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Adaptation of visual spectra and opsin genes in seabreams

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ABSTRACT

Three species of seabreams, *Acanthopagrus berda, Acanthopagrus schlegelii* and *Pagrus major*, living at different depths, were chosen to investigate how visual spectra and opsin genes evolve in response to various photic environments. The λmax of photoreceptors and opsin genes were measured and cloned from these species. Eight to twelve nm spectral shifts in the rod and blue cone cells were observed between the deep-sea, *P. major*, and shallow-sea species, *A. berda* and *A. schlegelii.* Furthermore, the deep-sea *P. major* has lost its red light vision. Six opsin genes, Rh1, Rh2A, Rh2B, SWS1, SWS2 and LWS, were identified from all three seabream species, with the LWS genes of *P. major* having undergone pseudogenization. These data indicate that the photic environment of habitats select for the physiology of visual spectra and coding of opsin genes.

1. Introduction

The photic conditions of aquatic environments vary greatly with many aspects of physical conditions, such as turbidity, colour and brightness. In clear oceanic water, light penetrates to the depths of 300–500 m with short wavelength light, i.e., blue light (470 nm). In contrast, penetration of light in coastal waters is only 30–50 m, with the maximum transmission in the region of 530–570 nm, i.e., green light. Photic conditions are also often correlated with the visual ability of fishes (Bowmaker, 1995). For example, maximal spectral sensitivity (λmax) of rod visual pigments of deep-sea fishes is around 480 nm, which matches the wavelength of deep penetrating blue light (Hunt, Dulai, Partridge, Cottrill, & Bowmaker, 2001; Partridge, Arche, & Lythgoe, 1988). To the contrary, fishes that live in shallow seas (e.g., black seabream *Acanthopagrus butcheri*) have blue cones that sense blue light with longer wavelengths (Shand et al., 2008) to match prevailing wavelengths predominate in shallow coastal waters. Additional examples of visual adaptation can be observed in some deep-sea Antarctic species, such as notothenioid fishes, which have lost their red-sensitive photoreceptors and narrowed their sensitivity range of green-sensitive photoreceptors to adapt to extremely dim environments (Pointer et al., 2005).

Opsins are visual pigments evolved for light detection. Opsin pigments comprise protein and chromophores (either 11-*cis*-retinal or 11-*cis*-3-dehydroretinal) derived from vitamin A. Each chromophore is covalently bonded to a protonated Schiff's base. Opsins are tuned for λmax by the electrostatic interaction between the chromophore and specific amino acids along binding pocket formed by seven transmembrane regions. In vertebrates, the λmax of opsins ranges from approximately 350 nm (ultraviolet) to 630 nm (red light spectrum) (Bowmaker, 2008; Yokoyama, 1994, 1997, 2000, 2002). There are five different types of opsins in vertebrates (Yokoyama, 1994, 1995, 1997). One of these opsins is known as rod opsin (Rh1) and is expressed exclusively in rod cells. Rh1 is responsible for vision in dim light and has a λmax ranging from well below 490 to approximately 510 nm (Yokoyama, 1997). The other four opsins are expressed in cone cells and their functions are in color/daylight vision in many animals. One of these opsins is known as the short-wavelength sensitive opsin 1 (SWS1). Depending on the species, SWS1 is sensitive to either UV or violet light ranging from 355 to beyond 450 nm. Another short-wavelength sensitive opsin (SWS2) has a λmax in the blue region raning from 415 to 480 nm. A third group of cone opsins is known as the rhodopsin-like opsins (Rh2), which have λmax values ranging from 470 to 530 nm. Finally, opsins that detect red light, from 495 to 570 nm, are known as long-wavelength sensitive opsins (LWS) (Bowmaker, 2008; Yokoyama, 2000).

Depth also shapes photic environments due to decreased availability of long-wavelength light, resulting in a blue-shifted light spectrum with increased depth (Denton, 1990). The effects of such changes in the photic environment on piscine opsins have previously been investigated in teleosts such as the coelacanth (Yokoyama & Tada, 2000; Yokoyama, Zhang, Radlwimmer, & Blow, 1999). These studies revealed blue-shifted Rh1 and Rh2 opsins, as well as

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pseudogenization of the UV opsin are found, in this deep-water dwelling species. Fishes in the suborder Cottoide, inhabiting in the depths of Lake Baikal, also possess blue-shifted Rh1 and SWS2 opsins (Bowmaker et al., 1994; Cowing, Poopalasundaram, Wilkie, Bowmaker, & Hunt, 2002; Hunt, Fitzgibbon, Slobodyyanyuk, & Bowmaker, 1996). Although the spectral shifts in response to depth are well known, few studies have explicitly examined this effects on the evolution of all five opsins by using closely related species (Bowmaker et al., 1994; Cowing, Poopalasundaram, Wilkie, Bowmaker, & Hunt, 2002; Hope, Partridge, Dulai, & Hunt, 1997; Hunt et al., 1996, 2001; Yokoyama & Tada, 2000; Yokoyama et al., 1999).

Seabreams (family Sparidae) are primarily marine, except for few species that have adapted to fresh- and brackish water habitats (Nelson, 1994). They are demersal inhabitants of the continental shelf and slope whose habitats are rather diverse, ranging from estuaries to coasts, shallow to deep sea, rocky reefs to muddy and sandy bottoms. Sparidae is one of the few teleost families with closely related species specialized for living at different depths in the ocean (Orrella & Carpenter, 2004). Niche segregation by depth makes sparids a suitable group to study how opsin genes have evolved in response to photic conditions at different depths. To date, opsin genes and spectral sensitivity of Sparidae have been characterized for only one species, the southern black beam (*A. butcheri*) (Shand, Hart, Thomas, & Partridge, 2002; Shand et al., 2008).

Here, seabreams that live at different depths are used as a model system to test the hypothesis that visual spectral sensitivities and the genes that code for opsin function are depth-dependent. Two seabreams in the genus *Acanthopagrus* were chosen as representatives of shallow-water species. *Acanthopagrus berda* and *Acanthopagrus schlegelii* both live in the estuaries and coastal waters of average depths less than 50 m. One deep-water dwelling seabream, *Pagrus major*, was selected for comparison. Habitats of *P. major* are rough substrates and reefs that range in depth down to 200 m (Frimodt, 1995). To test the predictions of our hypothesis, we cloned and sequenced opsin genes of these species and compared the λmax of their photoreceptors using microspectrophotometry (MSP).

2. Materials and methods

2.1. Samples collection

Three to five specimens of *A. schlegelii, A. berda,* and *P. major* were collected from I-Lan county and Keelung city located at the northeast area of Taiwan. Fishes were shipped to a holding facility in the Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, Taiwan for microspectrophotometry study. Individuals used for opsin cloning were obtained from local markets in Taipei, Taiwan.

2.2. Microspectrophotometry

Fishes were kept in complete darkness for at least 2 h before their eyes were enucleated under dim red light. The retinas were removed with the aid of night vision goggles and were immediately immersed in chilled phosphate buffered saline containing 6% sucrose (Sigma, USA). A small piece of each retina was transferred to a cover glass, cut into pieces, and squashed with a small glass cover slip that was sealed along the edges with silicone grease. This preparation was placed in a holder and transferred to the stage of a single-beam, computer-controlled microspectrophotometer (Loew, 1994). The outer-segment of individual photoreceptor cells was selected for measurement under infrared illumination, using an image converter. Baseline and sample spectra were obtained at 100 nm s^{-1} from 750 to 350 nm and back from 350 to 750 nm, with a step size of 1 nm (Loew, 1994). To verify that a photoreceptor being measured was functional, immediately after the MSP measurement, the same photoreceptor was exposed to white light for 120 s and scanned again for evidence of photobleaching. A positive photobleaching response indicated the MSP data obtained from the cell was valid. The selected absorbance curves and the λmax of the photoreceptors were obtained by a programmed statistical method described in Loew (1994). The λmax of the normalized visual pigment absorbance spectrum was acquired using the method of Mansfield, as presented by MacNichol (1986). The standard A1/A2 templates for visual pigment absorbance curves followed those of Lipetz and Cronin (1988). Estimates of best fit were made by selecting the template with the lowest standard deviation (SD). If the SD of λmax was smaller than 7.5 nm, then the spectrum was considered valid and stored by computer (Sillman, Carver, & Loew, 1999; Sillman, Johnson, & Loew, 2001). This process was repeated for each photoreceptor examined by the MSP. Final estimates of mean λmax ± SD were determined by averaging the λmax values of each photoreceptor measured.

2.3. Opsin genes cloning and sequencing

Genomic DNA was extracted from 2 ml of fresh fish blood using a Puregene genomic DNA isolation kit (Gentra Systems, Minneapolis, MN), following the manufacturer's instructions. Primers were designed from the alignment of various teleost species. In the case of rod opsins (*Rh1*), primers were designed from the known sequences of *Sparus aurata* (acc. no. EF439456). Primers for cone opsins were designed based on the published cone opsins of cichlids (Carleton, Harosi, & Kocher, 2000; Carleton & Kocher, 2001; Spady et al., 2005). A complete list of primers is available in Supplementary Table 1.

PCR products were obtained using an Ampliqon III PCR 2X polymerase kit (Ampliqon III, Bie & Bernsen A–S, Roedovre, Denmark) in 50 ml reactions by following the manufacturer's recommended reaction concentrations and using 2 mg of genomic DNA as the template. Each thermocycling reaction was run for 35 cycles at 95 °C for 60 s, 56 °C for 30 s, and 72 °C for 2.5 min using a MJ Research PTC 200 Peltier Thermal Cycler (MJ Research, Waltham, MA). In cases with multiple copies, such as the case of *Rh2*, PCR products were removed from the gel and cleaned using a Clean/Gel Extraction Kit (BioKit, Taipei, Taiwan) and then cloned using a yT&A Cloning kit (Yeastern Biotech, Taipei, Taiwan). Ten white colonies were selected and an additional round of PCR was performed for 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

In addition to the PCR reactions, primers were also used for sequencing the amplified products. The sequencing was performed with a Sequenase PCR Product Sequencing Kit (United States Biochemical Corp., Cleveland, OH). Commercial sequence kits (BigDye™ Terminator Cycle Sequencing Ready Reaction Kits of Applied Biosystems, Foster City, CA) and ABI model 377 automated DNA sequencers were used to obtain sequence data. The resulting sequences encompass only the exons that include the known spectral tuning sites.

2.4. Sequence analysis

The chromatograms were constructed into contigs using DNAStar software (DNAStar, Inc., Madison, WI). Exons and open reading frames were extracted by alignment with known opsins from other teleosts, such as cichlids (Carleton, Spady, & Cote, 2005; Spady et al., 2005). Sequences were aligned using the ClustalW function

in the MEGA 3.1 software (Kumar, Tamura, & Nei, 2004). The best-fit model of nucleotide evolution was determined by hierarchical likelihood ratio tests (LRT) using Model Test v3.7 (Posada & Crandall, 1998). Phylogenetic analysis of each opsin was performed using the best fit model. All phylogenies were constructed using the Neighbor-Joining method with 1000 bootstrap replicates.

3. Results

3.1. MSP measurements

The MSP results revealed three types of photoreceptor cells present in all species: rod, single cone, and double cone cells. Single and double cone cells were further classified into three classes of spectral sensitivities based on λmax, namely, blue, green and red cone (Fig. 1). Double cones were green/green and red/red doubles. There were no green/red doubles observed in this study. The absorbance spectra of the rod and cone cells are shown in Fig. 2.

Four classes of photoreceptor cells were found in *A. schlegelii*: rods and blue, green, and red cones with λmax values 499 ± 4.8, 471 ± 5.7, 528 ± 10 and 563 ± 8.4 nm, respectively (Table 1). In *A. berda*, the wavelengths of light absorbed by rods, blue and red cones were 501 ± 7.3, 472 ± 4.2 and 566 ± 8.1 nm, respectively (Table 1). Template fitting by MSP revealed chromophore usage of these two shallow-water species is A1 dominantly for rods and blue cones, and half A1 and half A2 for green and red cones. There were three classes of photoreceptor cells identified in the deep-sea species, *P. major*; with rods, blue and green cones with λmax values 491 ± 5.4, 460 ± 6.1 and 525 ± 4.7 nm, respectively (Table 1). The dominant chromophore of photoreceptor cells in *P. major* is A1.

Three significant differences in visual spectra were observed among the study species. First, the λmax of rod cells in *P. major*, the deep-sea species, showed a blue-shift of 8–10 nm compared to that of shallow-sea seabreams, *A. schlegelii* and *A. berda*. Second, blue cones showed a 10–12 nm spectral-shift between shallow- and deep-sea seabreams. Finally, red cones were absent from

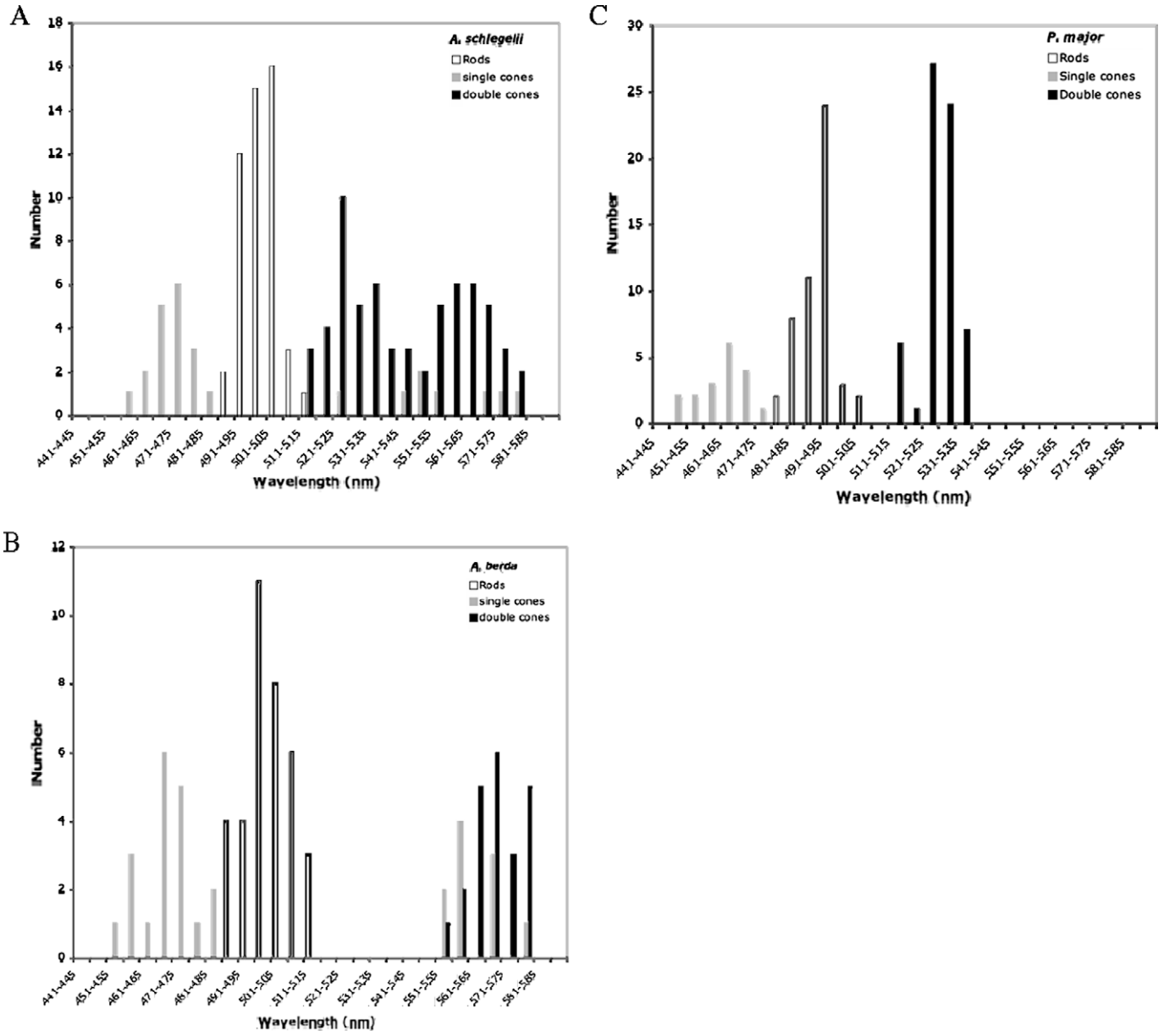

Fig. 1. Distribution histograms of λmax of individual photoreceptor cells of seabreams species used in this study. The empty, grey, and black bars indicate rod, single and double cone cells, respectively (A) *A. berda*, (B) *A. schlegelii*, (C) *P. major*.

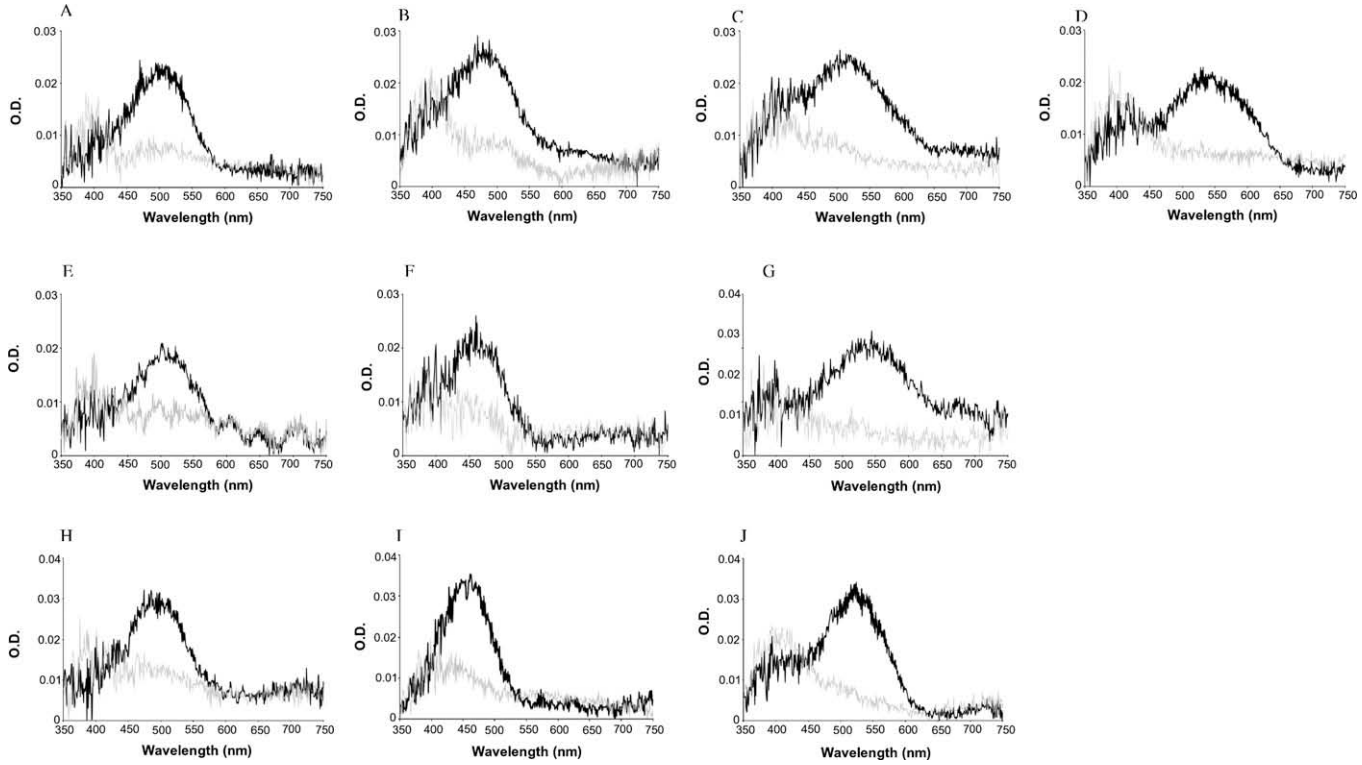

Fig. 2. Examples of absorbance spectra of rod and cone cells in seabreams. (A) Rod, (B) blue cone, (C) green cone, (D) red cone cells of *A. schlegelii*; (E) rod, (F) blue cone, (G) red cone cells of *A. berda*; (H) rod, (I) blue cone, (J) green cone cells of *P. major*. Black lines: before bleach; grey lines: after light-bleach.

Table 1
The λmax for rod and cone cells from seabreams measured by the MSP. All values are expressed in nanometers (nm) and where appropriate include nm ± SD. Numbers in parentheses indicate the number of the photoreceptor cells measured.

Species	Rods Rh1	Blue cones SWS2	Green cones Rh2A	Red cones LWS
A. schlegelii N = 3*	499 ± 4.8 (49)	471 ± 5.7 (17)	528 ± 10 (35)	563 ± 8.4 (35)
A. berda N = 5*	501 ± 7.3 (36)	472 ± 4.2 (18)	NA	566 ± 8.1 (33)
P. major N = 3*	491 ± 5.4 (50)	460 ± 6.1 (18)	525 ± 4.7 (65)	NA

NA indicates the λmax was not available.
* *N* indicates the number of the specimens examined.

P. major and green-light sensitive cone cells were absent in *A. berda*. The loss of red-light vision in *P. major* is consistent with the results of opsin gene cloning (see details below).

3.2. Opsin gene sequences of seabreams and amino acid substitutions at tuning sites

Partial opsin genes, from exon 2 to exon 5, were amplified and cloned from genomic DNA. All three seabreams possessed six opsin genes: Rh1, SWS1, SWS2, Rh2A, Rh2B and LWS. The coding region of the opsin genes was translated for alignment. All genes except the LWS gene of *P. major* have complete open reading frames. A pseudogene of LWS was discovered in *P. major* with a frameshift mutation. The opsin gene sequences of *A. butcheri*, a shallow-sea seabream, were downloaded from NCBI database for comparison.

In the Rh1 gene, there are seven amino acid sites important for spectral tuning: 83, 122, 211, 261, 265, 292 and 295 (Yokoyama, 2000). Except for site 83, there is homology among the other sites within seabreams (Table 2). In the shallow-sea species, asparagine is found at site 83, but in deep-sea species aspartate is found instead. In *P. major*, there is a substitution of D83 N (change from aspartate to asparagine), relative to the consensus sequence (Table 2). Earlier studies based on site-directed mutagenesis suggest that a substitution of D83 N could lead to the shift of λmax of Rh1 2–10 nm toward the blue spectrum (Archer, Hope, & Partridge, 1995; Fasick & Robinson, 1998; Nagata, Oura, Terakita, Kandori, & Shichida, 2002; Nathans, 1990). This observation is consistent with our MSP data. In the SWS1 gene, six critical amino acid sites are highly correlated with the λmax: 46, 52, 86, 97, 114 and 118 (Hunt, Wilkie, Bowmaker, & Poopalasundaram, 2001; Takahashi & Yokoyama, 2005). Among these sites, amino acid 86 is critical for the perception of UV- or violet-sensitivity in fishes. All teleosts with UV-vision have Phenyalanine (Phe) at this site (Hunt et al., 2004; Yokoyama & Shi, 2000). This suggests that seabream may have UV-vision because amino acid 86 appears to be conserved in all species tested.

Seabreams tested in this study have only one copy of the SWS2 gene instead of two copies that are observed in shallow-water *A. butcheri* (NCBI database). Phylogenetic analysis suggests that a single copy of SWS2 is in the same cluster with SWS2A of *A. butcheri* (Fig. 2). Amino acids 116, 117,118 and 295 of the SWS2 gene are involved in the spectral tuning of SWS2 gene (Chinen, Matsumoto, & Kawamura, 2005b; Yokoyama & Tada, 2003; Yokoyama, Takenaka, & Blow, 2007). All SWS2 genes of seabreams shared

Table 2
Comparisons of opsin sequences of seabream.

		Gene																															
		Rh1								SWS1								SWS2						Rh2						LWS					
		A.A. Sites								A.A. Sites								A.A. Sites						A.A. Sites						A.A. Sites					
		MSP λmax	83	122	207	211	265	292	295	MSP λmax	46	52	86	97	114	118	MSP λmax	Loci	116	118	265	292	MSP λmax	Loci	97	122	207	292	MSP λmax	164	181	261	269	292
	Consensus		D	E	M	H	W	P	A		F	A	F	Q	A	S			T	T	W	S			S	E	M	A		S	S	Y	Y	C
	A. butcheri	508*	.	.	.	.	.	.	.	NA	.	.	.	.	.	.	475*	SWS2A	.	.	.	.	527*	Rh2Aα	.	.	.	.	570*	.	.	.	.	.
																	425*	SWS2B	M	.	.	.	534*	Rh2Aβ	.	.	.	.						
Shallow sea	*A. schlegelii*	499	.	.	.	.	.	.	.	NA	.	.	.	.	.	.	471	SWS2	.	.	.	.	528	Rh2A	.	.	.	.	563	.	.	.	.	.
																								Rh2B	T	Q	.	.						
	A. berda	501	.	.	.	.	.	.	.	NA	.	.	.	.	.	.	472	SWS2	.	.	.	.	NA	Rh2A	.	.	.	.	566	.	.	.	.	.
																								Rh2B	T	Q	.	.						
Deep sea	*P. major*	491	N	.	.	.	.	.	.	NA	S	T	.	.	S	.	460	SWS2	.	.	.	.	525	Rh2A	.	.	.	.	NA	A	.	.	–	–
																								Rh2B	T	Q	.	.						

Sequences are compared to the consensus sequence with similar identity indicated by a dot. Dashed lines indicate deleted genes due to pseudogenization. NA indicates the MSP data were not available. Sites are numbered according to bovine rhodopsin. λmax from MSP (in nm) are listed for those genes that are expressed in sparids. The white and light grey rows indicate the sparid species that inhabit shallow and deep sea, respectively.
* indicates the opsin genes of *A. butcheri* expressed and measured by Shand et al. (2002, 2008).

the same elements of amino acids at the spectral tuning sites, except for SWS2B in *A. butcheri* (Table 2).

All seabreams have two copies of the *Rh2* opsin gene (Table 2). *A. butcheri* possesses two copies of Rh2A (Rh2Aα and Rh2Aβ), whereas species tested in this study had one copy of Rh2A and one copy of Rh2B. Amino acid substitutions at sites 97, 122, 207 and 292 could result in the λmax of the Rh2 gene (Takenaka & Yokoyama, 2007). In Rh2A and Rh2B genes, no differences were found among the tuning sites in seabream (Table 2).

In LWS, there are five critical amino acids sites, 164, 181, 261, 269 and 292, important for spectral tuning (known as the "Five Site Rule"; see Yokoyama & Radlwimmer, 1998). Sequences comparisons of LWS among shallow-sea species show that these sites are quite conserved (Table 2). A frameshift mutation was detected in the LWS opsin of the deep-sea species, *P. major*, which is caused by the insertion of two nucleotides (TG) at position 501 resulting in a premature stop codon at position 171. In the other two seabreams, a valine is coded at position 171. Without the TG insertion in LWS sequences, *P. major* would otherwise have a functional LWS protein after translation. This functional LWS would carry an S164A substitution (Table 2), which may result in a 7 nm shift toward the blue spectrum (Asenjo, Rim, & Oprian, 1994).

3.3. Opsin phylogeny

The opsin gene of *A. butheri* and the Rh2 and SWS2 genes of cichlid fishes were included in the phylogenetic analysis while those of goldfish and zebrafish were used as out-groups (Fig. 3). A neighbor-joining tree of opsin genes was constructed based on the best-fit model of nucleotide evolution. Except for Rh1, a similar pattern was found in the phylogeny of opsin genes as that of cytochrome-b (Orrella & Carpenter, 2004). Opsin genes in *Acanthopagrus* species clustered together to form a monophyletic group with one exception: Rh1 of *A. berda* clustered with *P. major* (Fig. 3D). SWS2 of the tested seabreams and SWS2A of *A. butcheri* form a monophyletic group closely related to the SWS2B group in cichlids. In contrast, SWS2A of *A. butcheri* clustered with SWS2B of cichlids (Fig. 3C). Rh2A genes of seabreams clustered together and formed a sister group of Rh2A in cichlids. The Rh2B genes of seabreams form a sister group to the Rh2A in cichlids (Fig. 3E). Opsin phylogeny of seabreams indicates that gene duplication and loss happened frequently during the evolutionary history of the Rh2 and SWS2 genes.

4. Discussion

4.1. The visual spectra of the seabreams

Spectral sensitivity of seabreams correlated with the photic conditions in which each species resides. The rod and blue cone cells of shallow-sea species, *A. berda* and *A. schlegelii*, absorb light with longer wavelengths, while those of the deep-sea *P. major* absorbs shorter wavelength light. The blue shifts of rod and blue cone cells, resulting from habitat depths, have also been reported for other species (Bowmaker et al., 1994; Hope et al., 1997; Hunt et al., 1996; Yokoyama & Tada, 2000; Yokoyama et al., 1999). Our observations in seabreams are consistent with earlier studies. Moreover, *P. major* has completely lost the cone cells used to sense red light. This loss of red cone cells has been reported in other deep-water species, such as coelacanth, cottoids, tuna, marlin and some coral reef fishes (Cowing, Poopalasundaram, Wilkie, Bowmaker, et al., 2002; Losey et al., 2003; Miyazaki, Kohbara, Takii, Ishibashi, & Kumai, 2008; Yokoyama & Tada, 2000; Yokoyama et al., 1999). These findings support our hypothesis that seabream adjust their visual spectra to match the photic environment present at different depths.

Shallow-sea seabreams, from estuaries and coastal waters, live in photic environments strongly influenced by turbidity. Comparisons among visual spectra of shallow-sea seabreams show that photoreceptors of *A. butcheri* absorb the longest wavelengths of light (except for red cones). *A. butcheri* lives in brackish waters of coastal rivers and lakes and occasionally enters freshwater habitats (Allen, Midgley, & Allen, 2002). The turbidity of these environments is high suggesting that turbidity could be an additional factor selecting for optimal visual spectra of shallow-sea seabream.

Green cones with λmax around 520 nm were found only in *A. schlegelii*, yet they were absent from another shallow-sea species, *A. berda*. This difference could result from ontogenetic changes in cone cells. The visual spectra of double cones ranged from 520 to 576 nm in juvenile *A. butcheri* (Shand et al., 2008), which is similar to the pattern observed in *A. schelegelii*. Moreover, gonads were not found in the specimens of *A. schlegelii* used for this study but were well developed in specimens of the other two seabream species. It is likely that the *A. schlegelii* chosen for this study were juveniles. Perhaps the visual spectra of *A. schlegelii* changes with maturation, resulting in the loss of green cones in adults. Further investigation is required to investigate the spectral tuning changes during the ontogeny of *A. schlegelii*.

4.2. Opsin genes of seabreams

The blue-shifted amino acid substitution D83 N in Rh1 occurs in deep-water cottoids in Lake Baikal but not in shallow-water cottoids (Hunt et al., 1996). Lake Baikal is extremely clear allowing light penetration to depths of up to 1000 m. Additional investigations using phylogenetically distant species revealed a D83 N substitution caused the blue shift of Rh1 in deep-sea fishes (Hope et al., 1997; Hunt et al., 2001) and the λmax of Rh1 toward blue in the European eel (Archer et al., 1995). Our seabream data are consistent with these observations. The same amino acid substitution was observed in *P. major* relative to that of their shallow-sea counterparts, *A. butcheri*, *A. berda* and *A. schlegelii*, which could cause a blue shift of 8–17 nm. The D83 N substitution in Rh1 appears to be a common mechanism among fishes of disparate taxa to adapt to the blue-shifted spectrum of deep-water environments.

All four seabreams in Table 2 have phenylalanine at site 86 indicating that these SWS1 opsins are UV sensitive (Cowing et al., 2002; Hunt et al., 2004; Yokoyama, 2000). However, UV vision is absent from these seabreams (Shand et al., 2002, 2008). Two possible mechanisms could lead to this phenomenon. First, the SWS1 gene could be expressed only at the larval or juvenile stages, since they feed at the surface. Many fishes detect food using UV spectra only at larval stages (Britt, Loew, & McFarland, 2001; Browman, Flamarique, & Hawryshyn, 1994; McFarland & Loew, 1994) but lose their UV vision as juveniles and adults (Allison et al., 2003; Couglin & Hawryshyn, 1994; Whitmore & Bowmaker, 1989). These findings raise the possibility that the tested seabreams could be UV-sensitive at larval or juvenile stages but lose UV-vision with growth. Another possibility is that SWS1 genes could be expressed in the pineal gland in addition to the retina. Expression of the SWS1 gene is detectable in the pineal gland of embryonic halibut (Forsell, Ekstrom, Flamarique, & Holmqvist, 2008; Forsell, Holmqvist, & Ekstrom, 2002).

In the case of SWS2 gene, we noticed that seabreams conserve most of the known tuning sites, except for SWS2B in *A. butcheri*. This observation is consistent with MSP data for the shallow-sea seabreams, but it is inconsistent with MSP data showing spectral shifts between deep- and shallow-sea species. Spectral shifts of at least 10 nm occur between deep- and shallow-sea species without any changes at the tuning sites. An alternative mechanism for this shift could be caused by the accumulation of substitutions located in regions distant to the binding pocket (Chinen, Matsumoto,

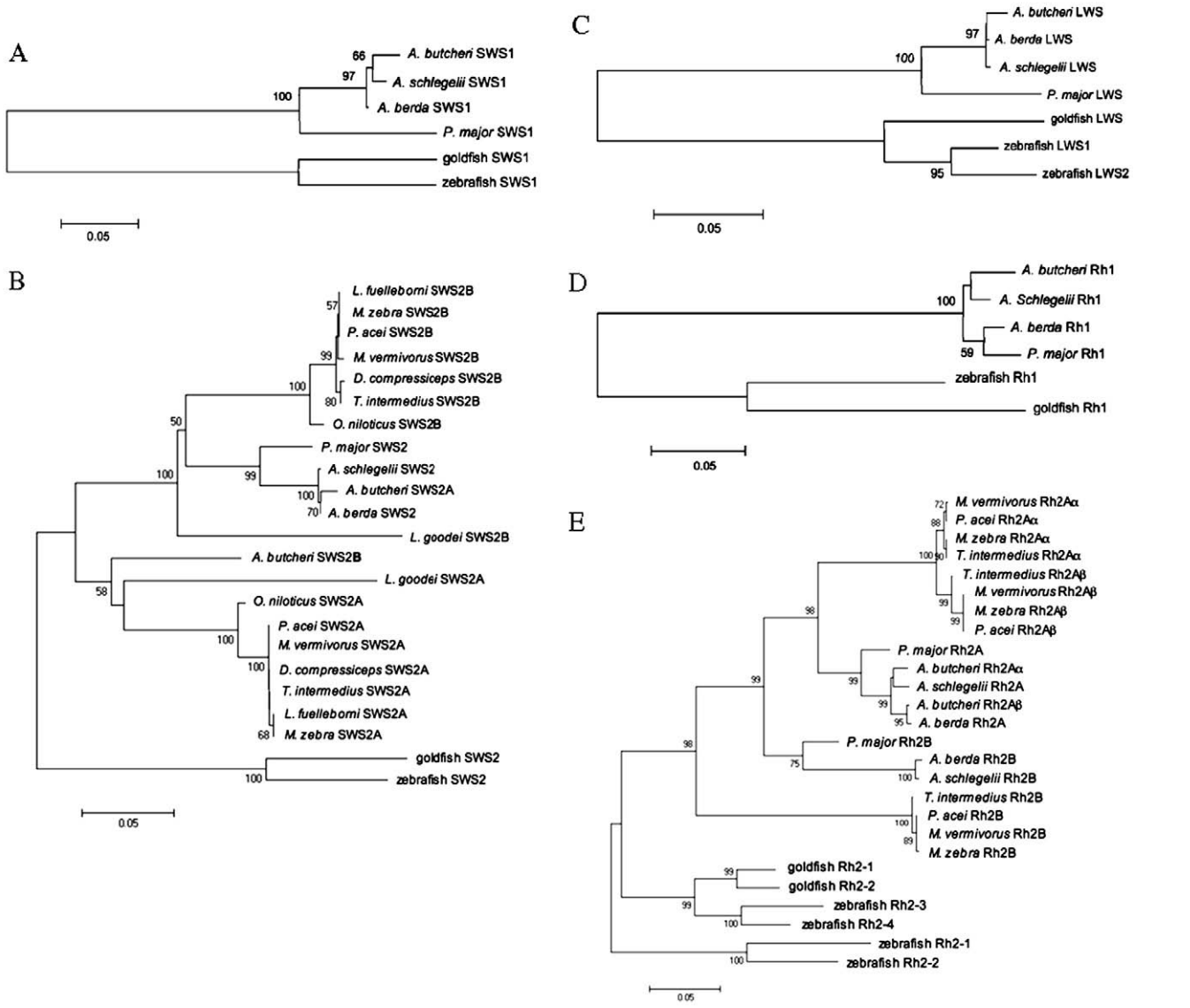

Fig. 3. Phylogenetic trees of the opsin genes in the seabreams. The neighbor-joining method was used in the construction of each species' phylogeny based on a designated model (see below) calculated from the Modeltest (Posada & Crandall, 1998). Bootstrap support from 1000 bootstrap replicates is shown for each node of the phylogenies. Corresponding genes of goldfish, *Carassius auratus*, and zebrafish, *Daino rerio*, were used as outgroups. (A), SWS1, HKY+G (Hasegawa, Kishino, & Yano, 1985). (B), SWS2, HKY+I+G (Hasegawa et al., 1985). (C), LWS, HKY+G. (D), Rh1, HKY+I+G. (E), Rh2, TrN+I+G (Tamura & Nei, 1993). The accession numbers of opsin genes used to construct the phylogenetic trees were list as following, goldfish: Rh1 L11863, Rh2-1 L11865, Rh2-2 L11866, SWS1 D85863, SWS2 L11864 and LWS L11867; zebrafish: Rh1 AB087811, Rh2-1 AB087805, Rh2-2 AB087806, Rh2-3 AB087807, Rh2-4 AB087808, SWS1 AB087810, SWS2 AB087809, LWS-1 AB087803 and LWS-2 AB087804; *A. butcheri*: Rh1 DQ354577, Rh2Aα EU090913, Rh2Aβ EU090914, SWS1 DQ354579, SWS2A DQ354580, SWS2B DQ354581 and LWS DQ354578; *Dimidiochromis compressiceps*: SWS2A AF247113 and SWS2B AF247117; *Labeotropheus fuelleborni*: SWS2A AF247115 and SWS2B AF247119; *Melanochromis vermivorus*: Rh2Aα DQ088631, Rh2Aβ DQ088634, Rh2B DQ088646, SWS2A DQ088637 and SWS2B DQ088640; *Metriaclima zebra*: Rh2Aα DQ088651, Rh2Aβ DQ088650, Rh2B DQ088652, SWS2A AF247114 and SWS2B AF247118; *Oreochromis niloticus*: SWS2A AF247116 and SWS2B AF247120; *Pseudotropheus acei*: Rh2Aα DQ088630, Rh2Aβ DQ088633, Rh2B DQ088645, SWS2A DQ088636 and SWS2B DQ088639; *Tramitichromis intermedius*: Rh2Aα DQ088632, Rh2Aβ DQ088635, Rh2B DQ088647, SWS2A DQ088638 and SWS2B DQ088641.

& Kawamura, 2005a; Chinen et al., 2005b; Takenaka & Yokoyama, 2007; Wang, Chung, Yan, & Tzeng, 2008) or other substitutions in the transmembrane domain. Another possibility is that more than one opsin gene is expressed in the blue cone cells. Examples of single photoreceptor cells expressing two or three opsin genes are known in fishes and mammals (Applebury et al., 2000; Shand et al., 2002; Temple, Veldhoen, Phelan, Veldhoen, & Hawryshyn, 2008). Finally, the possibility of different chromophore usage could not be ruled out. Chromophore replacement from A1 to A2 caused a 10 nm shift in SWS2 opsin of goldfish and salamander (Makino, Groesbeek, Lugtenburg, & Baylor, 1999; Parry & Bowmaker, 2000), which is similar to the shift observed here between seabream SWS2 opsins. Thus far the mechanism of the spectral shift by SWS2 genes in seabreams is not clear. Experiments of mutagenesis and QPCR of opsin genes would be helpful to explore these mechanisms further.

Phylogenetic analysis, amino acid sequence alignments, MSP data and the expression data of opsin genes of black bream and cichlids (Parry et al., 2005; Shand et al., 2008), all suggest that the opsin gene expressed in green cone cells could be Rh2A. No significant differences in λmax or tuning sites were observed among the Rh2A copies of the four seabreams. In the case of Rh2B, the E122Q and T97A substitutions are well-known substitutions that cause a spectral shift to the blue end of the spectrum (Takenaka

& Yokoyama, 2007; Yokoyama, 1995; Yokoyama & Radlwimmer, 2001). Like Rh2A, Rh2B of seabreams use the same elements in their tuning sites. These findings indicate that the Rh2B genes of the seabreams could share similar λmax values that should be shorter than λmax of Rh2A (Matsumoto, Fukamachi, Mitani, & Kawamura, 2006; Spady et al., 2005, 2006). Ontogeny influences expression of Rh2 in the black bream (Shand et al., 2008) and cichlids (Parry et al., 2005; Spady et al., 2006) and may therefore also influence the expression of Rh2A and Rh2B in seabreams.

Whereas shallow-sea seabreams have normal LWS genes, the deep-sea species has accumulated frameshifts of this gene. Long-wavelength opsins diverged between species in shallow- and deep-sea habitats. A functional LWS is translated without the frameshift mutation, suggesting that the pseudogenization is rather recent in this species. In addition, the functional LWS in *P. major* carries a S164A substitution, known to cause a blue shift (Asenjo et al., 1994). Together, these findings suggest that pseudogenization must have occurred after the genes had been blue-shifted. The pseudogenization of the LWS gene in *P. major* can be explained by the lack of red light in their habitat at depths of approximately 200 m (Bowmaker et al., 1994; Hope et al., 1997; Hunt et al., 1996). The LWS pseudogenization suggests that the ancestors of the deep-sea seabream lived in shallower habitat, where red light was available. Thus, adaptation of LWS to deep-water photic environments was likely a two-step process: a hypsochromic shift followed by its pseudogenization.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.visres.2009.04.023.

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