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The spectral tuning in the short wavelength-sensitive type 2 pigments

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Abstract

Using site-directed mutagenesis and multiple regression analysis, we have studied the molecular genetics and evolution of short wavelength-sensitive (SWS2) pigments in vertebrates. These analyses suggest that the SWS2 pigment in the vertebrate ancestor had the wavelength of maximum absorption (λ_{max}) of ~440 nm and that various λ_{max}'s of the contemporary SWS2 pigments in vertebrates are caused mainly by additive effects of amino acid replacements at ten sites.
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1. Introduction

Vision begins when photons are absorbed by visual pigments, which consist of a transmembrane protein, opsin, and an 11-*cis*-retinal chromophore. The Schiff base of 11-*cis*-retinal is usually protonated by the glutamate counterion at site 113 (Nathans, 1990a,b; Sakmar et al., 1989; Zhukovsky and Oprian, 1989). The protonated Schiff base-linked chromophore in solution absorbs light at 440 nm (Kito et al., 1968). Interacting with various opsins, however, the 11-*cis*-retinal chromophore may achieve the wavelength of maximal absorption (λ_{max}) from 360 to 635 nm (Kleinschmidt and Harosi, 1992). How these visual pigments detect such a wide range of color using the same 11-*cis*-retinal chromophore is a long-standing question in vision research (Nathans, 1990a,b).

Currently known visual pigments in vertebrates are classified into five groups: (1) rhodopsin (RH1); (2) RH1-like (RH2); (3) short wavelength-sensitive type 1 (SWS1); (4) SWS type 2 (SWS2); and (5) long wavelength- and middle wavelength-sensitive (LWS/MWS) pigments (e.g. see Yokoyama, 2000a; Ebrey and Koutalos, 2001). The RH1, RH2, SWS1, SWS2, MWS, and LWS pigments have λ_{max}'s of 480–510, 470–510, 360–430, 440–460, 510–530, and ~560 nm, respectively (e.g. see Yokoyama, 2000a). The spectral tuning of LWS/MWS and SWS1 pigments are controlled mainly by five (Yokoyama and Yokoyama, 1990; Neitz et al., 1991; Sun et al., 1997; Yokoyama and Radlwimmer, 1999, 2001) and ten (Yokoyama et al., 2000a; Wilkie et al., 2000; Yokoyama and Shi, 2000; Shi et al., 2001) amino acid sites, respectively. The molecular co-adaptation of the RH1 and RH2 pigments in the coelacanth (*Latimeria chalumnae*) to the depth of ~200 m has also been explained by a total of three amino acid replacements (Yokoyama et al., 1999; Yokoyama and Tada, 2000).

At present, the molecular basis of the spectral tuning in the SWS2 pigments is not known. So far, the SWS2 pigments of goldfish (*Carassius auratus*), salamander (*Ambystoma tigrinum*), bull frog (*Rana catesbeiana*), American chameleon (*Anolis carolinensis*), zebra finch (*Taeniopygia guttate*), chicken (*Gallus gallus*), and pigeon (*Columba livia*) are shown to have λ_{max}'s of 431–455 nm. Interestingly, these values are very close to the wavelength of absorption of the protonated Schiff base-linked chromophore, 440 nm. Thus, the other four paralogous groups of visual pigments may have shifted their λ_{max}'s from that of SWS2 pigment during vertebrate evolution (Ebrey and Takahashi, 2002).

Here, using site-directed mutagenesis and multiple regression analyses, we shall explore the molecular genetics and evolution of the seven SWS2 pigments. Our results suggest that the ancestral SWS2 pigment had a λ_{max} of 439 nm and goldfish, chameleon, and zebra finch pigments have maintained this λ_{max} (~440 nm) during the last ~450

Abbreviations: λ_{max}, wavelength of maximal absorption; SWS2, short wavelength-sensitive type 2.

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million years of vertebrate evolution. The major red-shift in the λ_{max} of the chicken and pigeon pigments occurred first in their common ancestral pigment by I49A, whereas the blue-shifts in the λ_{max} of the salamander and bull frog are caused by T93V and L207I, respectively.

2. Materials and methods

2.1. Background on site-directed mutagenesis of visual pigments

Mutagenesis studies on 'Structure and Function of Rhodopsin' by Doi et al. (1990) and subsequent papers by H.G. Khorana and his colleagues dramatically improved our understanding of the role of key amino acids in visual pigments (see also Nathans 1990a,b; Sakmar et al., 1989; Zhukovsky and Oprian, 1989). Virtually none of these mutations, however, has been found in nature and their significance in the spectral tuning in various visual pigments is not immediately clear (Yokoyama, 1995, 2000a). During the last decade, mutagenesis experiments based on actual amino acid polymorphism have also been conducted. These analyses show that amino acid changes at a total of 19 sites can shift the λ_{max}'s of visual pigments by more than 5 nm (Fig. 1; see Yokoyama, 2002).

2.2. Site-directed mutagenesis, regeneration of visual pigments, and spectral analyses

The SWS2 cDNA of American chameleon (*A. carolinensis*; Kawamura and Yokoyama, 1998), pigeon (*C. livia*, Kawamura et al., 1999), and goldfish (*C. auratus*) have been cloned. These cDNA clones were subcloned into the *Eco* RI and *Sal* I restriction sites of the expression vector pMT5 (Khorana et al., 1988). The mutants of the chameleon SWS2 opsin 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. The wild type and mutant 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) piperrazine-*N*′-2-ethane-sulfonic acid (HEPES; pH6.6), 140 mM NaCl, 3 mM $MgCl_2$, 20% (w/v) glycerol, and 0.1% dodecyl maltoside] (for more details, see Yokoyama, 2000b).

The absorption spectra of the resulting visual pigments 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).

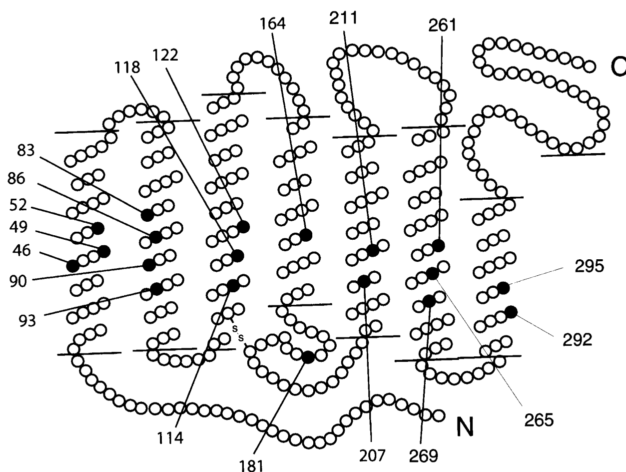

Fig. 1. Naturally occurring amino acid changes that shift the λ_{max} more than 5 nm (black circles) (see Yokoyama, 2000a; Shi et al., 2001). The topology of the bovine RH1 pigment is based on Palczewski et al. (2000).

2.3. Sequence data analyses

In Table 1, we can see the sources of the amino acid sequences and λ_{max}'s of the SWS2 pigments from seven vertebrate species, where both the amino acid sequences and λ_{max}'s of the pigments have been characterized. It is most likely that the five species have a phylogenetic relationship of (((((pigeon, chicken), zebra finch), chameleon), (salamander, bull frog)), goldfish) (see Yokoyama, 2000a; Yokoyama et al., 2000b). Given this tree topology, we inferred the ancestral sequences of the visual pigments by using a computer program, PAML, based on a likelihood-based Bayesian method (Yang et al., 1995; Yang, 1997). In the inference, RH1 (GenBank accession no. L11863), RH2 (L11865), SWS1 (D85863), and LWS (L11867) pigments of the goldfish were used as the outgroup.

Table 1
SWS2 pigments of vertebrates[a]

Visual pigment	Reference for the λ_{max} value	GenBank
Goldfish (P443)	This study	L11864
Salamander (P431)	Govardovskii et al. (2000)[b]	AF038946
Bull frog (P432)	Govardovskii et al. (2000)[b]	AB010085
Chameleon (P437)	Kawamura and Yokoyama (1998)	AF133907
Pigeon (P448)	Kawamura et al. (1999)	AF149238
Zebra finch (P440)	Yokoyama et al. (2000a)	AF222332
Chicken (P455)	Okano et al. (1989)[c]	M92037

[a] Bull frog, *Rana catesbeiana*; Chameleon, *Anolis carolinensis*; Chicken, *Gallus gallus*; Goldfish, *Carassius auratus*; Pigeon, *Columba livia*; Salamander, *Ambystoma tigrinum*; Zebra finch, *Taeniopygia guttata*.

[b] Estimated by microspectrophotometry.

[c] Estimated by a biochemical method.

3. Results

3.1. Amino acid replacements

Among the 19 currently known functionally important amino acid sites (Fig. 1), only sites 46, 49, 52, 93, 164, 207, and 269 are polymorphic among the seven contemporary SWS2 pigments, where amino acid site numbers are those of bovine RH1 pigment (GenBank accession no. U49742). The amino acid sequences of the ancestral pigments at nodes a–f (pigments *a–f*) in Figs. 2A,B were inferred by using JTT (Jones et al., 1992) and Dayhoff (Dayhoff et al., 1978) models of amino acid replacements. Amino acids with the two highest posterior probabilities at the seven sites are given in Table 2, where we can see that amino acids inferred by the two models agree most of the time. As may be expected, amino acid inferred at deeper nodes tend to have a probability of < 0.9. At node a, for example, I49, V52, T93, and A164 may have to be replaced by V49, A52, Vg3, and G164, respectively (Table 2). As we will see in Section 3.3, however, these ambiguous amino acids have little consequence in the estimation of the λ_{max} of pigment *a*.

Both the Dayhoff and JTT models predict that goldfish (P443) pigment has the smallest number of amino acid replacements (Figs. 2A,B), suggesting that the ancestral pigment *a* might have had a λ_{max} of ~440 nm. When the amino acids with the highest probabilities in the ancestral pigments and those of the contemporary pigments are compared, the Dayhoff and JTT models result in a total of 18 and 21 amino acid replacements, respectively (Figs. 2A, B). Thus, the amino acids inferred by the Dayhoff model is more parsimonious than those inferred by the JTT model. Both models predict that pigment *a* has amino acids F46/I49/V52/T93/A164/L207/A269 (denoted as FIVTALA).

3.2. Mutagenesis experiments

It is suspected that chameleon (P437) pigment has accumulated F46I, I49V, and A164G (Figs. 2A,B). When the reverse mutations I46F, V49I, G164A, and I46F/V49I/G164A are introduced into the chameleon pigment, the three mutant pigments have λ_{max} values of 438–439 nm (Fig. 3A, Table 3). Standard errors associated with these estimates are all within 1 nm. These mutagenesis results strongly suggest that the ancestral pigment *a* had a λ_{max} value of ~440 nm.

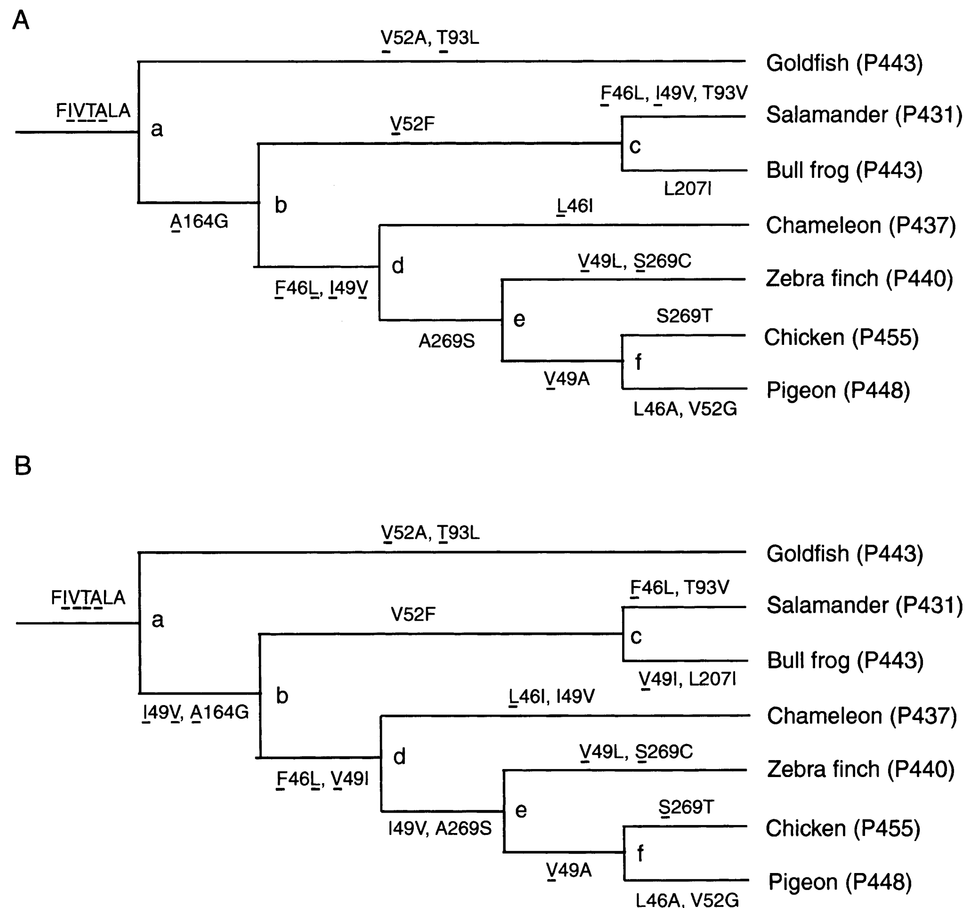

Fig. 2. Amino acid replacements in the SWS2 pigments inferred by the Dayhoff (A); and JTT (B) models. FIVTALA refers to the amino acids at sites 46, 49, 52, 93, 164, 207, and 269, respectively, for the ancestral SWS2 pigment. The numbers after P refer to λ_{max}'s. The ancestral amino acids that have a probability of < 0.9 are underlined.

Table 2
Amino acids with two highest posterior probabilities

Node	Amino acid site	Model			
		JTT		Dayhoff	
a	46	$F_{0.98}$	$L_{0.02}$	$F_{0.99}$	$L_{0.01}$
	49	$I_{0.61}$	$V_{0.39}$	$I_{0.86}$	$V_{0.14}$
	52	$V_{0.58}$	$A_{0.29}$	$V_{0.49}$	$A_{0.27}$
	93	$T_{0.68}$	$V_{0.09}$	$T_{0.63}$	$V_{0.18}$
	164	$A_{0.59}$	$G_{0.40}$	$A_{0.58}$	$G_{0.48}$
	207	$L_{1.0}$	–	$L_{1.0}$	–
	269	$A_{1.0}$	–	$A_{1.0}$	–
b	46	$F_{0.53}$	$L_{0.47}$	$F_{0.65}$	$L_{0.34}$
	49	$V_{0.75}$	$I_{0.25}$	$I_{0.52}$	$V_{0.48}$
	52	$V_{0.90}$	$I_{0.10}$	$V_{0.72}$	$I_{0.08}$
	93	$T_{0.97}$	$I_{0.01}$	$T_{0.95}$	$V_{0.05}$
	164	$G_{1.0}$	–	$G_{1.0}$	–
	207	$L_{1.0}$	–	$L_{1.0}$	–
	269	$A_{1.0}$	–	$A_{1.0}$	–
c	46	$F_{0.53}$	$L_{0.47}$	$F_{0.65}$	$L_{0.34}$
	49	$V_{0.73}$	$I_{0.27}$	$I_{0.53}$	$V_{0.47}$
	52	$F_{1.0}$	–	$F_{1.0}$	–
	93	$T_{0.97}$	$I_{0.03}$	$T_{0.94}$	$V_{0.06}$
	164	$G_{1.0}$	–	$G_{1.0}$	–
	207	$L_{0.98}$	$I_{0.02}$	$L_{0.98}$	$I_{0.02}$
	269	$A_{1.0}$	–	$A_{1.0}$	–
d	46	$L_{0.80}$	$F_{0.15}$	$L_{0.62}$	$F_{0.22}$
	49	$I_{0.99}$	$V_{0.01}$	$V_{0.69}$	$I_{0.29}$
	52	$V_{1.0}$	–	$V_{0.99}$	$I_{0.01}$
	93	$T_{1.0}$	–	$T_{1.0}$	–
	164	$G_{1.0}$	–	$G_{1.0}$	–
	207	$L_{1.0}$	–	$L_{1.0}$	–
	269	$A_{0.99}$	$S_{0.01}$	$A_{0.98}$	$S_{0.02}$
e	46	$L_{0.99}$	$F_{0.01}$	$L_{0.99}$	$I_{0.01}$
	49	$V_{0.75}$	$A_{0.09}$	$V_{0.52}$	$I_{0.17}$
	52	$V_{1.0}$	–	$V_{1.0}$	–
	93	$T_{1.0}$	–	$T_{1.0}$	–
	164	$G_{1.0}$	–	$G_{1.0}$	–
	207	$L_{1.0}$	–	$L_{1.0}$	–
	269	$S_{0.46}$	$A_{0.41}$	$S_{0.56}$	$A_{0.34}$
f	46	$L_{0.99}$	$A_{0.01}$	$L_{0.98}$	$A_{0.01}$
	49	$A_{0.98}$	$V_{0.01}$	$A_{0.99}$	$V_{0.01}$
	52	$V_{1.0}$	–	$V_{0.99}$	$G_{0.01}$
	93	$T_{1.0}$	–	$T_{1.0}$	–
	164	$G_{1.0}$	–	$G_{1.0}$	–
	207	$L_{1.0}$	–	$L_{0.99}$	$I_{0.01}$
	269	$S_{0.71}$	$T_{0.15}$	$S_{0.81}$	$T_{0.10}$

In order to explore the molecular basis of the spectral tuning in the seven SWS2 pigments, we need to consider F46L, F46I, F46A, I49V, I49L, I49A, V52A, V52G, V52F, T93L, T93V, A164G, L207I, A269C, A269T, and A269S (Figs. 2A,B). To evaluate the effects of these amino acid replacements on the λ_{max}-shift, we also introduced I46L/V49I/G164A, I46F/G164A, I46A/V49I/G164A, I46F/V49I/V52A/G164A, I46F/V49I/G164A/A269S, I46F/V49I/V52F/G164A, I46F/V49I/G164A/A269C, and I46F/V49I/G164A/A269T, into the chameleon (P437) pigment, A269S, A269C, A269T into goldfish (P448) pigment, and S269A, S269C, and S269T into pigeon (P448) pigment (Table 3).

Previously, the λ_{max} of the goldfish SWS2 pigment has been estimated to be 441 nm (Johnson et al., 1993). In the present analysis, the corresponding estimate is 443 nm (result not shown). To make the direct comparison to the mutagenesis results, we shall use the latter λ_{max} for the goldfish SWS2 pigment (Table 1). The λ_{max}'s of most of these mutant pigments are close to those of wild type pigments, but the mutant goldfish (P448), goldfish (P449), pigeon (P453), chameleon (P442), and chameleon (P444) pigments differ by ~5 nm (Table 3, Figs. 3A–C). The latter five pigments contain either A268S or A269T (Table 3). Thus, these results suggest that F46L, F46A, F46I, I49V, V52A, and A269C cause only small λ_{max}-shifts, if any, and that A269S and A269T increase the λ_{max} by ~5 nm.

3.3. Spectral tuning in the SWS2 pigments

Using the seven contemporary pigments and 18 mutant pigments, we can evaluate the effects of individual amino acid replacements on the λ_{max}-shift more rigorously. Following Yokoyama and Radlwimmer (1999, 2001), let θ_α and Z be the magnitude of the λ_{max}-shift caused by an amino acid replacement α and the λ_{max} of pigment a, respectively. Then, considering the amino acid compositions of a total of 25 SWS2 pigments in Table 3, the following relationships hold.

$$\theta_{V52A} + \theta_{T39L} + Z + \epsilon_1 = 443$$

$$\theta_{V52A} + \theta_{T93L} + \theta_{A269S} + Z + \epsilon_2 = 448$$

$$\theta_{V52A} + \theta_{T93L} + \theta_{A269C} + Z + \epsilon_3 = 444$$

$$\theta_{V52A} + \theta_{T93L} + \theta_{A269T} + Z + \epsilon_4 = 449$$

$$\theta_{F46L} + \theta_{I49V} + \theta_{V52F} + \theta_{T93V} + \theta_{A146G} + Z + \epsilon_5 = 431$$

$$\theta_{V52F} + \theta_{A164G} + \theta_{L207I} + Z + \epsilon_6 = 432$$

$$\theta_{F46A} + \theta_{I49A} + \theta_{V52G} + \theta_{A164G} + \theta_{A269S} + Z + \epsilon_7 = 448$$

$$\theta_{F46A} + \theta_{I49A} + \theta_{V52G} + \theta_{A146G} + Z + \epsilon_8 = 448$$

$$\theta_{F46A} + \theta_{I49A} + \theta_{V52G} + \theta_{A164G} + \theta_{A269C} + Z + \epsilon_9 = 450$$

$$\theta_{F46A} + \theta_{I49A} + \theta_{V52G} + \theta_{A164G} + \theta_{A269T} + Z + \epsilon_{10} = 453$$

$$\theta_{F46L} + \theta_{I49L} + \theta_{A164G} + \theta_{A269C} + Z + \epsilon_{11} = 440$$

$$\theta_{F46L} + \theta_{I49V} + \theta_{A164G} + \theta_{A269T} + Z + \epsilon_{12} = 455$$

$$\theta_{F46L} + \theta_{I49V} + \theta_{A164G} + Z + \epsilon_{13} = 437$$

$$\theta_{I49V} + \theta_{A164G} + Z + \epsilon_{14} = 439$$

$$\theta_{F46I} + \theta_{A164G} + Z + \epsilon_{15} = 439$$

$$\theta_{F46I} + \theta_{I49V} + Z + \epsilon_{16} = 438$$

$$Z + \epsilon_{17} = 438$$

$$\theta_{F46L} + Z + \epsilon_{18} = 438$$

$$\theta_{I49V} + Z + \epsilon_{19} = 439$$

$$\theta_{F46A} + Z + \epsilon_{20} = 438$$

$$\theta_{V52A} + Z + \epsilon_{21} = 439$$

$$\theta_{A269S} + Z + \epsilon_{22} = 442$$

$$\theta_{V52F} + Z + \epsilon_{23} = 438$$

$$\theta_{A269C} + Z + \epsilon_{24} = 439$$

$$\theta_{A269T} + Z + \epsilon_{25} = 444,$$

where ϵ_i's ($i = 1, 2, \ldots, 25$) denote random errors.

The multiple regression analysis (Searle, 1971) was then applied to these equations. The results show that the effects of most amino acid replacements, particularly those at sites 46 and 164, on the λ_{max}-shift are negligible (25 pigments, Table 4). The effect of I49A on the λ_{max}-shift is the largest (12 nm), followed by those of T93V, L207I, T93L, and A269T (Table 4). Note that the effect of A269T is 5 nm, which is much smaller than that in the bovine RH1 pigment, 14 nm (Chan et al., 1992), or that of the reverse mutation in the human LWS pigment, −16 nm (Asenjo et al., 1994). This regression analysis shows that the ancestral pigment *a* had a λ_{max} of 439 nm and that goldfish (P441), chameleon (P437), and zebra finch (P441) pigments have not changed their λ_{max}'s during vertebrate evolution. A major red-shift in the λ_{max}'s of chicken (P455) and pigeon (P448) pigments occurred first in their common ancestral pigment by I49A and the further red-shift occurred in the former pigment by A269T. On the other hand, the blue-shift in salamander (P431) and bull frog (P433) are caused mainly by T93V and L207I, respectively.

3.4. The expected λ_{max} of the contemporary pigments

Using the Z and θ values in Table 4, we can obtain the expected λ_{max}'s for all contemporary and mutant pigments. These expected and corresponding observed λ_{max}'s agree very well (Table 5). In fact, the largest difference (2 nm) can be found for pigeon (P448) pigment, where the expected value is 450 nm with a 95% confidence interval of 448–452 nm (Table 5). All of these results show that the spectral tuning in the currently known SWS2 pigments can be fully explained by the additive effects of amino acids at sites 49, 52, 93, 207, and 269.

4. Discussion

Considering the seven contemporary pigments and various mutant pigments, we have shown that the ancestral SWS2 pigment had a λ_{max} of ~440 nm. We have also seen that the spectral tuning in the currently known SWS2 pigments is determined by amino acids at sites 49, 52, 93, 207, and 269. Because of the small number of contemporary SWS2 pigments considered, however, these results should be interpreted with caution. Indeed, Takahashi and Ebrey (2003) have determined the λ_{max} of the SWS2 pigments of newt (*Cynops pyrrhogaster*) to be 474 nm, which is more than 20 nm higher than those of pigments considered here. Based on amino acid polymorphism, they also introduced P91S, S94A, I122M, C127S, S211C, Y261F, and A292S into the newt pigment and found that the λ_{max}'s of the respective pigments are 464, 460, 468, 472, 472, 469, and 466 nm. When these results are added to the multiple regression analysis, we obtain eight additional estimates: $\theta_{V52I} = -5$, $\theta_{S91P} = 10$, $\theta_{A94S} = 14$, $\theta_{M122I} = 6$, $\theta_{S127C} = 2$, $\theta_{C211S} = 2$, $\theta_{F261Y} = 5$, and $\theta_{S292A} = 8$ nm (33 pigments, Table 4). Otherwise, the estimates for Z and θ's are virtually identical to those obtained without the newt pigments (Table 4). θ_{T93L} is an exception. The change from 5 to −9 nm reflects the fact that goldfish (P443) pigment has also A94S in addition to V52A and T93L. Given θ_{A94S} of 14 nm, θ_{T93L} needs to be modified to explain the λ_{max}'s of the goldfish and newt pigments simultaneously. Thus, considering the two mutagenesis results of the SWS2 pigments, we can see that the λ_{max}'s of the eight contemporary SWS2 pigments can be shifted more than 5 nm by amino acids at sites 49, 52, 91, 93, 94, 122, 207, 261, 269, and 292.

So far, two types of the spectral tuning in visual pigments are known. One type has been found in RH1, RH2, and LWS/MWS pigments, where the effects of amino acid changes on the λ_{max}-shift can be detected separately and are mostly additive. For example, for the bovine RH1 pigment, the mutational effects of A164S (2 nm), F261S (10 nm), and A269T (14 nm) are additive (Chan et al., 1992). Similarly, during its adaptation to the depth of ~200 m, the coelacanth used mainly additive effects of A292S/E122Q and M207L/E122Q for the co-adaptation of its RH1 and RH2 pigments, respectively (Yokoyama et al., 1999; Yokoyama and Tada, 2000). Furthermore, the spectral tuning of 26 LWS/MWS pigments can be explained by amino acid differences at sites 164, 181, 261, 269, and 292, where mutations S164A, H181Y, Y261F, T269A, and A292S shift the λ_{max} of the pigments by −7, −28, −8, −15, and −27 nm, respectively, and S181Y and Y261F together increase the λ_{max} by ~10 nm (Yokoyama and Radlwimmer, 2001; see also Asenjo et al., 1994; Sun et al., 1997). Since S181Y and Y261F have been found only in four mammalian pigments, the λ_{max}'s of a majority of LWS/MWS pigments are also determined in an additive fashion.

For SWS1 pigments, an entirely different type of spectral tuning has been found. When single amino acid changes are introduced into the ultraviolet-sensitive pigments, virtually none of them causes any λ_{max}-shift (Yokoyama and Shi, 2000; Shi et al., 2001). For example, when the functionally

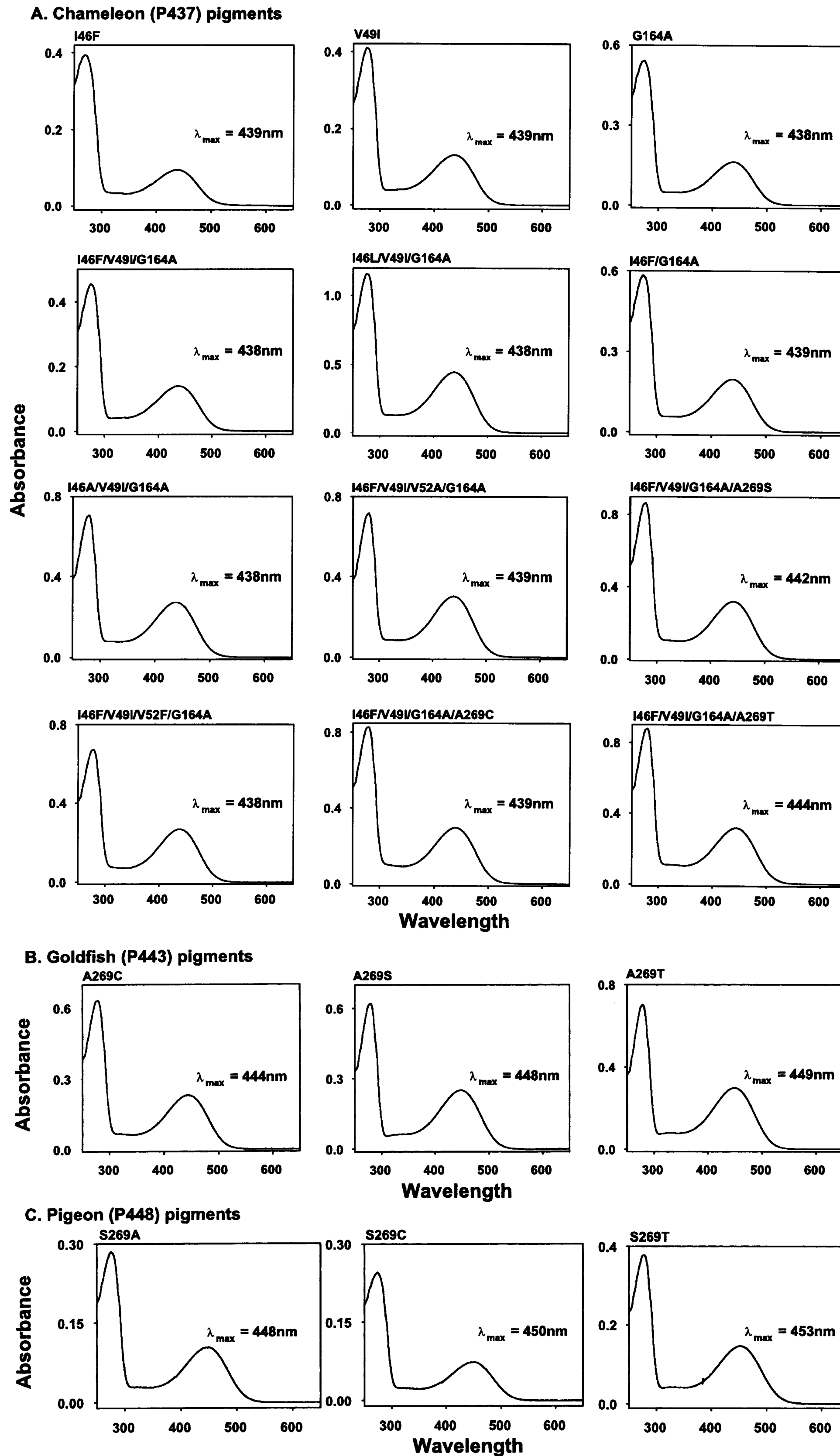

Fig. 3. Absorption spectra of the mutant forms of chameleon (P437) pigment (A); goldfish (P443) pigment (B); and pigeon (P448) pigment (C). Standard errors associated with these estimates are all within 1 nm.

Table 3
Amino acid compositions at the critical sites of the SWS2 pigments

Pigment	Site							Mutations in the chameleon pigment
	46	49	52	93	164	207	269	
Pigment *a*	F	I	V	T	A	L	A	–
Goldfish (P443)*	F	I	A	L	A	L	A	–
Goldfish (P448)	F	I	A	L	A	L	S	A269S
Goldfish (P444)	F	I	A	L	A	L	C	A269C
Goldfish (P449)	F	I	A	L	A	L	T	A269T
Salamander (P431)*	L	V	F	V	G	L	A	–
Bull frog (P432)*	F	I	F	T	G	I	A	–
Pigeon (P448)*	A	A	G	T	G	L	S	–
Pigeon (P448)	A	A	G	T	G	L	A	S269A
Pigeon (P450)	A	A	G	T	G	L	C	S269C
Pigeon (P453)	A	A	G	T	G	L	T	S269T
Zebra finch (P440)*	L	L	V	T	G	L	C	–
Chicken (P455)*	L	A	V	T	G	L	T	–
Chameleon (P437)*	I	V	V	T	G	L	A	–
Chameleon (P439)	F	V	V	T	G	L	A	I46F
Chameleon (P439)	I	I	V	T	G	L	A	V49I
Chameleon (P438)	I	V	V	T	A	L	A	G164A
Chameleon (P438)	F	I	V	T	A	L	A	I46F/V49I/G164A
Chameleon (P438)	L	I	V	T	A	L	A	I46L/V49I/G164A
Chameleon (P439)	F	V	V	T	A	L	A	I46F/G164A
Chameleon (P438)	A	I	V	T	A	L	A	I46A/V49I/G164A
Chameleon (P439)	F	I	A	T	A	L	A	I46F/V49I/V52A/G164A
Chameleon (P442)	F	I	V	T	A	L	S	I46F/V49I/G164A/A269S
Chameleon (P438)	F	I	F	T	A	L	A	I46F/V49I//V52F/G164A
Chameleon (P439)	F	I	V	T	A	L	C	I46F/V49I/G164A/A269C
Chameleon (P444)	F	I	V	T	A	L	T	I46F/V49I/G164A/A269T

*Contemporary pigments.

important seven amino acids of the mouse UV pigment are individually replaced by the corresponding amino acids of the orthologous human pigment (F46T, F49L, T52F, F86L, T93P, A114G, and S118T), they do not cause any λ_{max}-shift, but they jointly increase the λ_{max} by ~50 nm (Shi et al., 2001). One exception is F86Y, which increases the λ_{max} by 66 nm by itself (Fasick et al., 2002). It is most likely that the non-additive effects of amino acids in the spectral tuning of the UV pigment are due to unprotonated Schiff base-linked chromophore (e.g. Shi et al., 2001).

Here, we have seen that the spectral tuning in the SWS2 pigments can be explained by the additive effects of amino acid changes at a total of ten sites. The number of SWS2 pigment characterized is still rather small. As the number

Table 4
The λ_{max} values of the ancestral pigments *a* and the effects of amino acid changes on the λ_{max}-shift

Parameters		λ_{max} and $\Delta\lambda_{max}$ (nm)	
		25 pigments	33 pigments
Z		439 ± 1	439 ± 1
θ:	T93V, L207I	−6 ± 3	−6 ± 3
	V52I	–	−5 ± 7
	V52G	−3 ± 1	−3 ± 1
	F46L, F46A, F46I, V52F	−1 ± 1	−1 ± 1
	I49V, V52A, A164G, A269C	0 ± 1	0 ± 1
	S127C, C211S	–	2 ± 2
	A269S	3 ± 1	3 ± 1
	I49L	3 ± 2	3 ± 2
	T93L	5 ± 1	−9 ± 2
	A269T	5 ± 1	5 ± 1
	F261Y	–	5 ± 2
	122I	–	6 ± 2
	S292A	–	8 ± 2
	S91P	–	10 ± 2
	I49A	12 ± 2	12 ± 2
	A94S	–	14 ± 2

Table 5
The λ_{max}'s of visual pigments

Parameters	λ_{max} (nm)	
	Observed	Expected[a]
Goldfish (P443)	443 ± 1	444 (442–446)
Salamander (P431)	431[b]	431 (428–433)
Bull frog (P432)	432[b]	432 (429–434)
Chameleon (P437)	437 ± 2	438 (436–439)
Pigeon (P448)	448 ± 2	450 (448–452)
Zebra finch (P440)	440 ± 1	440 (437–443)
Chicken (P455)	455[b]	455 (453–457)

[a] Values in parentheses show 95% confidence intervals for both 25 and 33 pigment data sets.
[b] Standard errors are not available.

increases, we may find even more functionally important amino acid sites, which are involved in the spectral tuning of these pigments. Like RH1, RH2, and LWS/MWS pigments, however, it is most likely that the effects of amino acid changes on the λ_{max}-shift of the SWS2 pigments are additive.

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