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Evolution of visual pigments in geckos

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Abstract Most geckos are nocturnal forms and possess rod retinas, but some diurnal genera have pure-cone retinas. We isolated cDNAs encoding the diurnal gecko opsins, *dg1* and *dg2*, similar to nocturnal gecko *P521* and *P467*, respectively. Despite the large morphological differences between the diurnal and nocturnal gecko photoreceptor types, they express phylogenetically closely related opsins. These results provide molecular evidence for the reverse transmutation, that is, rods of an ancestral nocturnal gecko have backed into cones of diurnal geckos. The amino acid substitution rates of dg1 and dg2 are higher than those of P521 and P467, respectively. Changes of behavior regarding photic environment may have contributed to acceleration of amino acid substitutions in the diurnal gecko opsins.

Key words: Visual pigment; cDNA cloning; In situ hybridization; Diurnal gecko (*Phelsuma madagascariensis longinsulae*)

1. Introduction

The photoreceptor cells in vertebrate eyes have been categorized as rods or cones. In general, rods function under twilight conditions, while cones are most suited to daylight. The visual pigments, which absorb light and trigger the phototransduction cascade, are responsible for the spectral sensitivity of a photoreceptor cell. They are contained in the outer segment, and are tuned to their particular wavelength by the characteristics of the protein moiety (opsin).

Most geckos are nocturnal and possess rod retinas [1,2], and it has been proposed that the rods of nocturnal geckos have transmuted from the cones of an ancestral diurnal lizard (the transmutation theory) [3]. The retina of the nocturnal gecko, *Gekko gekko*, has three visual pigments, identified microspectrophotometrically as 'green' (P521), 'blue' (P467) and 'ultraviolet' (UV) (P364), with absorption maxima (λ_{max}) at 521 nm, 467 nm and 364 nm, respectively [4,5]. Two kinds of cDNAs encoding P521 and P467 have been isolated [6], and their deduced amino acid sequences are similar to those of chicken red-sensitive visual pigments (iodopsin) or *A. carolinensis lws*, and chicken green-sensitive visual pigments or *A. carolinensis rh2*, respectively. These observations provide strong support for the transmutation theory from a molecular point of view.

There are some diurnal geckos, such as *Phelsuma*, with pure-cone retinas. The transmutation theory also suggested that the rods of an ancestral nocturnal gecko have backed into the cones of diurnal geckos (the reverse transmutation) [3]. This theory has been supported by morphological studies of many gecko photoreceptors, and it has been concluded that the photoreceptor cells of *Phelsuma* have likely undergone the reverse transmutation [7,8]. However, the visual pigment genes of diurnal geckos have not been investigated at all.

In this study, we isolated and characterized two kinds of cDNAs, *dg1* and *dg2*, encoding visual pigments of the diurnal gecko, *Phelsuma madagascariens longinsulae*. The findings provide molecular evidence for the occurrence of the reverse transmutation, and suggest that changes in behavior regarding photic environment affect the molecular evolution of visual pigments.

2. Materials and methods

2.1. Isolation of dg1 cDNA fragments

Diurnal geckos (*Phelsuma madagascariensis longinsulae*) about 20 cm in length were obtained from a local supplier. Single-stranded cDNA was prepared with Superscript II reverse transcriptase (Gibco BRL) [9,10] and poly(G) tails were added to the cDNA with terminal deoxynucleotidyl transferase (TOYOBO). The partial cDNA fragments of *dg1* were amplified with RED-F2 and VVP-R2′ degenerate primers (corresponding to amino acid sequences PNYHIAP and YNP(I/V)VY, respectively) by polymerase chain reactions (PCRs), as described previously [11,12].

A retinal cDNA library was constructed using a Lambda-ZAPII *Eco*RI/CIAP Vector Kit (Stratagene), as described previously [13]. Briefly, double-stranded cDNA was ligated with an *Eco*RI-*Not*I adaptor, inserted into the *Eco*RI site of Lambda-ZAPII, and packaged into Giga-Pack II gold (Stratagene). The amplified fragments were used as probes for high stringency screening (hybridization, 50% formamide at 48°C; washing, 0.2×SSC 0.1% SDS at 55°C) [13,14]. Positive clones were transformed into plasmids by an EXASSIST-SOLR system (Stratagene).

2.2. Isolation of dg2 cDNA fragments by rapid amplification of both cDNA ends (RACE PCR)

The partial cDNA fragments of *dg2* are amplified using VVP-F5 (corresponding to amino acid sequences LN(Y/W)ILVN) and VVP-R2′ degenerate primers [11]. The *dg2* specific primers DG2-F1 (5′-ATAGGATCCGTCGCTTGGACCCCTTAT-3′, corresponding to the amino acid sequences VAWTPY) and DG2-R1 (5′-AGAAAGCTTGCAAAGAATCCTTCAA-3′, corresponding to the amino acid sequences AFEGFF) were used to obtain *dg2* cDNA covering the entire coding regions. The 3′ end of *dg2* cDNA fragment with a poly(A) tail was amplified by PCR, using DG2-F1 and T-amp primers [12] on annealing at 50°C. To amplify the 5′ end of *dg2* fragments, amplifications were carried out using DG2-R1 and C-amp primers [12] on annealing at 50°C.

2.3. Sequencing and data analysis

All sequences were determined for at least three independent clones to avoid PCR errors. Identities were calculated for 290 amino acids between the region corresponding to P23 and Q331 of dg1, and amino acid substitution rates (k) were estimated using the proportion of different amino acids between the two sequences (p), with a correction

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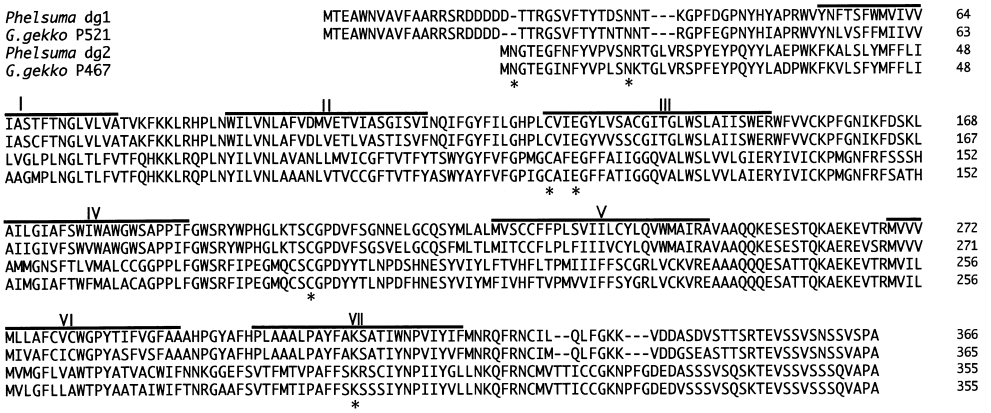

Fig. 1. Alignment of the deduced amino acid sequences of *P. m. longinsulae* opsins (*dg1* and *dg2*) and *G. gekko* opsins (*P521* and *P467*). Amino acids marked with asterisks are functionally important residues (see text). Gaps, denoted by dashes, were introduced to optimize sequence similarity. Putative transmembrane domains I–VII are indicated by horizontal lines. The sequences of *dg1* and *dg2* have been deposited in the EMBL nucleotide database with the accession numbers AF074043 and AF074044, respectively.

for multiple substitutions of $k = -\ln(1-p)$ [15]. A phylogenetic tree was constructed by the neighbor-joining (NJ) method [16,17]. Sequence data used in the present analyses were taken from GenBank and DDBJ databases, with the following accession numbers: *G. gekko* P521 (M92036), P467 (M92035); *Anolis carolinensis* lws (U08131), rh2 (S79167); chicken (*Gallus gallus*) red (M62903), green (M92038); medakafish (*Oryzias latipes*) KFH-R (AB001604), KFH-G (AB001603); goldfish (*Carassius auratus*) red (L11867) and green (L11865).

2.4. In situ hybridization

cDNA fragments of *dg1* (820 bases) and *dg2* (640 bases) were cloned into a pGEM-3Zf(+) plasmid vector (Promega), and linearized with appropriate endonucleases. Antisense cRNA riboprobes were synthesized by run-off transcription from the SP6 or T7 promoter with digoxigenin-UTP, as recommended in the manufacturer's protocol (Boehringer Mannheim). The preparation of *Phelsuma* retinal cryosections and methods for in situ hybridization were as described previously [12,13,18]. The dark-adapted eyes were fixed in 4% paraformaldehyde, and embedded in 33% OCT compound diluted with 20% sucrose in phosphate buffer. Five μm retinal cryosections were hybridized with 0.1–2.0 μg/ml (final concentration) cRNA probes. The hybridization signal was visualized using a nucleic acid detection kit (Boehringer Mannheim).

3. Results

3.1. The deduced amino acid sequences of dg1 and dg2

Two kinds of cDNAs, encoding the putative visual pigments (*dg1* and *dg2*) were isolated from the diurnal gecko *P. m. longinsulae*. The first ATG codons of the nucleotide sequences are followed by long open reading frames, which encode 366 (dg1) and 355 (dg2) amino acids (Fig. 1). Functionally important residues are conserved in the deduced amino acid sequences: the site of Schiff base linkages for the chromophore (K312 in dg1, K296 in dg2) [19], the Schiff base counterions (E129 in dg1, E113 in dg2) [20–22], sites for a disulfide bond (C126 and C203 in dg1, C110 and C187 in dg2) [23], *N*-glycosylation sites (N34 in dg1, N2 and N15 in dg2) [24] and the possible phosphorylation sites near the C-terminals [25]. Therefore, dg1 and dg2 are expected to be functional visual pigments.

The deduced amino acid sequence of *dg1* shows high identities with those of *P521* (86.6%) of the nocturnal gecko, *G.*

Table 1
The amino acid identities (%) and the estimated amino acid substitution rates per site (italics) among group-L (a) and group-ML (b) opsins

(a)	KFH-R	chicken red	*Anolis* lws	P521	dg1
goldfish red	88.6	84.5	85.9	78.6	75.0
	0.12	*0.17*	*0.15*	*0.24*	*0.29*
	KFH-R	85.9	88.3	81.4	76.6
		0.15	*0.12*	*0.21*	*0.28*
		chicken red	90.3	83.1	82.1
			0.10	*0.19*	*0.20*
			Anolis lws	81.7	79.7
				0.20	*0.23*
				P521	86.6
					0.14
(b)	KFH-G	chicken green	*Anolis* rh2	P467	dg2
goldfish green	76.6	78.6	77.2	75.2	74.1
	0.27	*0.24*	*0.29*	*0.29*	*0.30*
	KFH-G	69.7	70.0	71.0	65.5
		0.36	*0.36*	*0.34*	*0.42*
		chicken green	91.7	80.0	76.6
			0.09	*0.22*	*0.27*
			Anolis rh2	81.0	76.6
				0.21	*0.27*
				P467	83.8
					0.18

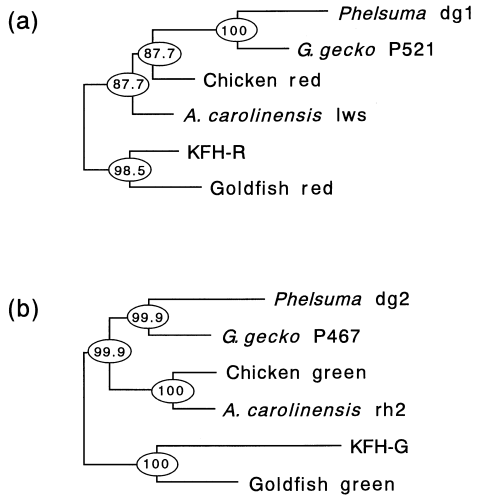

Fig. 2. An NJ tree of vertebrate group-L (a) and group-ML (b) opsins. Circled numbers indicate clustering percentage (%) obtained from 1000 bootstrap resamplings.

gekko, and *lws* (79.7%) of the diurnal lizard, *A. carolinensis* (Table 1). The deduced amino acid sequence of *dg2* is very similar to those of *G. gekko P467* and *A. carolinensis rh2*, with 83.8% and 76.6% identities, respectively. A phylogenetic tree constructed by the NJ method indicates that dg1 and dg2 belong to group-L and group-ML [26], respectively (Fig. 2).

3.2. *In situ localization of Phelsuma opsin mRNAs*

Diurnal geckos have three types of cones: (1) type A single cones; (2) type B double cones, consisting of a principal member with colorless oil-droplets and an accessory member without oil-droplets but with a prominent paraboloid; and (3) type C double cones, consisting of one thin and one thick member [8]. The distribution of the *dg1* and *dg2* mRNAs were investigated by in situ hybridization. In radial sections of the diurnal gecko retina, digoxigenin-conjugated *dg1* cRNA probes recognized the region around the outer limiting membrane (Fig. 3a). The signals were localized in the cell bodies and myoids of most photoreceptors: type A single cones; both members of type B double cones; and thick members of type C double cones. However, signals were not found in the thin members of type C double cones (Fig. 3b), so it is concluded that *dg1* is expressed in all photoreceptor types except the thin members of type C double cones.

Fig. 3c and d show the localization of *dg2* mRNA. Hybridization signals were found in places near the outer limiting membrane (Fig. 3c), and localized in the myoid regions of thin members of type C double cones (Fig. 3d). However, a small population of thin members of type C double cones are negative with the *dg2* cRNA probes (data not shown). It is likely

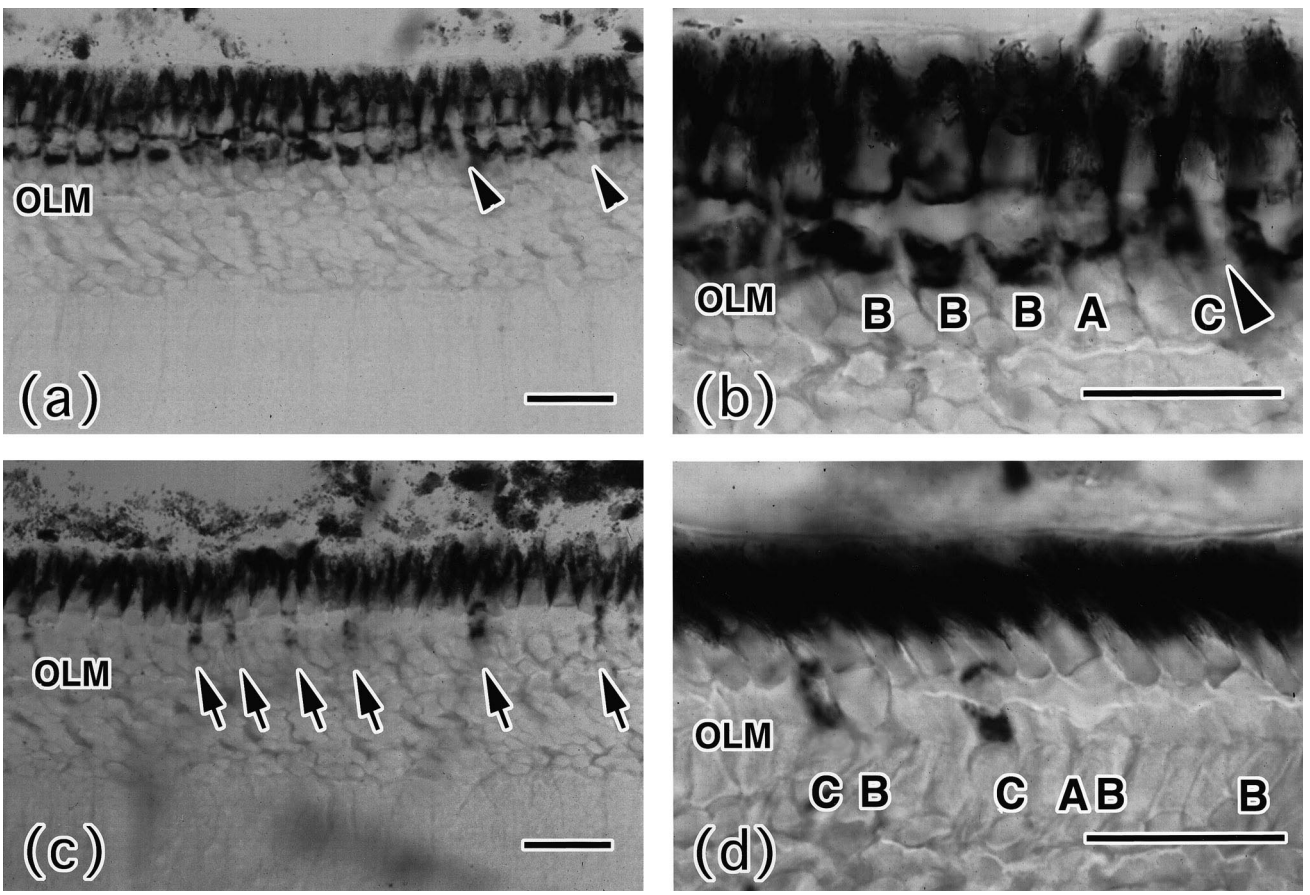

Fig. 3. (a) Distribution of the *dg1* mRNA in the retina of the diurnal gecko, *P. m. longinsulae*, and (b) magnified image. Arrowheads indicate the thin members of type C double cones negative with the *dg1* cRNA probes. All cells except the thin member (type A singles, both members of type B double, and the thick members of type C double) are recognized with the *dg1* cRNA probe. (c) Distribution of *dg2* mRNA, and (d) at a higher magnification image. *dg2* cRNA probes recognize only the thin members of type C double cones (arrows). OLM, outer limiting membrane; A, type A single cone; B, type B double cones; C, type C double cones. Scale bars = 20 μm.

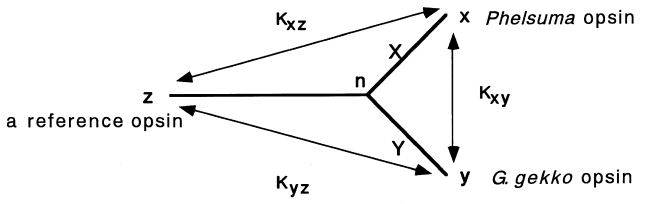

Fig. 4. Diagram illustrating calculation for the amino acid substitution rates. The length of branches leading to the diurnal (X) and nocturnal (Y) gecko opsins from node *n*, are estimated from the relationships $X=(K_{xy}+K_{xz}-K_{yz})/2$ and $Y=(K_{xy}-K_{xz}+K_{yz})/2$, where K_{xy}, K_{xz} and K_{yz} are the estimated substitution rates between a diurnal (x) and a nocturnal (y) gecko opsin, between a diurnal gecko and a reference opsin (z), and between a nocturnal gecko and a reference opsin, respectively.

that these cells express another type of visual pigment, possibly UV-sensitive: since it is known that a small population of thin members of type C double cones are UV-sensitive in the diurnal gecko *Gonatodes albogularis* [27].

Similar to diurnal geckos, photoreceptor cells of nocturnal geckos are classified into three types: (1) type A single rods, (2) type B double rods and (3) type C double rods. *G. gekko* P521 is expressed in all rods except the thin member of type C double rods, whereas P467 is expressed only in the thin members of type C double rods [4,5]. Despite the large morphological difference, the closely related visual pigments are expressed in similar photoreceptor types between the diurnal and nocturnal geckos. Our observations therefore provide molecular biological evidence favoring the reverse transmutation hypothesis.

4. Discussion

4.1. Spectral sensitivity of dg1

In group-L pigments, it is well known that certain amino acids contribute significantly to the tuning of spectral sensitivity. Mouse green pigment with a λ_{max} at 508 nm is the most blue-shifted member among group-L pigments, a shift greatly influenced by loss of the chloride binding residue, H197 [28]. The corresponding residue, H197, is conserved in dg1. Neitz et al. suggested that about 15 nm of the λ_{max} difference between human red- and green-sensitive pigments is mainly due to the amino acid substitution, T285A [29,30]. This residue corresponds to A284 in *G. gekko* P521 and to T285 in dg1. An electroretinogram recording shows a sensitivity peak at 560 nm in the retina of a closely related diurnal gecko, *Phelsuma inunguis* [31], so it is speculated that the *dg1* pigment has a λ_{max} longer than P521 of the nocturnal gecko.

4.2. Tree topology of dg1 and dg2

In Fig. 2, dg1 and dg2 are clustered with P521 and P467, respectively, but the tree topology is different between group-L and group-ML. In group-ML, *A. carolinensis* rh2 is clustered with chicken green with a high bootstrap probability of 100% (Fig. 2b). Judging from the similar topology, Kawamura and Yokoyama suggested that *A. carolinensis* rh2 and *G. gecko* P467 have been derived from duplicate ancestral genes [32]. In contrast, gecko group-L opsins are clustered with chicken red in our analysis, which shows a disagreement with both Kawamura's tree (LWS/MWS) [33] and the general phylogenetic tree of vertebrates. One of the causes for the disagreement of tree topology might be a large difference of amino acid substitution rates, as described in the following section.

4.3. Amino acid substitution rates

The amino acid identities of the diurnal gecko opsins show the highest identities with the nocturnal gecko opsins, suggesting that these gecko opsins are phylogenetically very close. However, it should be noted that all of the other opsins (goldfish, medakafish, *A. carolinensis* and chicken opsins) show higher identities with the nocturnal rather than the diurnal gecko opsins (Table 1). This suggests that the amino acid substitution rate is higher in the diurnal than in the nocturnal gecko opsins.

To analyze the substitution rates of diurnal and nocturnal gecko opsins, we compared the length of branches leading from node *n* to dg1 (X_L) and P521 (Y_L), and to dg2 (X_M) and P467 (Y_M) (Fig. 4), as described by Zhang and Yokoyama [34]. The X_L, Y_L, X_M and Y_M values calculated with each reference opsin are shown in Table 2a and b. These data suggest that amino acid substitution rates of diurnal gecko opsins are significantly higher than those of nocturnal gecko opsins.

Almost all of the descendral geckos (more than 75%) are nocturnal forms: there are only a few diurnal genera. The diurnal geckos are descended from nocturnal ancestors which probably lacked photopic visual specialization, and presumably re-evolved their present photopic visual capability [35].

Table 2
(a) The X_L, Y_L values for group-L opsins, and (b) X_M, Y_M values for group-ML opsins calculated with each reference opsin

(a) Reference opsins	dg1 (X_L)	P521 (Y_L)
Chicken red	0.075 ± 0.016	0.065 ± 0.015
Anolis lws	0.085 ± 0.017	0.055 ± 0.013
KFH-R	0.100 ± 0.019**	0.040 ± 0.012**
Goldfish red	0.095 ± 0.019*	0.045 ± 0.013*
(b) Reference opsins	dg2 (X_M)	P467 (Y_M)
Chicken green	0.130 ± 0.022**	0.040 ± 0.012**
Anolis rh2	0.115 ± 0.020*	0.055 ± 0.014*
KFH-G	0.125 ± 0.021**	0.045 ± 0.013**
Goldfish green	0.090 ± 0.018	0.080 ± 0.017

Standard errors were computed from $[p/\{n(1-p)\}]^{1/2}$, where p is the proportion of different amino acid residues and n is the number of aa sites involved [16,36].
*Difference in branch length X and Y is significant at 5% level.
**Difference in branch length X and Y is significant at 1% level.

The evolutionary pressure for the change in photic environment may have contributed to acceleration of the amino acid substitution rates of the diurnal gecko opsins.

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