

# Distribution of blue-sensitive photoreceptors in amphibian retinas

Yusuke Takahashi<sup>a,b</sup>, Osamu Hisatomi<sup>b</sup>, Shunsuke Sakakibara<sup>b</sup>, Fumio Tokunaga<sup>b,\*</sup>,  
Yasuo Tsukahara<sup>a</sup>

<sup>a</sup>Department of System Information Science, Graduate School of Information Science, Tohoku University, 2-1-1 Katahira, Aoba, Sendai, Miyagi 980-8577, Japan

<sup>b</sup>Department of Earth and Space Science, Graduate School of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Osaka 560-0043, Japan

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**Abstract** Previously, we reported that an opsin (Rc-MS) belonging to the SWS2 group opsins is expressed in bullfrog green rods [Hisatomi, O. et al., FEBS Lett., 1999, 447, 44–48]. An anti-Rc-MS antiserum recognized the cones of the Japanese common newt, *Cynops pyrrhogaster*, which has no green rods. We isolated a cDNA encoding an SWS2 group opsin (Cp-SWS2) from this newt and found that Cp-SWS2 is expressed in a small population of the cones. Our results suggest that SWS2 opsins can be expressed in either green rods or cones of caudata. It seems reasonable to suppose that green rods arose before amphibians were divided into caudata and anura. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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In situ hybridization; Immunohistochemistry;  
Japanese common newt (*Cynops pyrrhogaster*)

## 1. Introduction

Vertebrate photoreceptor cells are morphologically classified into rods and cones. At least four kinds of cones have been reported in amphibian retinas [1–6], namely principle and accessory members of double cones, long single cones, and short single cones. Generally rods, which mediate twilight vision, contain rhodopsins as their visual pigments with absorption maxima ( $\lambda_{\max}$ ) at about 500 nm. However, it is known that several amphibians possess not only conventional rods, called red rods, but also green rods containing short wavelength-sensitive visual pigments with  $\lambda_{\max}$  at about 430 nm. Green rods can be distinguished from red rods by some morphological features, such as a slenderer and shorter outer segment, a long and narrowing myoid, and a nucleus which is located among the cone nuclei. Green rods have been reported in the retinas of leopard frog (*Rana pipiens*) [1], bullfrog (*Rana catesbeiana*) [2,7], African clawed frog (*Xenopus laevis*) [8,9] and tiger salamander (*Ambystoma tigrinum*) [3,4] by morphological and spectroscopic studies [10–13]. The light response and sensitivity of green rods is similar to those of red rods in the toad retina, but is maximal at a shorter wavelength [14].

Most of the studies on green rods have investigated their morphological, spectroscopic and electrophysiological properties, and there have only been a few using molecular tech-

niques. Immunoreactivity of green rods has been investigated using an anti-bovine rhodopsin monoclonal antibody, 1D4, but it is not enough to characterize the green rod pigment [4,8]. Recently, a tiger salamander opsin, similar to chicken blue-sensitive cone opsin and belonging to the SWS2 (MS or M2) opsin group, has been cloned [15], but so far the opsin has not been localized to a specific photoreceptor. Previously, we have reported that a cDNA encoding the Rc-MS opsin is expressed in bullfrog green rods [7]. The Rc-MS pigment, expressed in HEK-293S cells and reconstituted with 11-*cis*-retinal, had its  $\lambda_{\max}$  at about 430 nm [16].

In this paper, we report the cloning of a cDNA encoding an opsin, Cp-SWS2, belonging to the SWS2 group opsins from the Japanese common newt (*Cynops pyrrhogaster*), and the localization of Cp-SWS2 by in situ hybridization and immunohistochemistry. Our results suggest that opsins belonging to the SWS2 group can be expressed in either green rods or cones in caudata. Moreover, we discuss the origin of green rods from the immunoreactivity of photoreceptors containing SWS2 opsins.

## 2. Materials and methods

### 2.1. Animals

We chose three members of the caudata order of amphibians to analyze the distribution of photoreceptors expressing SWS2 opsins. First, we selected the barred tiger salamander (*Ambystoma tigrinum mavortium*), because it is a subspecies of tiger salamander (*Ambystoma tigrinum*), and so probably has green rods in its retina. Secondly, we selected the black salamander (*Hynobius nigrescens*) which does not belong to the superfamily Salamandroideae. There have been a few investigations on the morphological features of caudata photoreceptors except for those of the tiger salamander. Thirdly, we selected the Japanese common newt (*C. pyrrhogaster*), which belongs to the superfamily Salamandroideae. It has been reported that there are no green rods in the retina of the fire salamander (*Salmandra salamandra*) [17,18] which belongs to the same family Salamandroideae. So, we expected there to be no green rods in *C. pyrrhogaster* retinas as well.

### 2.2. Western blot analysis

Dark-adapted eyes were enucleated from the three kinds of amphibians. The retinas were immersed into a sodium dodecyl sulfate (SDS) sample buffer and sonicated briefly. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to standard methods using 12.5% acrylamide mini-gels. Proteins were transferred to PVDF membranes (Bio-Rad) using a semi-dry transfer cell (Transblot SD, Bio-Rad) in the presence of 100 mM Tris, 192 mM glycine, and 15% methanol at 1.7 mA/cm<sup>2</sup> for 2 h. Membranes were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min, and were incubated with a 1000-fold dilution of anti-Rc-MS antiserum in PBS containing 3% BSA for 2 h. Alkaline phosphatase-conjugated anti-mouse IgG (Chemicon International) was reacted

\*Corresponding author. Fax: (81)-668-505480.

E-mail address: tokunaga@ess.sci.osaka-u.ac.jp (Fumio Tokunaga).

as recommended by the manufacturer, and antibody binding was visualized with an NBT/BCIT stock solution (Boehringer Mannheim).

### 2.3. Immunohistochemistry

Immunohistochemical procedures were carried out as described by Hisatomi et al. [5,7]. Briefly, paraffin sections (4  $\mu$ m) of each kind of amphibian retina were incubated with a 100-fold dilution of the anti-Rc-MS antisera, washed with PBS, incubated with biotin-conjugated anti-mouse antibodies, and reacted with horseradish peroxidase-conjugated avidin–biotin complex (*Elite* ABC kit, Vectastain). The localization of opsin similar to Rc-MS was visualized with diaminobenzidine (DAB) developing solution, and identified using Nomarski optics (Olympus-BX50). For immunofluorescent observations, washed sections were reacted with fluorescein-conjugated anti-mouse IgG (Jackson), and counterstained with 0.1% Evans blue (Wako) solution. Fluorescence was detected and imported images using a confocal microscope (Olympus-Fluoview). After that, identical bright field images were superposed on the fluorescent images using Adobe Photoshop (Adobe Systems) to clarify the morphological features of the photoreceptors.

### 2.4. Isolation of a newt cDNA encoding Cp-SWS2

A cDNA fragment encoding a putative opsin of the Japanese com-

mon newt was amplified from a retinal cDNA pool with degenerate oligonucleotides (SWS-F1: 5'-ACAGAATTCTGYGGNCCNGAYTGGTAY-3' and VVP-R2': 5'-CGAAGCTTAYRTANAYNAYNGGRTTATA-3'; Y=C+T, R=A+G, N=A+C+G+T; corresponding to the amino acid sequences CGPDWYT and YNPV(I/I)V(I/I)Y, respectively) [19,20] as primers. The amplified cDNA fragments were used as a probe to screen a newt retinal cDNA library. The construction and the high stringency screening of the newt retinal cDNA library was carried out as described by Hisatomi et al. [21,22]. Positive clones were picked up and transformed into the plasmid by an Ex-assist-Solr system (Stratagene), and sequenced in both directions according to the cycle sequencing method (Hitachi SQ-5500, Pharmacia).

The putative opsin sequence was named Cp-SWS2. The deduced amino acid sequence of Cp-SWS2 was aligned with those of other vertebrate and invertebrate opsins. Amino acid identities were calculated for 290 amino acids from P32 to Q321 of Cp-SWS2 (corresponding to P23 and Q312 of bovine rhodopsin, respectively) as described by Hisatomi et al. [7].

### 2.5. In situ hybridization

The Cp-SWS2 cDNA fragment was cloned into a pGEM-3Zf(+) plasmid vector (Promega), and linearized with *Eco*RI restriction en-

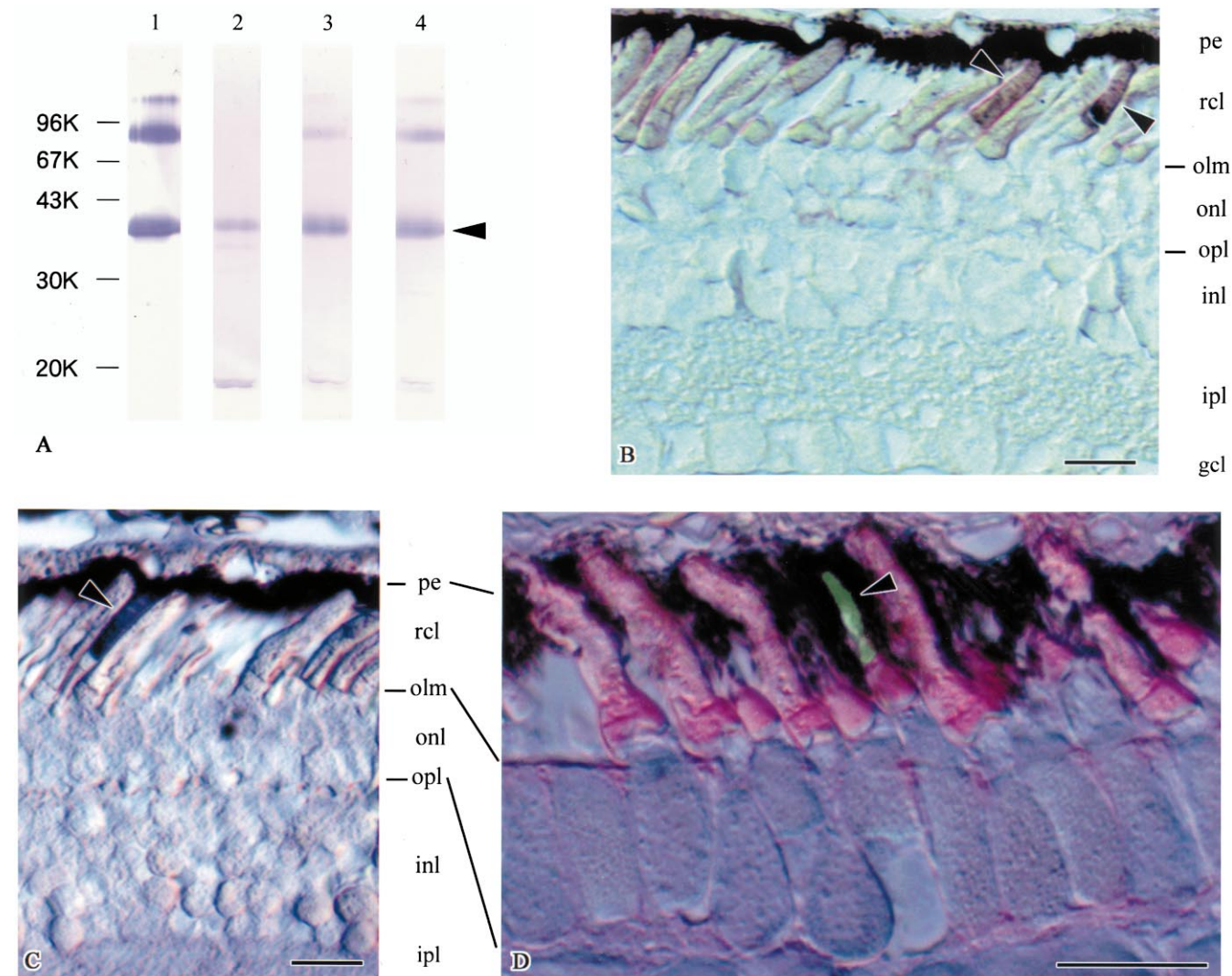


Fig. 1. A: Western blot analysis of amphibian retinal homogenates of bullfrog (lane 1), Japanese common newt (lane 2), barred tiger salamander (lane 3) and black salamander (lane 4) with anti-Rc-MS antiserum. Anti-Rc-MS antisera recognized major bands of about 37 kDa in all retinal homogenates (arrowhead). Immunoreactivity of anti-Rc-MS antiserum in paraffin sections of (B) barred tiger salamander, (C) black salamander and (D) Japanese common newt retinas. Arrowheads indicate positive signals. Abbreviations: pe, pigment epithelium; rcl, receptor cell layer; olm, outer limiting membrane; onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer; scale bar = 20  $\mu$ m.

zyme. Antisense cRNA riboprobes (675 bases in length) were synthesized by run-off transcription from a T3 promoter with digoxigenin-UTP, as recommended in the manufacturer's protocol (Boehringer Mannheim). Preparation of the retinal cryosections and methods for in situ hybridization were described by Imanishi et al. [23]. Retinal cryosections of 15  $\mu$ m were hybridized with 1  $\mu$ g/ml (final concentration) cRNA probes, and the hybridization signal was visualized using the HNPP set (Boehringer Mannheim). Fluorescence was detected using a confocal microscope (Olympus-Fluoview).

### 3. Results

#### 3.1. Western blot analysis

We have prepared an antiserum, anti-Rc-MS, raised against the fusion protein including the C-terminal portion of bullfrog green rod opsin (Rc-MS) and reported that the anti-Rc-MS antiserum selectively recognizes green rods in the bullfrog retina [7]. To clarify the distribution of green rods in caudata retinas, we carried out Western blot analysis of retinal homogenates.

The anti-Rc-MS antiserum recognized a major band of about 37 kDa and a minor band of about 80 kDa, in the retinal homogenate of bullfrog (Fig. 1A, lane 1). The species shown with higher molecular weight bands, may be oligomers of opsins. Fig. 1A, lanes 2–4 indicate that the anti-Rc-MS antiserum also recognized almost the same molecular weight bands in the three other amphibian retinal homogenates. These results suggested that opsins similar to Rc-MS are expressed in these caudata retinas.

#### 3.2. Immunohistochemistry

In paraffin sections of the barred tiger salamander and black salamander retinas, the anti-Rc-MS antisera recognized the cylindrical outer segment of a very small fraction (less than 1%) of the photoreceptors (Fig. 1B,C). These cells could

be identified as green rods by their morphological features. These results were in good agreement with published tiger salamander data [3,4] and also suggested that most caudata possess green rods including a species that does not belong to the superfamily Salamandroideae, the black salamander. However, the antisera recognized only the outer segments of the single cones in the newt retinal sections (Fig. 1D). We did not observe any stained cells which had the morphological features of green rods. These results suggested that an opsin similar to Rc-MS is expressed only in cones in the newt retina.

#### 3.3. The deduced amino acid sequence of Cp-SWS2

To identify the newt opsin which reacted with the anti-Rc-MS antiserum, we tried to isolate the opsin expressed in the newt cones. The cDNA fragment encoding the newt opsin was amplified using degenerate oligonucleotides (SWS-F1 and VVP-R2') corresponding to amino acid sequences highly conserved in short wavelength-sensitive vertebrate opsins belonging to what we call the SWS1 and SWS2 opsin groups. The amplified fragment showed high amino acid identities to other SWS2 opsins. Therefore, we named this cDNA fragment Cp-SWS2 (*C. pyrrhogaster*-SWS2 group) cDNA. The complete coding region of Cp-SWS2 cDNA was isolated by screening a newt retinal cDNA library. The Cp-SWS2 cDNA consists of 1694 bases, which encodes 363 amino acids with a molecular mass of about 40 kDa (Fig. 2A). Its deduced amino acid sequence includes typical opsin features, such as the retinal binding site (K291) [24], the counterion of the protonated Schiff base (E108) [25], the sites for a disulfide bond (C105 and C182) [26], an N-glycosylation site (N12), and possible phosphorylation sites near the C-terminal [27]. Therefore, Cp-SWS2 cDNA seems to encode a functional opsin.

Cp-SWS2 has high identity with the amino acid sequences of other SWS2 opsins, such as tiger salamander (80.1%) [15],

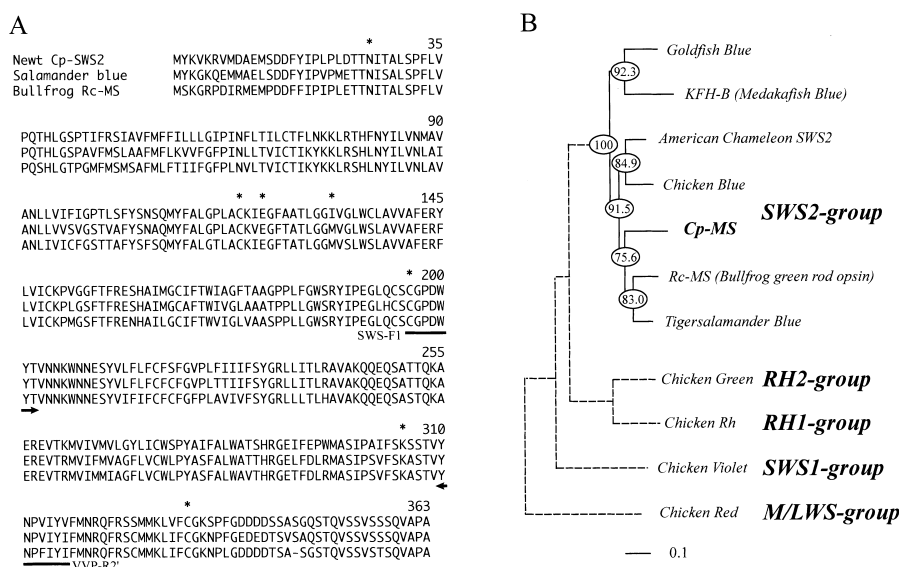


Fig. 2. A: Alignment of the deduced amino acid sequence of amphibian SWS2 opsins. Arrows indicated the position of the degenerate primers, SWS-F1 and VVP-R2' respectively. Asterisks represent the several important amino acids for opsins (N26, C119, E122, I131, C196, K305 and C332, see text). The nucleotide sequence of Cp-SWS2 has been submitted to the EMBL nucleotide sequence database with accession number AB040148. B: An unrooted molecular phylogenetic tree of SWS2 group opsins generated by the neighbor-joining method [41]. The branches except SWS2 group are shown in broken lines. The references for the sequences (accession numbers) are chicken red (P22329), blue (P28682), green (P28683), violet (P28684), rhodopsin (P22328), tiger salamander blue (AAC96069), American chameleon (*Anolis carolinensis*) SWS2 (AAD42779), Medaka fish KFH-B (AB001602), goldfish blue (P32310), bullfrog green rod opsin (Rc-MS, AB010085) respectively. The scale bar is calibrated in substitution per site.



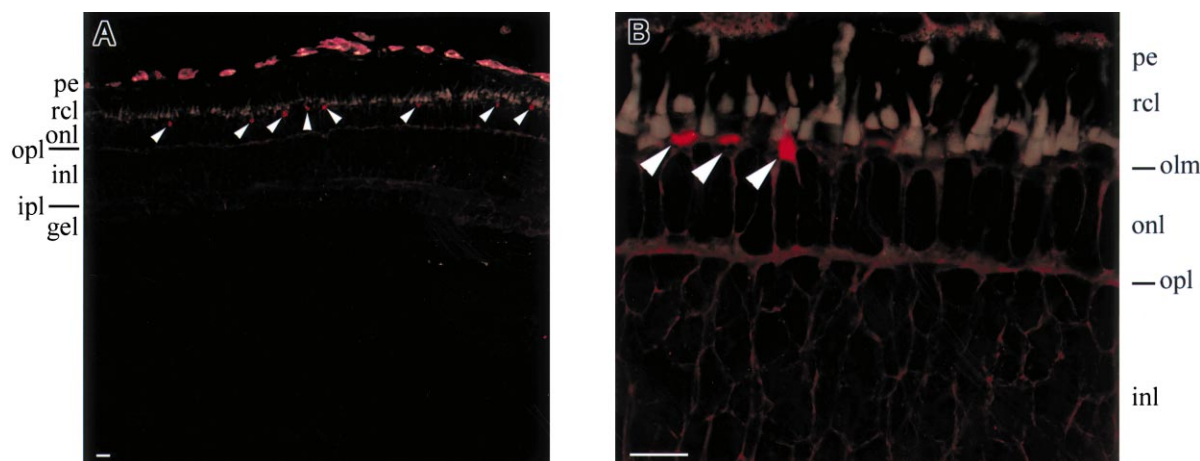


Fig. 3. Localization of Cp-SWS2 mRNA in the newt retinal sections. (A) Low and (B) high magnification using antisense Cp-SWS2 cRNA as a probe. Arrowheads indicate the positive hybridization signals. Abbreviations as in Fig. 1. Scale bar = 20  $\mu$ m.

chicken (76.9%) [28], goldfish (70.4%) [29], frog Rc-MS (75.2%) [7] and American chameleon SWS2 (75.9%) [30] opsins, but had less identity (less than 60%) with opsins in the other opsin groups (the M/LWS, SWS1, RH1 and RH2 groups; about the terminology of opsin groups, please refer to the review [31]). The phylogenetic tree clearly indicates that Cp-SWS2 belongs to the SWS2 group (Fig. 2B).

### 3.4. *In situ* localization of Cp-SWS2 mRNA

The localization of Cp-SWS2 mRNA was investigated by *in situ* hybridization. The digoxigenin-conjugated Cp-SWS2 cRNA probe recognized the outer nuclear layer (or the cell bodies and myoid regions) of single cones in radial sections (Fig. 3A,B). Fluorescence was not observed with the sense probe (negative control, data not shown). In bullfrog retinas, the SWS2 opsin (Rc-MS) was expressed only in green rods [7]. In newt retinas, however, the SWS2 opsin (Cp-SWS2) was expressed only in cones, and furthermore, we have not observed any green rods. Approximately 8% of all newt photoreceptors had positive fluorescence signals. The morphology

of the positive cells shows a certain correspondence to anti-Rc-MS immunopositive cells. Moreover, the amino acid sequence of the C-terminal region of Rc-MS is 75.5% identical with that of Cp-SWS2 and less than 52.0% identical with those of other newt opsins [32]. Therefore, we conclude that anti-Rc-MS antiserum recognized Cp-SWS2. We suggest that the other single cones contain the other opsins, belonging to the M/LWS, SWS1 or RH2 groups [32].

## 4. Discussion

In this paper, we have determined the type of photoreceptors expressing SWS2 opsins in several caudata retinas. Generally the amino acids which relate to spectral tuning of opsins were highly conserved in the same group [33], so we expect that opsins belonging to the same group show similar absorption maxima, for example, SWS2 opsins show absorption maxima about at 430–450 nm. However some specific amino acids which relate to spectral tuning, Phe270 and Ser301 are not conserved in Cp-SWS2; there they are Tyr270 and Ala301

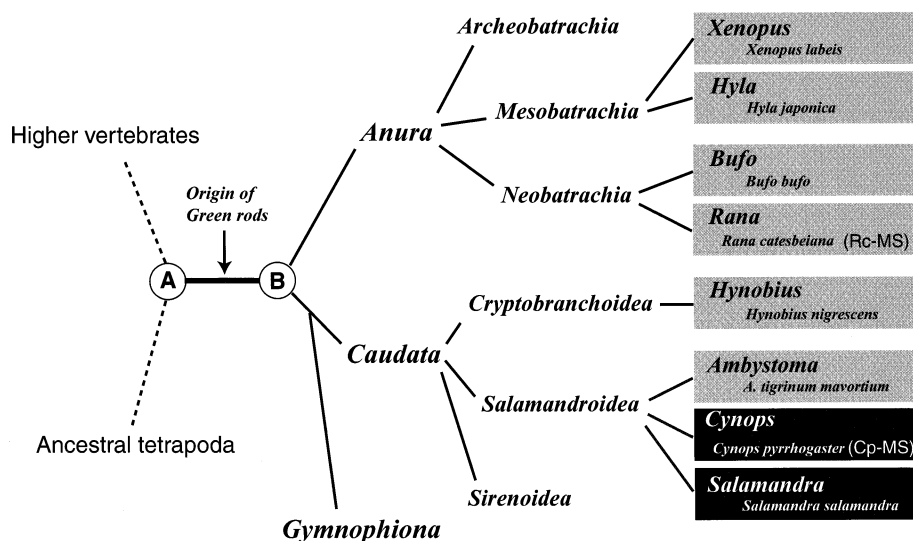


Fig. 4. The phylogenetic relationships of the subtypes of vertebrate blue-sensitive (SWS2 or MS group opsin expressing) photoreceptors in amphibian retina. The lengths of the lines have no special meaning. Species having green rods (cones) which probably express SWS2 opsins are indicated by gray (black) rectangles.

respectively. These two amino acids correspond to Tyr261 and Ala292 of bovine rhodopsin, and it has been reported that each of the site-directed mutants of bovine rhodopsin, F261Y and S292A, shows a red shift of absorption maximum (approximately 10 nm) in comparison to the wild-type [34–37]. We expected that Cp-MS opsin may have an absorption maximum red-shifted compared to the Rc-MS opsin.

Comparing amphibian SWS2 opsins, the tiger salamander (caudata) blue opsin makes a cluster with the green rod opsin of the bullfrog (anura), but not with the Cp-SWS2 opsin of the newt (caudata) (Fig. 2B). Even if we altered the comparative region and species of opsins, the topology of the tree is not changed. SWS2 opsins of the tiger salamander and bullfrog are expressed in rods but Cp-SWS2 is expressed in cones. It may be that there are some motifs in the amino acid sequences of SWS2 opsins that cause there to be expressed in rod cells or some increase of the amino acid substitution rate similar to those found in a diurnal gecko opsins during the reverse transmutation [38].

Fig. 4 shows the phylogenetic relationships and the morphological features of amphibian photoreceptor cells expressing SWS2 opsins. There are three points to consider in a discussion of the origin of green rods. (1) From the phylogenetic analysis of mitochondrial DNA sequences, it has been suggested that ancestral anura and caudata diverged from ancestral amphibia, and gymnophiona diverged from caudata [39]. (2) There are blue-sensitive (SWS2 opsin containing) cones in fish, reptile and bird retinas, so we expect that the ancestral amphibia also possessed SWS2 cones in their retina (node A). (3) Our results suggest on the molecular level that green rods of caudata express SWS2 opsins as well as in anura. It is unlikely that several different caudata have independently evolved green rods after the divergence between anura and caudata. Therefore, we speculate that green rods were obtained before the caudata–anura divergence, that is between A and B nodes.

Also, our results suggest that SWS2 opsins are expressed in two kinds of morphologically different photoreceptor subtypes (green rods and cones) in Salamandroideae. According to the transmutation theory which was proposed by Walls [40], all photoreceptors gradually transmuted to morphological rods or cones in nocturnal or diurnal reptilian retinas respectively. Blue-sensitive photoreceptors in amphibian retinas have been transmuted to green rods. After that, the subspecies of Salamandroideae such as the Japanese common newt would be reverse-transmuted from green rods back to cones. However, only blue-sensitive photoreceptors change their morphology in both cases. There may be a switching mechanism of photoreceptor development or opsin expression between green rods and cones.

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