UV visible spectra were recorded at 20° C using a Hitachi U-3000 dual beam spectrophotometer. Visual pigments were bleached for 3 min using a 60 W standard light bulb equipped with a Kodak Wratten #3 filter at a distance of 20 cm. Data were analyzed using Sigmaplot software (Jandel Scientific, San Rafael, CA).

2.2. Site-directed mutagenesis

Mutant opsins were generated by using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All DNA fragments that were subjected to mutagenesis were sequenced to rule out spurious mutations.

2.3. Sequence data analyses

We considered a total of 38 representative fish pigments from the five pigment groups: RH1 pigments (goldfish 1, GenBank accession no. L11863; zebrafish 1, AF109368; cavefish 1, U12328; medaka 1, AB001606; and bluefin killifish, AY296738), RH2 pigments (goldfish 2-A, L11865; goldfish 2-B, L11866; zebrafish 2–1, AB087805; zebrafish 2–2, AB087806; zebrafish 2–3, AB087807; zebrafish 2–4, AB087808; cavefish 2; S75255; medaka 2-A, AB223053; medaka 2-B, AB223054; medaka 2-C, AB223055; and bluefin killifish 2, AY296739), SWS1 pigments (goldfish S1, D85863; zebrafish S1, AB087810; medaka S1, AB223058; tilapia S1, AF191221; and bluefin killifish S1, AY296735), SWS2 pigments (goldfish S2, L11864; zebrafish S2, AB087809; cavefish S2, AF134762; medaka S2-A, AB223056; medaka S2-B, AB223057; tilapia S2-A, AF247116; tilapia S2-B, AF247120; bluefin killifish S2-A, AY296737; and bluefin killifish S2-B, AY296736), and M/LWS pigments (goldfish L, L11867; cavefish L, M38625; cavefish M, M38619; zebrafish L-1, AB087803; zebrafish L-2, AB087804; medaka L-A, AB223051; medaka L-B, AB223052; and bluefin killifish L, AY296740). The phylogenetic tree of these visual pigments has been constructed by applying the neighbor-joining (NJ) method (Saitou and Nei, 1987) to the nucleotide sequences between codon positions 9 and 320. On the basis of this tree topology, we inferred the ancestral amino acid sequences of visual pigments by using a computer program, PAML, based on a likelihood-based Bayesian method (Yang, 1997).

3. Results

3.1. Absorption spectra of the SWS1 and SWS2 pigments of bluefin killifish

The SWS1, SWS2-A, and SWS2-B opsins were expressed in the COS1 cells transiently and were reconstituted with the 11-*cis*-retinal. The *in vitro* assays show that the SWS1, SWS2-A, and SWS2-B pigments have λ_{max}'s of 354 nm ± 0 (referred to as bfin killifish S1 (P354)), 448 ± 1 nm (bfin killifish S2-A (P448)), and 397 ± 0 nm (bfin killifish S2-B (P397)), respectively (Fig. 1). These values are close to the corresponding MSP estimates of 359 ± 2, 454 ± 3, and 405 ± 2 (Fuller et al., 2003), but our estimates are consistently 5–8 nm shorter than the MSP values. In particular, the λ_{max} of bfin killifish S2-B (P397) is lowest among all currently known SWS2 pigments; in fact, it is almost identical to the λ_{max} of 393 nm of the pigeon violet pigment, which is lowest among those of the currently known violet pigments in the paralogous SWS1 pigment group (Yokoyama, 2000a). Consequently, the observed λ_{max} difference of 51 nm between the SWS2-A and SWS2-B pigments of bluefin killifish is largest among those of the currently known pairs of SWS2 pigments within a species.

3.2. The phylogenetic tree of fish visual pigments

At present, the *in vitro* assays of visual pigments in fish have been performed for goldfish (*Carassius auratus*), zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), cavefish (*Astyanax fasciatus*), tilapia (*Oreochromis niloticus*), and bluefin killifish. Applying the NJ method to the nucleotide sequences of 38 pigments (see Materials and methods), we have constructed the unrooted phylogenetic tree (Fig. 2). A majority of bootstrap values in Fig. 2 are > 0.95 and most bifurcation patterns are highly reliable. The NJ tree shows that the visual pigments are classified into RH1, RH2, SWS1, SWS2, and M/LWS groups and that in each group, the goldfish and zebrafish pigments form one cluster and medaka, tilapia, and bluefin killifish pigments form another cluster; the cavefish pigment seems to belong to the goldfish-zebrafish

pigment cluster (see also Yokoyama, 2000a;Matsumoto et al., 2006;Spady et al., 2006). The exact phylogenetic relationships of medaka, tilapia, and bluefin killifish pigments, however, cannot be established. When the amino acid sequences are considered, the tree topologies of the SWS1 and SWS2 pigments are identical to those in Fig. 2. However, the cavefish 1 is most distantly related to the other RH1 pigments, and the medaka 2-A in the RH2 group is more closely related to the medaka-bluefin killifish cluster. Since the bootstrap values of these nodes in the amino acid tree are much lower than 0.95, the two tree topologies are consistent with each other. Because of its higher bootstrap values at different nodes, the tree topology in Fig. 2 seems to be more reliable than the amino acid tree.

3.3. Molecular bases of spectral tuning in the SWS1 and SWS2 pigments of bluefin killifish

Based on the tree topology in Fig. 2, we inferred amino acid sequences of all ancestral pigments at all nodes using the JTT, Dayhoff, and WAG models of amino acid replacements (Yang, 1997). At present, certain amino acid replacements at 25 sites (46, 49, 52, 83, 86, 90, 91, 93, 94, 97, 109, 113, 114, 116, 118, 122, 164, 181, 207, 211, 261, 265, 269, 292, and 295) are known to have modified the λ_{max}'s of various visual pigments in nature (see the Discussion section), where amino acid site numbers correspond to those of the bovine RH1 pigment. At these sites, no amino acid replacement has occurred in bfin killifish S1 (P354). Since the ancestral vertebrate SWS1 pigment had a λ_{max} of ~360 nm (Shi and Yokoyama, 2003), like many other fishes, bfin killifish S1 (P354) must have inherited its UV-sensitivity directly from the vertebrate ancestor. Because of its small shift, we did not explore the exact molecular basis for the decrease in the λ_{max} of bfin killifish S1 (P354).

Of the 25 critical amino acid sites, bfin killifish S2-A (P448) and bfin killifish S2-B (P397) differ at eight positions: the former pigment contains amino acids L46, A94, S97, A109, I116, T118, W265, and A292 and the latter pigment contains F46, C94, C97, G109, T116, G118, Y265, and S292. The amino acid sequences of these pigments and their inferred ancestral pigments suggest that these amino acid differences might have been generated by F46L, M116I, and S292A (denoted as M46L/M116I/S292A), A94C/S97C/M116T, and A109G/T118G/W265Y at branches b - bfin killifish S2-A (P448), a – c, and c - bfin killifish S2-B (P397), respectively (Table 1). Thus, F46L/M116I/S292A and A94C/S97C/A109G/M116T/T118G/W265Y seem to have occurred in bfin killifish S2-A (P448) and bfin killifish S2-B (P397), respectively.

In order to evaluate the effects of these amino acid replacements on the λ_{max}-shift, we introduced mutations L46F, A94C, S97C, A109G, I116T, T118G, W265Y, and A292S into the bfin killifish S2-A (P448) individually, where the two independent events of M116I in bfin killifish S2-A (P448) and M116T in bfin killifish S2-B (P397) are combined into one step. If we follow the patterns of amino acid replacements in Table 1, then A94C, S97C, A109G, T118G, and W265Y are forward mutations and L46F and A292S are reverse mutations. The *in vitro* assays show that with the exception of A109G, these mutations decrease the λ_{max} at least by 4 nm (Table 2). Although it is not included among the 25 critical sites, we also found that T44M decreases the λ_{max} by 4 nm (Table 2).

In theory, if the effects of these 9 amino acid changes on the λ_{max}-shift were additive, then they would have decreased the λ_{max} of bfin killifish S2-A (P448) by 100 nm, predicting that the bfin killifish S2-B (P397) should have a λ_{max} of 348 nm, which is ~50 nm lower than the actual value. In practice, however, when the nine amino acid changes are introduced into bfin killifish S2-A (P448) together, they decrease the λ_{max} by 41 nm, explaining a 80% of the entire λ_{max} difference between the two SWS2 pigments (Table 2). We cannot explain the remaining 10 nm difference between the λ_{max}'s of the two SWS2 pigments, but it is clear that the major portion of spectral difference between bfin killifish S2-A (P448) and bfin killifish S2-B (P397) is modulated by both individual and interactive amino acid replacements.

To see the extent of the synergistic effects of the critical amino acid replacements on the λ_{max}-shift, we first considered A94C and W265Y, which reduced the λ_{max} by 16 and 29 nm, respectively (Table 2). When both of these mutations were introduced into the bfin killifish S2-A (P448), they reduced the λ_{max} by 32 nm. Thus, the interaction between A94C and W265 (denoted as A94CxW265) increases the λ_{max} by 13 nm (Table 3). T118G decreases the λ_{max} by 15 nm and the interactions between T118G and A94C/W265Y (the sum of T118G x A94C, T118G x W265Y, and T118G x A94C x W265Y, or T118GxA94C/W265) increase the λ_{max} by 14 nm; similarly, the interactions between A292S and A94C/T118G/W265 (A292SxA94C/T118G/W265) increase the λ_{max} by 11 nm (Table 3). On the other hand, the interactions between T44M and A94C/T118G/W265Y/A292S cause only a trivial λ_{max}-shift of 2 nm (Table 3). These examples show that the larger the individual effects of amino acid changes on the λ_{max}-shift are, the more the synergistic effects of these amino acid changes on the λ_{max}-shift tend to be generated.

4. Discussion

At present, certain amino acid changes at a total of 25 sites are known to have modified the λ_{max} of various visual pigments during vertebrate evolution. They include sites 83, 122, 211, 261, 265, 292, and 295 in RH1 pigments (e. g. Yokoyama, 2000a), sites 49, 122 and 207 in RH2 pigments (Yokoyama et al., 1999; Chinen et al., 2005a), sites 46, 49, 52, 86, 90, 91, 93, 97, 109, 113, 114, 116, and 118 in SWS1 pigments (e. g. Yokoyama, 2000a; Babu et al., 2001; Shi and Yokoyama, 2003; Fasick et al., 2002; Takahashi and Yokoyama, 2005), sites 91, 94, 116, 122, 261, 292, and 295 in SWS2 pigments (Takahashi and Ebrey, 2003; Chinen et al., 2005b), and sites 164, 181, 261, 269, and 292 in M/LWS pigments, which correspond to sites180, 197, 277, 285, and 308 of the human red- and green-sensitive pigments (Yokoyama and Radlwimmer, 2001). Our analyses of the bluefin killifish SWS2 pigments show that site 44 is also involved in the spectral tuning of visual pigments, increasing the total number of critical amino acid sites to 26.

At present, the mechanisms of spectral tuning in visual pigments in nature can be distinguished into two major groups: the absorption spectra of visual pigments are determined largely either 1) by a small number of individual amino acid changes, each causing a significant λ_{max}-shift, or 2) by interactions among different critical amino acids, each causing a relatively small or no λ_{max}-shift. The extreme examples of the first group can be seen in several amino acid changes at sites 86 and 90 in SWS1 pigments. That is, Y86F in bovine (Fasick et al., 2002; Cowing et al., 2002) and flying squirrel (Carvalho et al., 2006) pigments as well as F86Y in mouse (Fasick et al., 2002) and goldfish (Cowing et al., 2002) pigments shift the λ_{max} by 60–75 nm; V86F in guinea pig pigment (Parry et al., 2004) and S86F in elephant pigment (Yokoyama et al., 2005) decrease the λ_{max} by 52–53 nm. S90C and C90S in several avian and African clawed frog pigments also shift the λ_{max} by 35–45 nm (Yokoyama et al., 2000; Wilkie et al., 2000; Dukkipati et al., 2002). The interactions of amino acids at these sites with others seem to cause significantly smaller magnitudes of λ_{max}-shifts (Yokoyama et al., 2005). Although the magnitudes of changes are much smaller, S164A, H181Y, Y261F, T269A, and A292S in the LWS pigments and the reverse changes in the MWS pigments also shift the λ_{max} by 7–30 nm individually. These amino acids act mostly in an additive fashion, but a significant interaction seems to occur only between sites 164 and 181 in rodent lineages (Yokoyama and Radlwimmer, 2001). Amino acid replacements D83N, E122Q, M207L, and A292S in various RH1 (Nathans, 1990; DeCaluwe et al., 1995; Sugawara et al., 2005; Fasick and Robinson, 1998; Yokoyama, 2000b; Jans and Farrens, 2001; Sun et al., 1997; Lin et al., 1998), RH2 (Yokoyama et al., 1999; Chinen et al., 2005a; Imai et al., 1997), SWS2 (Takahashi and Ebrey, 2003), and M/LWS (Fasick and Robinson, 1998; Sun et al., 1997) pigments also belong to the same group.

The second group is represented by certain critical amino acid changes in RH2 (Chinen et al., 2005a), SWS1 (Shi et al., 2001), and SWS2 (Chinen et al., 2005b) pigments. The most dramatic example of this type of spectral tuning can be seen in the mouse UV pigment, where F46T, F49L, T52F, F86L, T93P, A114G, and S118T cause no λ_{max}-shift individually, but they together increase the λ_{max} by 52 nm (Shi et al., 2001).

In the bluefin killifish, the nine amino acid changes are involved in generating a major λ_{max} difference between bfin killifish S2-A (P448) and bfin killifish S2-B (P397). Out of the nine critical amino acid changes, A94C, S97C, T118G, and W265Y decrease the λ_{max} by >15 nm individually, but the overall λ_{max} of the latter pigment is also modulated by equally strong interactions among them. Thus, the molecular basis of spectral tuning in bfin killifish S2-B (P397) differs from the two currently known mechanisms of spectral tuning in visual pigments. It's generality, however, remains to be tested by additional mutagenesis analyses using the other SWS2 and paralogous pigments.

We have seen that the medaka, tilapia, and bluefin killifish SWS2 pigments have different λ_{max}'s (Fig. 2). The λ_{max}'s of medaka (Matsumoto et al., 2006) and bluefin killifish pigments were evaluated from dark spectra and those of tilapia (Spady et al., 2006) from dark-light spectra. It should be noted that the λ_{max}'s of bfin killifish S1 (P354), bfin killifish S2-A (P448), and bfin killifish S2-B (P397) evaluated from dark-light spectra are 343 ± 0, 462 ± 0, and 425 ± 0 nm, respectively. Thus, the λ_{max}'s evaluated from dark and dark-light spectra can differ by more than 10 nm and should not be compared directly. Thus, only the corresponding λ_{max}'s of SWS2 pigments of medaka and bluefin killifish are coparable. Since their divergence, medaka S2-A (P439) and bfin killifish S2- A (P448) seem to have accumulated M116A and M44T/F46L/M116I/S292A, respectively (Table 1). Although we don't know the effects of M116A and M116I on the λ_{max}-shift, M44T, F46L, and S292A are all expected to increase the λ_{max} of the latter pigment (Table 2) and, therefore, bfin killifish S2-A (P448) should have a higher λ_{max} than medaka S2-A (P439). This explains the observed spectral difference. Similarly, F46V/A109G/T118A/W265Y and A109G/T118G/W265Y occurred in medaka S2-B (P405) and bfin killifish S2-B (P397), respectively (Table 1). Since T118A decreases the λ_{max} of bovine RH1 pigment by 16 nm (Jans and Farrens, 2001), it is likely that A109G/T118AW265Y in medaka S2-B (P405) and A109G/T118G/W265Y in bfin killifish S2-B (P397) decrease the λ_{max} by the same extent. Therefore, the spectral difference between medaka S2-B (P405) and bfin killifish S2-B (P397) might have been generated by F46V in medaka S2-B (P405) and/or other unidentified amino acid replacements.

In 1990, we proposed that the combination between the evolutionary prediction of amino acid changes which might have been involved in the functional adaptation of visual pigments, followed by experimental tests of such evolutionary hypotheses, would be useful in studying adaptive evolution of color vision in vertebrates (Yokoyama and Yokoyama, 1990; see also Yokoyama and Yokoyama, 1996; Yokoyama, 1997). Indeed, this approach has contributed significantly in elucidating not only the molecular mechanisms of the adaptation of organisms to various color environments but also the molecular bases of spectral tuning of visual pigments. As noted earlier, it has been established that specific amino acid differences at sites 164, 181, 261, 269, and 292 modulate the λ_{max}'s of M/LWS pigments (e.g. Yokoyama and Yokoyama, 1990; Neitz et al., 1991; Asenjo et al., 1994; Sun et al., 1997; Yokoyama and Radlwimer, 2001). This genetic information has been used to establish a seemingly contradictory observation that red and green color vision as well as color blindness have undergone adaptive evolution in different lineages (Yokoyama and Takenaka, 2005). At the other end of the spectrum, using the evolutionary arguments, followed by mutagenesis analyses, it has been established that amino acid differences at more than 13 sites are involved in the differentiation of the absorption spectra of certain UV- and violet-sensitive pigments (e. g., Takahashi and Yokoyama, 2005). The evolution of these SWS1 pigments also reflects the

ecological and physiological requirements for UV vision (Shi and Yokoyama, 2003). Furthermore, certain fish species have adapted to their deeper habitats by modifying a relatively small number of amino acids, such as E122Q, E122M, M207L, and A292S (Yokoyama et al., 1999; Yokoyama and Takenaka, 2004; Hunt et al., 2001; Sugawara et al., 2005). Indeed, we may claim that these analyses of dim-light and color vision have produced "the deepest body of knowledge linking differences in specific genes to differences in ecology and to the evolution of species (Carroll, 2006)."

To further deepen our understanding of the molecular bases of spectral tuning in visual pigments, it seems most effective to consider macroevolutionary changes of distantly related visual pigments, where a significant number of critical amino acid changes have accumulated, providing solid grounds in the molecular genetic analyses of dim-light and color vision in different organisms. The microevolutionary results of the visual pigments in the bluefin killifish by Fuller and her colleagues add an additional important dimension to the evolutionary analyses of organismal adaptation; that is, adaptive changes in color vision within a species can arise through differential expression of the same sets of opsin genes (Fuller *et al.* 2003, 2004). In the future, therefore, the molecular evolutionary genetic analyses of adaptive evolution of dim-light and color vision have to be understood by studying not only the coding regions of opsin genes but also the regulatory elements that modulate the expression of various opsin genes.

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Abbreviations

λ_{max}
: wavelength of maximal absorption

SWS2
: short wavelength-sensitive type 2

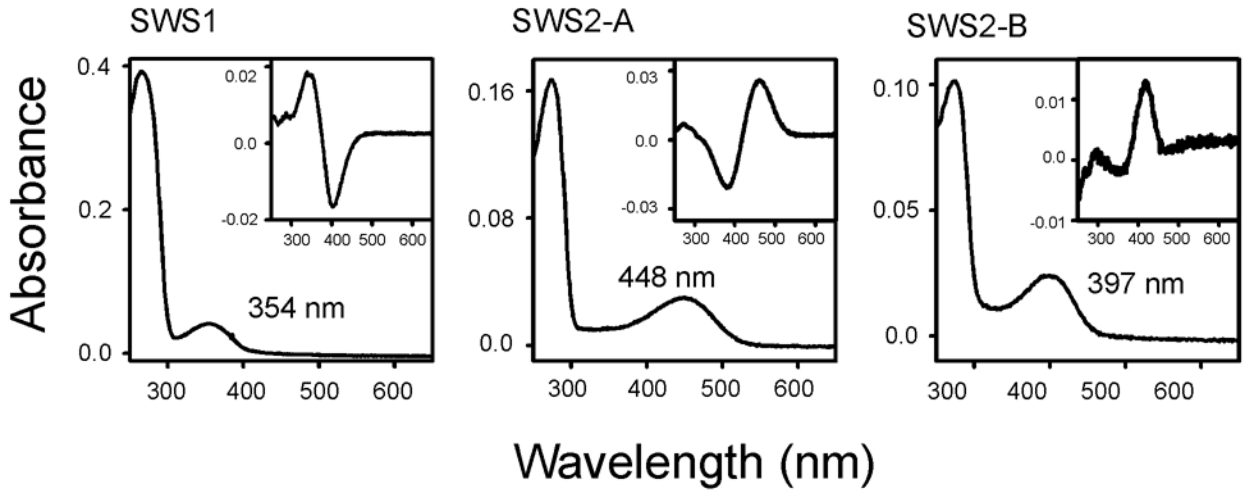

Fig. 1.
The absorption spectra of the bluefin killifish SWS1, SWS2-A, and SWS2-B pigments evaluated by the *in vitro* assay. The dark-light difference spectra are shown in the inset.

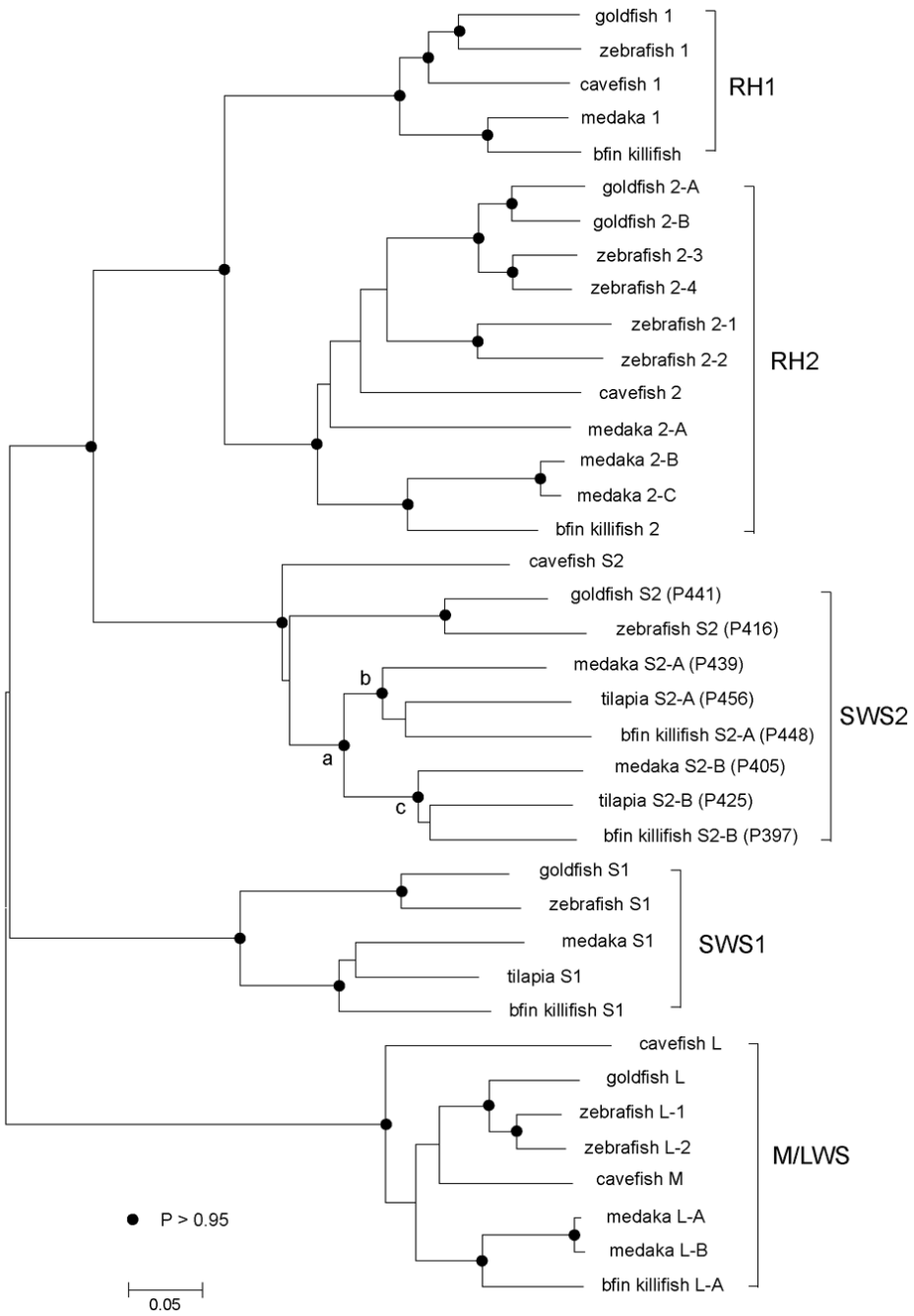

Fig. 2.

The phylogenetic tree of some representative fish visual pigments. The numbers after P in the SWS2 pigments refer to λ_{max}'s evaluated by the *in vitro* assay. The filled circles at different nodes indicate that their bootstrap values are >0.95; otherwise, the bootstrap values are <0.95. The bar at the bottom indicates evolutionary distance measured as the number of nucleotide substitutions per site.

Table 1

Amino acid replacements at the 25 critical sites that may cause λ_{max}-shifts in the SWS2 pigments, which are inferred by three different amino acid replacement models[a]

Branch	Models		
	JTT	**Dayhoff**	**WAG**
a – b	-	-	-
b – medaka S2-A (P439)	$M_{0.58}116A$	$M_{0.53}116A$	$M_{0.77}116A$
b – tilapia S2-A (P456)	$V_{0.90}49I$	$V_{0.88}49I$	$V_{0.90}49I$
b – bfin killifish S2-A (P448)	$M_{1.0}44T$[b]	$M_{1.0}44T$[b]	$M_{1.0}44T$[b]
	$F_{1.0}46L$	$F_{1.0}46L$	$F_{1.0}46L$
	$M_{0.70}116I$	$M_{0.63}116I$	$M_{0.85}116I$
	$S_{0.99}292A$	$S_{0.99}292A$	$S_{0.99}292A$
a - c	$A_{1.0}94C_{0.99}$	$A_{0.99}94C_{1.0}$	$A_{0.99}94C_{1.0}$
	$S_{0.99}97C_{0.99}$	$S_{1.0}97C_{1.0}$	$S_{1.0}97C_{1.0}$
	$M_{0.57}116T_{1.0}$	$M_{0.52}116T_{1.0}$	$M_{0.76}116T_{1.0}$
c – medaka S2-B (P405)	$F_{1.0}46V$	$F_{1.0}46V$	$F_{1.0}46V$
	$A_{0.53}109G$	$A_{0.54}109G$	$A_{0.53}109G$
	$T_{0.90}118A$	$T_{0.89}118A$	$T_{0.94}118A$
	$W_{0.76}265Y$	$W_{0.81}265Y$	$W_{0.78}265Y$
c – tilapia S2-B (P425)	-	-	-
c – bfin killifish S2-B (P397)	$A_{0.53}109G$	$A_{0.55}109G$	$A_{0.53}109G$
	$T_{0.90}118G$	$T_{0.89}118G$	$T_{0.94}118G$
	$W_{0.76}265Y$	$W_{0.81}265Y$	$W_{0.78}265Y$

[a] Subscripts indicate the posterior probabilities; for nodes a, b, and c, see Fig. 1.

[b] The effect of amino acid replacement on the λ_{max}-shift has been identified by mutagenesis analyses.

Table 2

The effects of amino acid changes on the λ_{max}-shift

Pigment	Mutation	λ_{max} (nm)	$\delta \lambda_{max}$ (nm)
bfin killifish S2-A (P448)	-	448 ± 0	
	T44M	444 ± 2	-4
	L46F	442 ± 1	-6
	A94C	432 ± 2	-16
	S97C	431 ± 1	-17
	A109G	446 ± 1	-2
	I116T	441 ± 1	-7
	T118G	433 ± 1	-15
	W265Y	419 ± 1	-29
	A292S	444 ± 0	-4
	A94C/W265Y	416 ± 2	-32
	A94C/T118G/W265Y	415 ± 1	-33
	A94C/T118G/W265Y/A292S	422 ± 0	-26
	T44M/A94C/T118G/W265Y/A292S	420 ± 1	-28
	T44M/L46F/A94C/S97C/A109G/I116T/ T118G/ W265Y/A292S	407 ± 0	-41
bfin killifish S2-B (P397)	-	397 ± 0	

Table 3

The effects of some amino acid interactions on the λ_{max}-shift

Interaction	λ_{max}-shift (nm)
A94C x W265Y	13
T118G x A94C/W265Y	14
A292S x A94C/T118G /W265Y	11
T44M x A94C/T118G /W265Y/A292S	2