

Adaptive evolution of the African and Indonesian coelacanths to deep-sea environments

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Received 10 July 2000; received in revised form 22 August 2000; accepted 25 September 2000

Received by G. Bernardi

Abstract

We have PCR amplified and sequenced the rhodopsin (RH1) and evolutionarily closely related RH2 genes of the Indonesian coelacanth, now referred to as *Latimeria menadoensis*. When the RH1 and RH2 coding sequences are constructed, expressed in cultured cells, and reconstituted with 11-*cis*-retinal, the resulting visual pigments have wavelengths of maximal absorption (λ_{\max}) of 485 and 479 nm, respectively. These λ_{\max} values are identical to those of the African coelacanth, *Latimeria chalumnae*, showing that the Indonesian coelacanths also detect a narrow range of color. Statistical analyses show that the adaptation of the coelacanths toward the deep-sea started as early as 200 million years ago. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Visual pigments; Wavelength absorption; Color vision; Vertebrates

1. Introduction

How natural selection operates at the molecular level is a major focus in evolutionary biology. Unfortunately, it is not an easy task to elucidate the molecular mechanisms of adaptive evolution in vertebrates. This is because it is extremely difficult to find genetic systems where the functional effects of possible adaptive mutations can be rigorously assessed experimentally. The photosensitive molecules, visual pigments, present one of a very few model systems for studying adaptive evolution in vertebrates (Golding and Dean, 1998).

A visual pigment consists of the chromophore, 11-*cis*-retinal, and a membrane protein, an opsin that is encoded by a distinct opsin gene. Visual pigments in vertebrate retinas are classified into five evolutionarily distinct groups: (i) rhodopsin (RH1), (ii) RH1-like (RH2), (iii) short wavelength-sensitive (SWS1), (iv) SWS1-like (SWS2), and (v) long wavelength-sensitive (LWS) or middle wavelength-sensitive (MWS) (LWS/MWS) pigment clusters (Yokoyama, 1994; 1995; 1997; 2000a). RH1 pigments are usually expressed in rod photoreceptor cells and the other four classes of pigments in cone photoreceptor cells. The

origin of these five groups of pigments seems to be old and they already coexisted prior to the divergence of various vertebrates (Yokoyama and Yokoyama, 1996).

The coelacanths, *Latimeria chalumnae*, live near the Comoros islands in the western Indian Ocean (Fricke and Hissmann, 1990; Schliwen et al., 1993; Fricke et al., 1995). Living at a depth of about 200 m, these African coelacanths receive only a narrow range of light, at about 480 nm. To detect the entire, albeit narrow, range of 'color' available at this depth, the coelacanths use RH1 and RH2 pigments with the optimum light sensitivities (λ_{\max}) at 478 and 485 nm, respectively (Yokoyama et al., 1999). Compared to the corresponding orthologous pigments, these λ_{\max} values are shifted about 20 nm toward blue. Furthermore, SWS1, SWS2, and LWS/MWS genes have been inactivated. Clearly, all of these molecular changes are geared toward the adaptation of the coelacanths to their contemporary habitats (Yokoyama et al., 1999). The adaptation of the RH1 pigment has been accomplished through two amino acid replacements E122 → Q122 and A292 → S292 (E122Q/A292S) and that of the RH2 pigment through E122Q/M207L (Yokoyama et al., 1999).

The population size of these African coelacanths is suspected to have dwindled to a few hundred individuals (Hissmann et al., 1998). Therefore, the recent discovery of a new coelacanth species, *Latimeria menadoensis*, in Indonesian waters (Erdmann et al., 1998; Pouyaud et al., 1999;

Abbreviations: λ_{\max} , wavelength of maximal absorption; RH1, rhodopsin; RH2, RH1-like

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Holder et al., 1999) has generated hope for the existence of additional coelacanth populations (Gordon, 1998; Fricke et al., 2000). The Indonesian coelacanths also provide a rare opportunity to study processes of natural selection. Here, we show that the Indonesian coelacanth also uses the RH1 and RH2 pigments with identical light-sensitivities to those of the African coelacanth. We also present a method of computing the time of the migration of the coelacanths toward the deep-sea. The results show that the adaptation of the coelacanth to the deep-sea started as early as 200 million years (Myr) ago.

2. Materials and methods

2.1. Genomic DNA, PCR amplification, and sequencing

The Indonesian coelacanth heart tissue was originally sent to Dr Chris Amemiya (Boston Univ. School of Medicine) from Drs Mark Erdman and Roy Caldwell (Univ. California, Berkeley). Dr Amemiya isolated the genomic DNA from this tissue and provided it to us. PCR was performed by using 30 cycles at 92°C for 45 s, 55°C for 60 s, and 72°C for 90 s. At each cycle, the duration of the extension reaction was progressively extended by 3 s. The PCR products were sequenced by cycle sequencing reactions using the Sequitherm Excell II Long-Read kits (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).

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

The mutants were generated by using Quick Change site-directed mutagenesis kit from Stratagene. The DNA fragments that were subjected to mutagenesis were sequenced to

rule out spurious mutations. Then, these cDNAs were ligated into an expression vector pMT separately, expressed in cultured COS1 cells, and regenerated with 11-*cis*-retinal (Storm Eye Institute, Medical University of South Carolina), and the resulting visual pigments were purified using an antibody (The Cell Culture Center, Minneapolis, Minnesota; see also Yokoyama, 2000b). UV-visible spectra were recorded at 20°C by using a Hitachi U3000 dual beam spectrophotometer (dark spectrum). The visual pigments were bleached for 3 min by using a 60 W standard light bulb at a distance of 20 cm (light spectrum). Data were analyzed with SIGMA PLOT software (Jandel, San Rafael, CA). The λ_{\max} values were evaluated by subtracting the light spectrum from the dark spectrum.

3. Results

3.1. RH1 and RH2 genes of the Indonesian coelacanth

Using the sequence information of the RH1 and RH2 genes of the African coelacanth (Fig. 1), we have PCR amplified and sequenced the entire coding regions of the corresponding genes of the Indonesian coelacanth. When the 1065 nucleotide sites of the coding region of the two coelacanth RH1 genes are compared, we can find three nucleotide differences (0.3%), one of which results in an amino acid F at site 24 (F24) in the African coelacanth and L24 in the Indonesian coelacanth (Table 1). The two coelacanth RH2 genes show nine nucleotide differences out of 1065 sites (0.8%), again resulting in one amino acid difference: I216 in the African coelacanth and V216 in the Indonesian coelacanth (Table 1).

If we consider the tetrapod RH1 pigments with λ_{\max} values between 497–503 nm, the typical range for the RH1 pigments (Yokoyama and Yokoyama, 1996; Yokoyama, 1997), we can find 12 tetrapod pigments (Table 2; see Yokoyama, 2000c, and references therein).

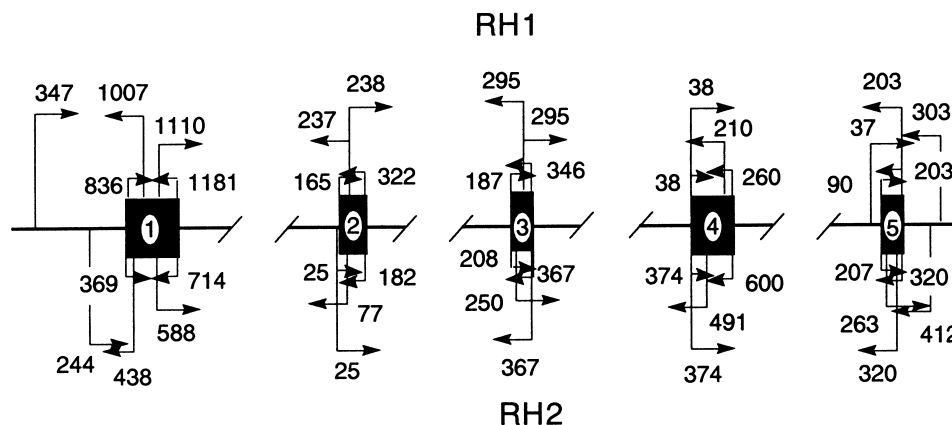


Fig. 1. The sequencing strategy for the RH1 and RH2 genes of the Indonesian coelacanth. The five numbered black boxes indicate the putative coding regions. The arrows indicate the direction of each primer, and the numbers associated correspond to the nucleotide sites of the published sequences for the corresponding African coelacanth genes (RH1 gene: GenBank AF13123–AF131257; RH2 gene: AF131258–AF131262). For each gene, the 11 pairs of adjacent forward and reverse primers with similar heights are used for PCR amplification.

Table 1
Nucleotide sequence differences between the RH1 and RH2 genes of the African and Indonesian coelacanths

Gene	Exon	Site	African	Indonesian
RH1	1	70 ^a	T	C
	1	357	A	T
	2	528	T	G
RH2	1	312	C	A
	1	354	C	T
	1	357	A	T
	2	528	T	A
	3	531	A	G
	3	646 ^b	A	G
	4	699	A	T
	5	987	A	C
	5	1062	A	C

^a These nucleotides cause an amino acid difference in the African coelacanth (F24) and Indonesian coelacanth (L24).

^b These nucleotides cause an amino acid difference in the African coelacanth (I216) and Indonesian coelacanth (V216).

Table 2
RH1 and RH2 pigments in vertebrates^a

Pigments	GenBank accession no.	Pigments	GenBank accession no.
[RH1]		Rat (P498)	Z46957
Marine lamprey (P500)	U67123	Bovine (P500)	M21606
River lamprey (P500)	M63632	Rabbit (P502)	U21688
Goldfish (P492)	L11863	Macaque monkey (P500)	S76579
John Dory (P492)	Y14484	Human (P497)	U49742
Afr. Coelacanth (P485)	AF131253	[RH2]	
Ind. Coelacanth (P485)	This study	Goldfish (P511)	L11865
Leopard frog (P502)	S49004	Goldfish (P506)	L11866
Bullfrog (P500)	S79840	Afr. Coelacanth (P478)	AF131258
Clawed frog (P502)	L07770	Ind. Coelacanth (P479)	This study
Alligator (P499)	U23802	Chicken (P508)	M92038
Chicken (P503)	D00702	Pigeon (P503)	AF149232
Pigeon (P502)	AF149230	Chameleon (P495)	AF134189
Mouse (P498)	Z46957	Gecko (P467)	M92035

^a The numbers after P refer to λ_{\max} values. Afr. coelacanth, *Latimeria chalumnae*; Alligator, *Alligator mississippiensis*; Bovine, *Bos taurus*; Bullfrog, *Rana catesbeiana*; Chameleon, *Anolis carolinensis*; Chicken, *Gallus gallus*; Clawed frog, *Xenopus laevis*; Gecko, *Gekko gekko*; Goldfish, *Carassius auratus*; Human, *Homo sapiens*; Ind. coelacanth, *Latimeria menadoensis*; John Dory, *Zeus faber*; Leopard frog, *Rana pipiens*; Macaque monkey, *Macaca fascicularis*; Marine lamprey, *Lamptera marinus*; Mouse, *Mus musculus*; Pigeon, *Columba livia*; Rabbit, *Oryctolagus cuniculus*; Rat, *Rattus norvegicus*; River lamprey, *Lamptera japonica*.

Table 3
Proportion of different nucleotides (amino acids) per site for selected pairs of RH1 and RH2 genes (pigments)^a

		1	2	3	4	5	6	7	8	9
1.	Afr. Coelacanth (P485)		0.003	0.19	0.21	0.21	0.28	0.29	0.28	0.29
2.	Ind. Coelacanth (P485)	0.003		0.19	0.21	0.22	0.29	0.29	0.28	0.30
3.	Clawed frog (P502)	0.15	0.15		0.19	0.22	0.29	0.29	0.28	0.30
4.	Chicken (P503)	0.15	0.16	0.13		0.15	0.28	0.28	0.27	0.29
5.	Human (P497)	0.17	0.18	0.16	0.12		0.27	0.27	0.25	0.29
6.	Afr. Coelacanth (P478)	0.26	0.26	0.27	0.26	0.25		0.009	0.23	0.25
7.	Ind. Coelacanth (P479)	0.26	0.26	0.27	0.26	0.25	0.003		0.23	0.25
8.	Chicken (P508)	0.27	0.28	0.28	0.27	0.29	0.20	0.19		0.16
9.	Chameleon (P495)	0.28	0.29	0.28	0.28	0.29	0.19	0.19	0.23	

^a Values above the diagonal are the proportions of different nucleotides per site; values below the diagonal are the proportions of different amino acids per site.

Similarly, we can find four tetrapod RH2 pigments with known λ_{\max} values (Table 2). Excluding insertions and deletions, we can compare 996 nucleotide sites (and the corresponding 332 amino acid sites) that are common to the RH1 and RH2 genes of the coelacanths and other vertebrates. At the nucleotide level, the RH1 and RH2 genes differ by less than 25% within each group, whereas the two groups of opsin genes differ at about 30% (Table 3). At the amino acid level, the RH1 and RH2 pigments differ by less than 23% and 29% within and between the two groups of pigments, respectively (Table 3). Thus, the RH1 and RH2 pigments are evolutionarily relatively closely related (see also Yokoyama and Yokoyama, 1996; Yokoyama, 1997; 1999; Yokoyama, 2000a,c).

3.2. Absorption spectra of the RH1 and RH2 pigments

The RH1 and RH2 coding sequences of the Indonesian coelacanth were synthesized by introducing nucleotide

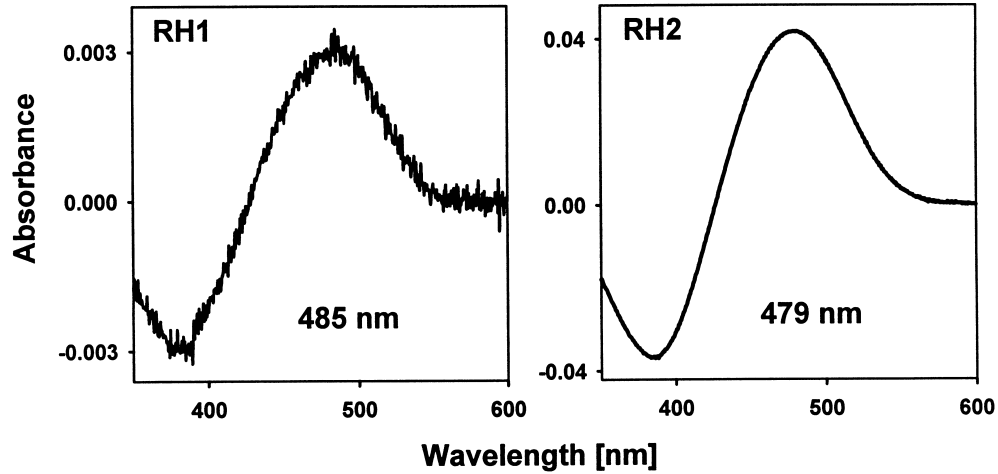


Fig. 2. Absorption spectra of the RH1 and RH2 pigments evaluated by the dark-light difference.

changes T7°C and A646G into the respective minigenes of the African coelacanth. When these minigenes are expressed in cultured cells and reconstituted with 11-*cis*-retinal, the resulting RH1 and RH2 pigments attain λ_{\max} values at 485 ± 1 and 479 ± 1 nm, respectively (Fig. 2). These values are virtually identical to those of the African coelacanth (Yokoyama et al., 1999), showing that the Indonesian coelacanths are also best fitted to detect a specific narrow range of color at around 480 nm. Given the same color-sensitivities of the two coelacanth species and the high amino acid sequence identities of the pigments, it is clear that the adaptive change of the coelacanth vision was achieved prior to the separation of the two coelacanth species. Where did the coelacanth ancestor live? Oceanographic data show that water from the Mindanao Current directly flows into the Suawesi Sea in Indonesia and eventually reaches to the vicinity of the Comoros Islands (Gordon, 1998). Furthermore, geological evidence suggests that the age of the African population is less than 100,000 years (Fricke et al., 2000). Thus, it is most likely that this ancestor lived near Indonesia, from which the African coelacanths must have been derived.

3.3. Adaptation of the coelacanths to the deep-sea

Fig. 3 shows a probable evolutionary scheme for the African coelacanth (A), Indonesian coelacanth (I), tetrapod (T), and fish (F) pigments. The coelacanths in the early Carboniferous period lived in rivers and swamps (Maisey, 1996) and, therefore, the migration of the coelacanth toward the deep-sea must have occurred since then. T_m Denotes the time in million years (Myr) of the migration of the coelacanth ancestor toward the deep-sea, while a and b denote the rates of amino acid replacement per site per year in the coelacanth pigments after and before the migration, respectively. Similarly, c denotes the corresponding rate for the fish pigments.

Once the coelacanths start their descent toward the deep-

sea, they do not have to maintain the λ_{\max} values of the RH1 and RH2 pigments at about 500 nm any more and the level of purifying selection will be less stringent. Thus, it is expected that a is larger than b . By the same token, when the coelacanths accomplish the adaptation, the purifying selection should resume and the evolutionary rates of the visual pigments slow down. Indeed, the RH1 and RH2 pigments in the common ancestor of the African and Indonesian coelacanths appear to have accomplished the adaptation to the deep-sea. Unfortunately, the time of the completion of this adaptation cannot be evaluated from the available data. Thus, a may be interpreted as a lower limit, whereas T_m , which is roughly inversely related to a , may present an upper limit.

Let t_1 , t_2 , and t_3 be the divergence times in Myr between the two coelacanth pigments, between the coelacanth and tetrapod pigments, and between tetrapod and fish pigments, respectively. If we let d_{ij} be the number of amino acid replacements per site between i and j pigments ($i, j = A, I, T$, and F), $d_{AI} = 2(t_1 \times 10^6)a$, $d_{AT} = d_{IT} = [(a - b)T_m + 2bt_2] \times 10^6$, $d_{AF} = d_{IF} = [(a - b)T_m + (b + c)t_3] \times 10^6$, and $d_{TF} = (b + c)t_3 \times 10^6$.

Knowing t_1 and t_2 values, a , b , and T_m can be estimated by

$$a = [d_{AI}/(0.002t_1)] \times 10^{-9} \quad (1)$$

$$b = [(-d_{AF} - d_{IF} + d_{AT} + d_{IT} + 2d_{TF})/(0.004t_2)] \times 10^{-9} \quad (2)$$

$$T_m = A/B \quad (3)$$

where

$$A = 2t_2(d_{AF} + d_{IF} - 2d_{TF}) \text{ and}$$

$$B = 2(t_2/t_1)d_{AI} + d_{AF} + d_{IF} - d_{AT} - d_{IT} - 2d_{TF}$$

For both RH1 and RH2 pigment sequences, we computed the proportion of different amino acids between two pigments i and j , and from this proportion (p_{ij}), d_{ij} was

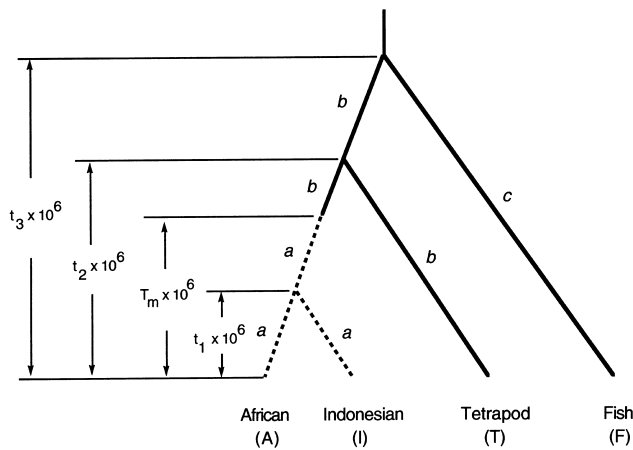


Fig. 3. Plausible phylogenetic tree for African coelacanth (A), Indonesian coelacanth (I), tetrapod (T), and fish (F) pigments.

estimated by $d_{ij} = -\ln(1 - p_{ij})$. For RH1 pigments, in addition to goldfish (P492) and John Dory (P492) pigments, marine lamprey (P500) and river lamprey (P500) pigments were used as the fish pigment sequences simply because the two lamprey pigments have evolved with significantly slower rates than other orthologous pigments (Zhang and Yokoyama, 1997). We have therefore computed the d_{ij} values for each of these pigments and used the averages of the values to compute d_{AF} , d_{IF} , and d_{TF} . Similarly, for RH2 pigments, the RH1 pigments of marine lamprey and river lamprey and the two goldfish RH2 pigments were used as the fish pigment sequences.

The time of divergence between the African and Indonesian coelacanths has been estimated to be 4.7–6.3 million years ago (Holder et al., 1999). Thus, a is given by $0.24\text{--}0.32 \times 10^{-9}$. If we assume that $t_1 = 5$, then a is given by $(0.30 \pm 0.301) \times 10^{-9}$. Table 4 shows the results for b and T_m for the RH1 pigments by assuming that $t_1 = 5$

Table 4

The rate (b) of amino acid replacement in the RH1 pigment and the time (T_m) of the migration of the coelacanth toward the deep-sea^a

Tetrapod	$b (\times 10^{-9} \text{ /site/year})$	T_m
Clawed frog (P502) ^b	0.21 ± 0.076	147
Leopard frog (P502)	0.23 ± 0.078	189
Bullfrog (P500)	0.20 ± 0.076	272
Alligator (P499)	0.19 ± 0.076	220
Pigeon (P502)	0.17 ± 0.075	269
Chicken (P503)	0.18 ± 0.075	249
Human (P497)	0.24 ± 0.078	271
Macaque monkey (P500)	0.24 ± 0.078	136
Mouse (P498)	0.23 ± 0.076	170
Rat (P498)	0.24 ± 0.078	272
Rabbit (P502)	0.23 ± 0.077	274
Average	0.21	224

^a The estimates obtained using the bovine RH1 pigment as a tetrapod sequence are not shown because T_m was 492.

^b Numbers after P refer to λ_{\max} values.

and $t_2 = 365$ (Benton, 1993; 1997). Interestingly, the b values are nearly the same for various tetrapod pigment sequences with the average value of 0.21×10^{-9} . T_m values are more variable, ranging from 136 to 274, with the mean of 224 ± 55 . For the RH2 pigments, $a = (0.30 \pm 0.301) \times 10^{-9}$, $b = (0.28 \pm 0.079) \times 10^{-9}$, and $T_m = 196$ are obtained only when chameleon (P495) pigment was used as a tetrapod pigment sequence. Otherwise, T_m values are estimated to be much larger than its upper limit 365. The acceptable a , b , and T_m values are similar to those obtained for the RH1 pigments. These results suggest that the migration of the coelacanth ancestor toward the deep-sea occurred as early as 200 Myr ago.

3.4. Evolution of the SWS1 pseudogenes

SWS1 and SWS2 pigments have λ_{\max} values at 360–455 nm, whereas LWS/MWS pigments have λ_{\max} values at 510–610 nm (Yokoyama, 2000c) and, therefore, these pigments cannot detect light at around 480 nm efficiently. Thus, these opsin genes in the coelacanths became unnecessary and, consequently, must be inactivated. Today, the LWS/MWS genes cannot be detected by Southern hybridization method and, furthermore, the SWS2 gene cannot be cloned from the coelacanth genome (Yokoyama et al., 1999). Thus, these two groups of opsin genes must have become pseudogenes in the early stage of the adaptation of the coelacanths to the deep-sea.

Previously, we have cloned and sequenced exons 1–4 of the SWS1 gene of the African coelacanth (Yokoyama et al., 1999). Using this sequence information, we have PCR amplified and sequenced most parts of the corresponding exons 1–4 from the Indonesian coelacanth (Fig. 4). When the 691 nucleotide sites of the pseudogenes of the African and Indonesian coelacanths are compared, only four sites differ. It is surprising to see that the level of the sequence divergence (0.6%) between the two pseudogenes is lower than that between the two functional RH2 genes (0.8%). Assuming that the two coelacanth species diverged 5 Myr ago, the rates of nucleotide substitution for the RH1, RH2, and SWS1 genes are given by $(0.3 \pm 0.17) \times 10^{-9}$, $(0.9 \pm 0.30) \times 10^{-9}$, and $(0.6 \pm 0.29) \times 10^{-9}$ per site per year, respectively. Thus, the SWS1 pseudogenes in the coelacanths are evolving with an order of magnitude lower than those of the α and β globin pseudogenes (Li et al., 1981). At present, the cause of the slow evolution of the coelacanth SWS1 pseudogenes is not clear.

4. Discussion

We have seen that the adaptation of the coelacanth vision to the deep-sea has been accomplished by shifting the λ_{\max} values of their RH1 and RH2 pigments about 20 nm toward blue. These blue-shifts in the λ_{\max} values are not limited to the coelacanths. Although most RH1 and RH2 pigments have λ_{\max} values at about 500 nm (Yokoyama and

(P485) pigment and bottle-nose dolphin (P488) pigment are known to cause 8 nm (Yokoyama et al., 1999) and 28 nm (Fasick and Robinson, 1998) of blue-shifts in the λ_{\max} values, respectively. These mutagenesis experiments strongly suggest that most of the blue-shifted λ_{\max} values of the RH1 pigments of Conger eel, marine eel, John Dory, chameleon, and two dolphin species can be explained by these three amino acid replacements.

The RH2 pigments of two coelacanth species and gecko have blue-shifted λ_{\max} values (Table 2). Why did the gecko not use SWS1 or SWS2 pigments for its blue vision ($\lambda_{\max} = 467$ nm), like other vertebrates? The answer may lie in the fact that the gecko retinas contain only rod photoreceptor cells (Walls, 1934). In other words, the blue-shifted λ_{\max} value may be related to the efficiency of the evolutionary adaptation of the cone-specific RH2 pigment to the pure-rod retina of the gecko. The RH2 pigments are evolutionarily much more closely related to the RH1 pigments than the SWS1 and SWS2 pigments. Thus, the RH2 pigment might be allowed to adapt to the pure-rod retina more easily than the SWS1 or SWS2 pigments (Yokoyama, 2000c).

For the RH2 pigments, E122Q occurred in the common ancestor of the coelacanth and tetrapod pigments, followed by M207L in the coelacanth pigment and D83N in gecko (P467) pigment (Yokoyama, 1999, 2000a,c). The mutagenesis experiments using African coelacanth (P478) pigment suggest that both E122Q and M207L shift the λ_{\max} values toward blue (Yokoyama et al., 1999). As we saw already, D83N has the same effect on the λ_{\max} -shift. Constructing chimeric pigments, the cause of the blue-shift in the λ_{\max} value of gecko (P467) pigment was localized somewhere between transmembrane helices I–III (Kojima et al., 1996). Therefore, D83N and E122Q, in transmembrane helices I and III, respectively, seem to be the major cause of the blue-shifted λ_{\max} value of the gecko pigment.

All of these observations strongly suggest that the adaptive blue-shifts in the λ_{\max} values of RH1 and RH2 pigments in vertebrates have been achieved by a small number of amino acid replacements. For example, during the last 200 Myr, each of the RH1 and RH2 pigments of the coelacanth has accumulated about 20 amino acid changes [$(0.30 \times 10^{-9}$ per site per year) \times (200 Myr) \times (355 sites)]. Only 10% of these changes are used for the adaptation of the coelacanths to the new environment. Small numbers of amino acid differences are also known to cause the functional differentiation of the LWS/MWS pigments in a wide range of vertebrates (Yokoyama and Radlwimmer, 1998, 1999) and the violet and ultraviolet pigments in birds (Yokoyama et al., 2000). Furthermore, the same conclusion has been reached in a survey of other adapted molecules (Golding and Dean, 1998). In order to elucidate the molecular bases of natural selection, therefore, it is necessary to identify amino acid changes that are actually used in natural selection. This can be accomplished most effectively through comparative sequence analyses followed by site-directed mutagenesis

experiments (Golding and Dean, 1998; Yokoyama and Yokoyama, 1996; Yokoyama, 1997, 2000c).

Acknowledgements

We thank Drs M. Erdmann, R. Caldwell (Univ. California, Berkeley), C. Amemiya (Boston Univ. School of Medicine) for giving us access to the genomic DNA of the Indonesian coelacanth. Comments by R. Yokoyama, J. Belote, and two anonymous reviewers were greatly appreciated. This work was supported by a National Institutes of Health grant GM42379.

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