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Mechanisms of spectral tuning in the RH2 pigments of Tokay gecko and American chameleon

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Abstract

At present, molecular bases of spectral tuning in rhodopsin-like (RH2) pigments are not well understood. Here, we have constructed the RH2 pigments of nocturnal Tokay gecko (*Gekko gekko*) and diurnal American chameleon (*Anolis carolinensis*) as well as chimeras between them. The RH2 pigments of the gecko and chameleon reconstituted with 11-*cis*-retinal had the wavelengths of maximal absorption ($\lambda_{\max}$'s) of 467 and 496 nm, respectively. Chimeric pigment analyses indicated that 76–86%, 14–24%, and 10% of the spectral difference between them could be explained by amino acid differences in transmembrane (TM) helices I–IV, V–VII, and amino acid interactions between the two segments, respectively. Evolutionary and mutagenesis analyses revealed that the $\lambda_{\max}$'s of the gecko and chameleon pigments diverged from each other not only by S49A (serine to alanine replacement at residue 49), S49F (serine to phenylalanine), L52M (leucine to methionine), D83N (aspartic acid to asparagine), M86T (methionine to threonine), and T97A (threonine to alanine) but also by other amino acid replacements that cause minor $\lambda_{\max}$ -shifts individually.

Keywords

Visual pigments; absorption spectra; mutagenesis; reptile

1. Introduction

The nocturnal Tokay gecko (*Gekko gekko*) has pure-rod retinas and uses 11-*cis*-retinal as the chromophore for its visual pigments (Crescitelli, 1972; Crescitelli et al., 1977). Various microspectrophotometry (MSP) analyses show that the gecko uses RH2, SWS1 (short wavelength-sensitive type 1), and MWS (middle wavelength-sensitive) pigments with the wavelengths of maximal absorption ($\lambda_{\max}$'s) of 445–470, 364, and 520–540 nm, respectively (Liebman, 1972; Crescitelli et al., 1977; Govardovskii et al., 1984; Loew, 1994). In contrast, the diurnal American chameleon (*Anolis carolinensis*) not only has pure-cone retinas but uses 11-*cis*-3, 4-dehydroretinal as the chromophore (Crescitelli, 1972; Crescitelli et al., 1977; Yu and Fager, 1982; Fowlkes et al., 1984). The corresponding RH2, SWS1, and LWS (long wavelength-sensitive) pigments of chameleon have $\lambda_{\max}$'s of 503, 365, and 625 nm (Provencio et al., 1992; E. R. Loew and L. J. Fleishman, personal communication), respectively. When the chameleon RH2, SWS1, and LWS opsins are reconstituted with 11-*cis*-retinal, the resulting pigments have $\lambda_{\max}$'s of 495, 358, and 560 nm, showing that 11-*cis*-3, 4-dehydroretinal absorbs

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a longer wavelength than 11-*cis*-retinal (Kawamura and Yokoyama, 1998). Thus, when we compare the orthologous pigments of the two species that are reconstituted with identical 11-*cis*-retinal, the SWS1 pigments have similar UV-sensitivities, but the $\lambda_{\max}$'s of RH2 and M/LWS pigments are more blue-shifted in gecko than in chameleon. This makes sense because many nocturnal animals, including gecko, are active in twilight where the distribution of light is blue-shifted (Lythgoe, 1979). Compared with the $\lambda_{\max}$ of 560 nm of chameleon LWS pigment, the gecko MWS pigment has a $\lambda_{\max}$ of ~530 nm (Blow, 2003). This spectral difference can be explained fully by three amino acid differences between the two pigments: A164 (alanine at residue 164, residue numbers hereafter follow those in bovine rhodopsin), F261 (phenylalanine 261), and A269 (alanine 269) in the gecko pigment and S164, Y261, and T269 in the chameleon pigment (e.g. Yokoyama, 2000a, 2002).

At present, the molecular basis of spectral difference between the gecko and chameleon RH2 pigments is not known. In fact, molecular analyses of spectral tuning in RH2 pigments are limited to those of chicken (*Gallus gallus*), coelacanth (*Latimeria chalumnae*), and zebrafish (*Danio rerio*) (Imai et al., 1997; Yokoyama et al., 1999; Chinen et al., 2005). Since they are reasonably distantly related to these species, reptiles provide an opportunity to explore the general molecular mechanisms of spectral tuning in RH2 pigments. To identify the relevant amino acid replacements to the spectral shifts in reptile RH2 pigments, we constructed the gecko and chameleon RH2 pigments and a series of chimeras between them, and applied site-directed mutagenesis to these pigments.

2. Materials and Methods

2.1. RH2 pigment constructions

The full-length RH2 opsin cDNA of gecko was obtained by RT-PCR using two primers (Forward: 5' - CTCCTCGAATTCCACCATGAATGGAACAGAAGGT - 3' and Reverse: 5' - TCCATAGTCGACGCAGGTGCCACCTGGCTGGAG - 3'), which were based on the 5' and 3' flanking regions of the RH2 opsin cDNA sequence of gecko (GenBank accession no. M92035). Similarly, the full-length medaka 2-A opsin cDNA was obtained by RT-PCR using two primers (Forward: 5' - ATCCTAGAATTCCACCATGGAGAACGGCACAGAG - 3' and Reverse: 5' - CGTGGTGTGTCGACGCAGCAGTAGAGACTTC - 3'), which were based on the medaka RH2-A gene (AB223053). The amplified cDNA fragments were cloned into the *EcoRI* and *SalI* restriction sites of the expression vector pMT5 (Khorana et al., 1988). The full length RH2 opsin cDNA of American chameleon has been subcloned into pMT5 in our laboratory (Kawamura and Yokoyama, 1998). The cDNA clones were sequenced to rule out spurious mutations by using Sequitherm Excel II long-read kit (Epicentre Technologies, Madison, WI) with dye-labeled M13 forward and reverse primers. Reactions were run on a LI-COR 4200LD automated DNA sequencer (LI-COR, Lincoln, NE).

These plasmids 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 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 MgCl₂, 20% (w/v) glycerol and 0.1% dodecyl maltoside) (for 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. Construction of chimeric RH2 pigments and site-directed mutagenesis

All mutant pigments with single and multiple amino acid changes were generated by using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the primers were designed according to the manufacture protocol. Various chimeric pigments were constructed by recombining their cDNA fragments at restriction enzyme recognition sites, *Hind*III, *Rsa*I, *Bsu*36I, *Pvu*II, and *Bst*YI (Fig. 1). DNA sequences of all chimeric pigments were confirmed by sequencing and the desired mutants were cloned into expression vector pMT5.

2.3. Sequence data analyses

We considered the RH2 pigments of goldfish (*Carassius auratus*; goldfish 2-1 (P511) and goldfish 2-2 (P506)), zebrafish (*Danio rerio*; zebrafish 2-1 (P467), zebrafish 2-2 (P476), zebrafish 2-3 (P488), and zebrafish 2-4 (P505)), bluefin killifish (*Lucania goodie*; bfin killifish 2), takifugu (*Takifugu rubripes*; takifugu 2), medaka (*Oryzias latipes*; medaka 2-A (P452)), cavefish (*Astyanax fasciatus*; cavefish 2), coelacanth (*Latimeria chalumnae*; coelacanth 2 (P478)), chameleon (*Anolis carolinensis*; chameleon 2 (P496)), gecko (*Gekko gekko*; gecko 2 (P467)), pigeon (*Columba livia*; pigeon 2 (P503)), zebra finch (*Taeniopygia guttata*; zebra finch 2 (P505)), and chicken (*Gallus gallus*; chicken 2 (P508)), where the numbers after P denote $\lambda_{\max}$'s. We then considered the phylogenetic relationship [((((goldfish 2-1 (P511), goldfish 2-2 (P506)), (zebrafish 2-3 (P488), zebrafish 2-4 (P505)), (zebrafish 2-1 (P467), zebrafish 2-2 (P476))), ((bfin killifish 2, takifugu 2), medaka 2-A (P452)), cavefish 2), (coelacanth 2 (P478), ((chameleon 2 (P496), gecko 2 (P467)), (pigeon 2 (P503), zebra finch 2 (P505), chicken 2 (P508)))))]. This tree topology was constructed by applying the neighbor-joining (NJ) method (Saitou and Nei, 1987) to the first 996 nucleotide sites. With the exception of the ancestral node between the goldfish and zebrafish RH2 pigments, all bifurcation events that led to the contemporary fish pigments have the bootstrap values of > 90%. When more sequences are added, the ancestral node of the goldfish and zebrafish pigments has a bootstrap value of 95% (Spady et al., 2006). The phylogenetic positions of the coelacanth and vertebrate pigments were also supported by other molecular and morphological data (e.g., Kumar and Hedges, 1998).

On the basis of this tree topology, we inferred the amino acid sequences of all ancestral RH2 pigments by using a computer program, PAML, based on a likelihood-based Bayesian method (Yang, 1997). In this inference, paralogous RH1 pigments in goldfish (L11863), frog (*Xenopus laevis*) (L07770), chicken (D00702), and bovine (*Bos Taurus*) (U49742) were used as the outgroup.

3. Results

3.1. Absorption spectra of the chameleon and gecko RH2 pigments

When the chameleon and gecko RH2 opsins were reconstituted with 11-*cis*-retinal, the corresponding visual pigments had $\lambda_{\max}$'s of 496 ± 0 nm (referred to as chameleon 2 (P496)) and 467 ± 0.5 nm (gecko 2 (P467)), respectively (Fig. 2). The $\lambda_{\max}$ of chameleon 2 (P496) was virtually identical to the previous estimate of 495 nm (Kawamura and Yokoyama, 1998). The absorption spectra can also be evaluated indirectly by subtracting a spectrum measured after photobleaching from a spectrum measured before light exposure. These dark-light difference spectra for chameleon 2 (P496) and gecko 2 (P467) had $\lambda_{\max}$'s of 499 and 472 nm, respectively (Fig. 2, insets), which differ slightly from the corresponding dark spectra. Because of their direct estimation procedures, dark spectra give more accurate $\lambda_{\max}$ estimates of visual pigments than dark-light difference spectra. In the following, therefore, we shall use the $\lambda_{\max}$'s of 496 and 467 nm for chameleon 2 (P496) and gecko 2 (P467), respectively.

The $\lambda_{\max}$ of gecko 2 (P467) is more specific than the MSP estimates of 445~470 nm (Liebman, 1972; Crescitelli et al., 1977; Govardovskii et al., 1984). Using the empirical formulae developed by Whitmore and Bowmaker (1989) and Harosi (1994), we can convert the $\lambda_{\max}$ of chameleon 2 (P496) into the actual $\lambda_{\max}$ of chameleon RH2 pigment with 11-*cis*-3, 4-dehydroretinal. The converted values were ~520 nm and were roughly 15~20 nm higher than the MSP estimate of 503 nm (see also Kawamura and Yokoyama, 1998). It should be noted that the conversion formulae had been derived by considering a small number of species and their applicability may be limited. Furthermore, the MSP estimate for gecko 2 (P467) had a large standard deviation (Provencio et al., 1992). Thus, the actual $\lambda_{\max}$ predicted from the *in vitro* assay and MSP for the chameleon RH2 pigment are roughly consistent with each other.

3.2. Ancestral RH2 pigments

To identify the amino acid replacements that caused the absorption difference between gecko 2 (P467) and chameleon 2 (P496), we need to know the amino acid sequence of their ancestor. For that purpose, we aligned a total of 16 representative RH2 pigments (see Materials and Methods), where the first 322 amino acids were comparable (Fig. 1). Using the tree topology in Fig. 3, therefore, we inferred the amino acid sequences of all ancestral pigments by using the JTT model of amino acid replacements (Yang, 1997). Most amino acids inferred for the ancestral reptilian pigment had posterior probabilities of >0.95 and were highly reliable. In Fig. 1, for example, we can see that amino acids at only 17 out of 322 residues of the reptilian ancestor had posterior probabilities of <0.95. When the Dayhoff model of amino acid replacements was used, similar results were obtained (see also Yokoyama and Blow, 2001). By comparing the amino acid sequences of the gecko, chameleon, and their ancestral RH2 pigments, we can see that the number of amino acid replacements (47) in gecko 2 (P467) is about four times larger than that (12) in chameleon 2 (P496) (Fig. 1). The total number of amino acid replacements and the number of amino acid replacements that actually cause $\lambda_{\max}$ -shifts are highly correlated (see below), suggesting that the accelerated evolution of gecko 2 (P467) helped the Tokay gecko to switch from diurnal life-style to nocturnal life-style.

3.3. Chimeras between the gecko and chameleon RH2 pigments

The $\lambda_{\max}$'s of gecko 2 (P467) and chameleon 2 (P496) differ by 29 nm. To evaluate the effects of amino acid replacements on the $\lambda_{\max}$ -shift, we constructed a total of eight chimeric pigments by recombining the two pigments at five restriction enzyme sites (Fig. 1): 1) TMs I-, II-, III~IV-, V-, VI-, and VII-containing segments (or simply TMs I, II, III~IV, V, VI, and VII) of chameleon 2 (P496) were replaced by those of gecko 2 (P467) individually (pigments 1~6) and 2) the TMs I~IV and V~VII segments of chameleon 2 (P496) were replaced by those of gecko 2 (P467) (pigments 7 and 8) (Fig. 4).

If we assume that pigment 7, pigment 8, and gecko 2 (P467) were derived from chameleon 2 (P496), then the $\lambda_{\max}$ -shifts caused by replacing the TMs I~IV, V~VII, and I~VII of chameleon 2 (P496) by those of gecko 2 (P467) are -25, -7, and -29 nm, respectively (Fig. 4). Thus, these effects, denoted as $\theta_{\text{I-IV}}$, $\theta_{\text{V-VII}}$, and $\theta_{\text{I-VII}}$, respectively, are given by -25, -7, and -29 nm. Since $\theta_{\text{I-VII}}$ consists of $\theta_{\text{I-IV}}$, $\theta_{\text{V-VII}}$, and $\theta_{\text{I-IV} \times \text{V-VII}}$, which is the $\lambda_{\max}$ -shift caused by interactions between TMs I~IV and V~VII, $\theta_{\text{I-IV} \times \text{V-VII}}$ is 3 nm. On the other hand, if we assume that pigment 7, pigment 8, and chameleon 2 (P496) were derived from gecko 2 (P467), then the corresponding $\theta_{\text{I-IV}}$, $\theta_{\text{V-VII}}$, and $\theta_{\text{I-IV} \times \text{V-VII}}$ are given by 22, 4, and 3 nm, respectively. Hence, different amino acids in TMs I~IV and V~VII between gecko 2 (P467) and chameleon 2 (P496) cause 76~86% and 14~24% of spectral difference, respectively, and their interactions between the two regions modify the $\lambda_{\max}$ by ~10%. This result is consistent with the observation of Kojima et al. (1996), where they detected a 22 nm blue-shift in the $\lambda_{\max}$ by replacing the TMs I~III of bovine RH1 pigment by those of gecko 2 (P467).

It should also be noted that the sum of the $\lambda_{\max}$ -shifts caused by replacing the TMs I, II, and III~IV of chameleon 2 (P496) by those of gecko 2 (P467) individually (pigments 1, 2, and 3) was about the same to the $\lambda_{\max}$ -shift caused by replacing the TMs I~IV of chameleon 2 (P496) by those of gecko 2 (P467) together (pigment 7); similarly, the sum of the $\lambda_{\max}$ -shifts caused by replacing TMs V, VI, and VII of chameleon 2 (P496) by those of gecko 2 (P467) (pigment 4, 5, and 6) was about the same to the $\lambda_{\max}$ -shift caused by replacing the TMs V~VII of chameleon 2 (P496) by those of gecko 2 (P467) (pigment 8) (Fig. 4). Within TMs I~IV and V~VII, therefore, the effects of TM replacements on the $\lambda_{\max}$ -shift are roughly additive.

3.4. Spectral tunings in the gecko and chameleon RH2 pigments

During vertebrate evolution, certain amino acid changes at a total of 25 residues (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) have caused significant $\lambda_{\max}$ -shifts in various visual pigments (Yokoyama et al., 2007). Among these residues, amino acids differ at positions 49 and 52 in TM I, 83, 86, and 97 in TM II, and 164 in TM IV of gecko 2 (P467) and chameleon 2 (P496) (Fig. 1). Amino acid comparison of gecko and chameleon RH2 pigments with that of the ancestral RH2 pigment indicated that S49A, L52M, D83N, M86T, and T97A occurred in gecko 2 (P467) and S49F and A164S occurred in chameleon 2 (P496) (Fig. 1). Based on these evolutionary considerations, we introduced F49A, L52M, D83N, M86T, and T97A into chameleon 2 (P496) and A164S into gecko 2 (P467). Note that L52M, D83N, M86T, T97A, and A164S are forward mutations, which actually took place during the evolution of two pigments, and that F49A did not actually occur, but it combines two separate processes of S49A and S49F into one step.

The mutagenesis results indicated that neither F49A nor L52M caused any $\lambda_{\max}$ -shift individually, but they together reduced the $\lambda_{\max}$ of chameleon 2 (P496) by 4 nm (Table 1), which explained the 5 nm difference between the $\lambda_{\max}$'s of chameleon 2 (P496) and pigment 1 (Fig. 4). Although the effect of M86T on the $\lambda_{\max}$ -shift could not be evaluated, D83N caused no $\lambda_{\max}$ -shift, and T97A decreased the $\lambda_{\max}$ by 8 nm, D83N/M86T/T97A together decreased the $\lambda_{\max}$ of chameleon 2 (P496) by 15 nm (Table 1), which fully explained the $\lambda_{\max}$ difference between chameleon 2 (P496) and pigment 2 (Fig. 4). When the TMs III~IV of chameleon 2 (P496) were replaced by those of gecko 2 (P467), the change decreased the $\lambda_{\max}$ by 4 nm (Fig. 3). In this region, gecko 2 (P467) and chameleon 2 (P496) have 10 different amino acids, one of which consists of A164 in gecko 2 (P467) and S164 in chameleon 2 (P496) (Fig. 1). It is known that A164S in bovine rhodopsin increases the $\lambda_{\max}$ by 2 nm (Chan et al., 1992). For M/LWS pigments, A164S increases the $\lambda_{\max}$ by 2~6 nm and S164A decreases it by 7 nm (Asenjo et al., 1994; Yokoyama et al., 2005). However, our mutagenesis analysis showed that A164S in gecko 2 (P467) did not cause any $\lambda_{\max}$ -shift (Table 1). In addition, when we introduced S164A into chameleon 2 (P496), the mutation decreased the $\lambda_{\max}$ only by 1 nm (Table 1). Thus, the $\lambda_{\max}$ difference between the chameleon 2 (P496) and pigment 3 remains to be explained.

In summary, the F49A/L52M/D83N/M86T/T97A/S164A replacements in chameleon 2 (P496) explained about 75% of the $\lambda_{\max}$ -shift caused by replacing the TMs I~IV of chameleon 2 (P496) by those of gecko 2 (P467), and 65% of the entire $\lambda_{\max}$ difference between gecko 2 (P467) and chameleon 2 (P496). When the TMs V, VI, and VII of chameleon 2 (P496) were replaced by those of gecko 2 (P467), the $\lambda_{\max}$ was decreased by 2~4 nm (Fig. 4). These results indicate that there exist additional amino acids that are involved in the spectral tuning of the two RH2 pigments, and they contribute very small $\lambda_{\max}$ -shifts individually.

4. Discussion

4.1. Spectral tuning of RH2 pigments

To date, only E122Q and M207L are known to cause significant $\lambda_{\max}$ -shifts in RH2 pigments; that is, Q122E in the RH2 pigments of chicken (Imai et al., 1997), coelacanth (Yokoyama et al., 1999), and zebrafish (Chinen et al., 2005) increase the $\lambda_{\max}$ by 13~16 nm, while L207M in coelacanth 2 (P478) increases the $\lambda_{\max}$ by 6 nm (Yokoyama et al., 1999). Our new mutagenesis analyses also showed that Q122E in gecko 2 (P467) and chameleon 2 (P496) increased the $\lambda_{\max}$ by ~15 nm (Table 1). Our analyses also show that T97A can decrease the $\lambda_{\max}$ of RH2 pigments significantly.

Among currently known RH2 pigments, medaka 2-A (P452) has the lowest $\lambda_{\max}$. Our evolutionary analyses based on the amino acid sequences of ancestral pigments indicated that E122Q and A292S occurred in this pigment (Fig. 3). Interestingly, the same amino acid replacements also occurred independently in the paralogous RH1 pigment of coelacanth and decreased the $\lambda_{\max}$ by ~30 nm (Yokoyama et al., 1999). When we introduced the reverse changes S292A and Q122E/S292A into medaka 2-A (P452), these mutations increased the $\lambda_{\max}$ by 7 and 17 nm, respectively (Table 1). Thus, we have identified the fourth amino acid replacement, A292S, that shifts the $\lambda_{\max}$ of a RH2 pigment. However, since the ancestral vertebrate RH2 pigment seems to have had a $\lambda_{\max}$ of 500 nm or higher (see the next section), additional currently unknown amino acid replacements must be involved in the spectral tuning of medaka 2-A (P452), which remain to be identified.

These results suggest that the variable $\lambda_{\max}$'s of currently known RH2 pigments were generated by two groups of amino acid replacements: 1) T97A, E122Q, M207L, and A292S, which cause significant $\lambda_{\max}$ -shifts and 2) amino acid replacements such as S49A, S49F, L52M, D83N, A164S, and others, each causing a rather trivial $\lambda_{\max}$ -shift individually (e.g., Chinen et al., 2005). It should be cautioned, however, that depending on their amino acid backgrounds of RH2 pigments, the effects of D83N and A164S on the $\lambda_{\max}$ -shift can be much larger (e.g., Yokoyama 2000a; Yokoyama et al., 2005).

4.2. Evolution of RH2 pigments in vertebrates

To evaluate the $\lambda_{\max}$'s of RH2 pigment in the vertebrate ancestor, we need to reconstruct them by introducing a large number of amino acid changes into extant RH2 pigments and measure their absorption spectra (e.g., Yokoyama and Radlwimmer, 2001; Shi and Yokoyama, 2003). However, we can speculate an approximate $\lambda_{\max}$ of the ancestral pigment using four sets of mutagenesis results. First, Chinen et al. (2005) engineered the RH2 pigment of the goldfish and zebrafish ancestor. Their results show that this ancestral pigment had a $\lambda_{\max}$ of 506 nm. If we assume that no critical amino acid replacement occurred before that (Fig. 3), then the RH2 pigment in the vertebrate ancestor had a $\lambda_{\max}$ of 506 nm. Second, if we assume that only E122Q and M207L generated the $\lambda_{\max}$ of coelacanth 2 (P478) (Yokoyama et al. 1999), then the ancestral pigment had a $\lambda_{\max}$ of 499 nm. Third, we may assume that chameleon 2 (P496) evolved from the ancestral pigment only by S49F/E122Q/A164S (Fig. 3). When we introduced F49S/Q122E/S164A into chameleon 2 (P496), the mutant pigment had a $\lambda_{\max}$ of 511 nm (Table 1), suggesting that the ancestral vertebrate RH2 pigment had a $\lambda_{\max}$ of 511 nm. Fourth, if we assume that the $\lambda_{\max}$'s of the extant avian RH2 pigments were generated only by E122Q, decreasing the $\lambda_{\max}$ by 15 nm (Table 1), then the $\lambda_{\max}$ of the ancestral pigment is ~520 nm.

These results suggest that the RH2 pigment in the vertebrate ancestor had a $\lambda_{\max}$ of 500~520 nm and, therefore, the $\lambda_{\max}$'s of many extant RH2 pigments have been blue-shifted. In goldfish and zebrafish, goldfish 2-2 (P506), goldfish 2-1 (P511), and zebrafish 2-4 (P505) seem to have

inherited their $\lambda_{\max}$'s from the common ancestral pigment, but zebrafish 2-1 (P467), zebrafish 2-2 (P476), and zebrafish 2-3 (P488) decreased their $\lambda_{\max}$'s by 20~40 nm.

One evolutionarily critical question then is why these variable $\lambda_{\max}$'s have been generated. As we can see in Fig. 3, E122Q decreased the $\lambda_{\max}$'s of zebrafish 2-3 (P488), the ancestral pigment of zebrafish 2-1 (P467) and zebrafish 2-2 (P476), medaka 2-A (P452), and the ancestral pigment of the coelacanth and tetrapod pigments independently (Fig. 3). Comparison of amino acids at all residues of the ancestral and contemporary RH2 pigments show that glutamic acid (E) has changed to alanine (A), aspartic acid (D), glutamine (Q), glycine (G), valine (V), and lysine (K) with probabilities 0.1, 0.5, 0.25, 0.05, 0.05, and 0.05, respectively. Therefore, the probability that E122Q occur in four separate occasions is 0.004. These parallel amino acid replacements may be taken as a supportive evidence for the adaptive evolution of RH2 pigments. Certainly, the $\lambda_{\max}$'s of gecko 2 (P467) and coelacanth 2 (P478) must be blue-shifted because the animals are active in the blue-shifted light environments. However, the adaptive significance of gene duplication events and the blue-shifts in the $\lambda_{\max}$'s of zebrafish and medaka RH2 pigments are not apparent. To fully appreciate any adaptive changes of RH2 pigments, we must not only elucidate the molecular bases of spectral tuning in RH2 pigments but also relate the $\lambda_{\max}$ -shifts to the changes in their ecological and physiological environments as well as behavioural characteristics.

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Abbreviations

TM	transmembrane
$\lambda_{\max}$	wavelength of maximal absorption
RH2	rhodopsin-like
SWS1	short wavelength-sensitive type 1
MWS	middle wavelength-sensitive
and LWS	long wavelength-sensitive

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Fig. 1.

Aligned amino acid sequences of the gecko and chameleon RH2 pigments and their ancestral pigment. Dots indicate the identity of the amino acids with those of the ancestral pigment. The ancestral amino acids that have a probability of 95% or less are underlined. *HindIII*, *RsaI*, *Bsu36I*, *PvuII*, and *BstYI* denote the positions of five restriction enzyme recognition sites. Stars (*) indicate the critical amino acid residues, where certain changes are known to cause significant $\lambda_{\max}$ -shifts. Seven putative TM regions (Palczewski et al., 2000) are boxed.

Fig. 2.

Absorption spectra of the chameleon and gecko RH2 pigments measured in the dark. The dark-light difference spectra are shown in the insets. The units for the absorbance spectra are same for the two pigments.

Fig. 3.

A composite phylogenetic tree of representative RH2 pigments. Goldfish 2-1 (P511), GenBank accession no. L11865), goldfish 2-2 (P506) (L11866), zebrafish 2-1 (P467) (AB087805), zebrafish 2-2 (P476) (AB087806), zebrafish 2-3 (P488) (AB087807), zebrafish 2-4 (P505) (AB087808), bfin killifish 2 (AY296739), takifugu 2 (AF226989), medaka 2-A (P452) (AB223053), cavefish 2 (S75255), coelacanth 2 (P478) (AF131258), chameleon 2 (P496) (AF134189), gecko 2 (P467) (M92035), pigeon 2 (P503) (AF149232), zebra finch 2 (P505) (AF222330), and chicken 2 (P508) (M92038). Amino acid changes that cause significant $\lambda_{\max}$ -shifts are shown at appropriate branches. The $\lambda_{\max}$'s of zebrafish and medaka RH2 pigments are taken from Chinen et al. (2003) and Matsumoto et al. (2005), respectively, and those of others from Yokoyama (2000a).

	I	II	III-IV	V	VI	VII	$\lambda_{\max}$ (nm)	$\Delta\lambda$ (nm)
chameleon 2 (P496)	○	○	○	○	○	○	496 ± 0	0
pigment 1	●	○	○	○	○	○	491 ± 0.7	-5
pigment 2	○	●	○	○	○	○	482 ± 0.4	-14
pigment 3	○	○	●	○	○	○	492 ± 1.5	-4
pigment 4	○	○	○	●	○	○	494 ± 1.2	-2
pigment 5	○	○	○	○	●	○	493 ± 0.8	-3
pigment 6	○	○	○	○	○	●	492 ± 0.4	-4
pigment 7	●	●	●	○	○	○	471 ± 0.5	-25
pigment 8	○	○	○	●	●	●	489 ± 0.5	-7
gecko 2 (P467)	●	●	●	●	●	●	467 ± 0.5	-29

Fig. 4.

Schematic representation of the chimeric pigments between the gecko and chameleon RH2 pigments. Open circles indicate TM helix-containing segments of the chameleon pigment, whereas solid circles indicate those of the gecko pigment. The $\Delta\lambda$ indicates the effect of TM-containing segment of the gecko pigment on the $\lambda_{\max}$ -shift.

Table 1The effects of amino acid changes on the $\lambda_{\max}$ -shift ($\Delta\lambda$)

Visual pigment	Amino acid change	$\lambda_{\max}$ (nm)	$\Delta\lambda$ (nm)
chameleon 2 (P496)	WT	496 $\pm$ 0	
	F49A	496 $\pm$ 0.5	0
	L52M	496 $\pm$ 1.8	0
	F49A/L52M	492 $\pm$ 0.8	-4
	D83N	496 $\pm$ 0.7	0
	M86T	ND	
	T97A	488 $\pm$ 0.4	-8
	D83N/M86T/T97A	481 $\pm$ 1.1	-15
	S164A	495 $\pm$ 0.5	-1
	Q122E	512 $\pm$ 0.7	+16
	F49S/Q122E/S164A	511 $\pm$ 0.8	+15
gecko 2 (P467)	WT	467 $\pm$ 0.5	
	A164S	467 $\pm$ 0.4	0
	Q122E	480 $\pm$ 0.8	+13
medaka 2-A (P452)	WT	452 $\pm$ 1.0 ^a	
	S292A	459 $\pm$ 0.5	+7
	Q122E/S292A	469 $\pm$ 0.8	+17

ND – the mutant pigment was unstable and its $\lambda_{\max}$ could not be evaluated.^aMatsumoto et al., 2006.