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Ph.D. Dissertation

鯉科魚類視覺基因的適應演化
Adaptive Evolution of Visual Genes in Cyprinids

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摘　　要

鯉科是淡水魚類最大的一群，絕大多數都是體色樸素的種類，但少數的亞科、屬具有鮮豔的體色。其中鮈亞科中一些特化的魚種其體色鮮豔並具有婚姻色，適合用來研究視覺基因與體色的相關性。此外鯉科魚類棲息的環境差異很大，從清澈的小溪到混濁湖泊都可以發現其蹤跡，因此，鯉科魚類也是用來研究棲地光環境與視覺基因相關性的好材料。本研究主要是利用鯉科魚類棲地與體色的多樣性來測試兩個假說：一，魚類的視覺能力與視覺基因是否與體色上的差異具有相關性；二，鯉科魚類的視覺能力及其相對應的視覺基因是否會受到棲地光環境的影響而產生適應演化的現象。

我們定序了鮈亞科裡的長鰭鱲(*Opsariichthys evolans*)、粗首鱲(*Opsariichthys pachycephalus*)與馬口魚(*Candidia barbatus*)三種體色獨特且鮮豔的台灣特有鯉科魚類的視覺基因（分別是吸收UV光的SWS1、藍光的SWS2、綠光的Rh2、紅光的LWS以及感應明暗視覺的rhodopsin），並且測量這三種魚的視網膜吸收光譜與體表反射光譜。結果發現，鯉科魚類的體色跟視覺基因有很高的相關性，各魚種之體色上在藍色的反射光譜有明顯的差異；而在視覺能力方面，各魚種在藍色與綠色視覺基因的最大吸收光波則有不同程度的光譜位移。這三種鮮豔的鯉科魚類體色的差異反應了視覺能力的不同，而這樣的差異是由視覺基因序列的改變、視覺基因表現的不同與基因序列改變所造成。

我們並定序了其他四種體色樸素且生活習性截然不同的鯉科魚類（台灣石鱝*Acrossocheilus paradoxus*、台灣鏟頷魚*Onychostoma barbatula*、羅漢魚*Pseudorasbora parva*以及唇鯝*Hemibarbus labeo*）的視覺基因，同時測量其視網膜吸收光譜。結果發現，棲地光環境的改變影響了感應明暗、藍光、綠光與紅光的視覺基因之表現，並在這些基因的λmax上發現明顯的光譜位移。生活在清澈水域的鯉科魚類使用較短波長的視覺基因，而在混濁水域的種類則使用較長波長的視覺基因。這樣的差異，在rhodopsin、Rh2 與 LWS 等視覺基因中可能是因

為使用不同的chromophore或者累積了基因序列上比較次要的改變所造成的，而在 SWS1 與 SWS2則是由基因序列改變所造成的。由以上的結果，我們認為體色與棲地光環境的差別影響了鯉科魚類的視覺能力，而鯉科魚類視覺基因也因為這樣的差異而產生了適應演化的現象。

關鍵字：視覺基因、最大吸收光波長、鯉科、光譜位移、適應演化

Abstract

Cyprinidae is the largest freshwater fish family, and their habitats are diverse from clear to turbid water. Several specific genera from this group exhibit unique nuptial coloration, although most of them are drab in their coloration. This study was aimed to test two hypotheses. First, differences in nuptial coloration among colorful cyprinids could reflect differences in color vision and opsin gene sequences. Second, photic environments of habitats could affect the visual abilities and the opsin genes of cyprinids. To test the first hypothesis, genes encoding the visual pigments of three colorful cyprinids (*Opsariichthys evolans*, *Opsariichthys pachycephalus* and *Candidia barbatus*) were cloned and sequenced, the λmax of cone photoreceptor absorption spectra and the reflectance spectra of their body coloration were measured. It was found that the differentiation of spectral sensitivities and unique nuptial coloration of the colorful cyprinids might have evolved through sexual selection. The results also indicated that the spectral shift among colorful cyprinids could result from differential expression of opsin genes and amino acid substitutions. At the same time, other four dull-colored cyprinid (*Acrossocheilus paradoxus, Hemibarbus labeo, Pseudorasbora parva* and *Onychostoma barbatula*) lived in different habitats were tested to test the second hypothesis. The photic environments of habitats where the cyprinids lived could affect their visual abilities in blue-, green-, and red-sensitive photoreceptors. The cyprinids inhabited clear water use photoreceptors with shorter wavelength opsins, yet the species lived in turbid water possess photoreceptors with longer wavelength opsins. The alternative chromophore usage and accumulation of the complex interactive substitutions could dominate the obvious spectral differences of rhodopsin, Rh2, and LWS between the cyprinids inhabited two distinct photic environments. The amino acid substitutions may be responsible for differences in

the absorbance of SWS1 and SWS2. These results support our hypotheses that nuptial coloration and photic environment of habitats could be tied to visual sensitivities and opsin genes.

Keywords: spectral shift, nuptial coloration, photic environment, λmax, reflectance spectra, opsin gene

Contents

1. Introduction

The photic environments of aquatic environments are very diverse, ranging from total darkness in the deep sea to the brightness at the surface of a clear, shallow stream. Visual abilities of fishes are influenced by ecological photo-environments (Bowmaker, 1995). For example, in deep sea fishes, the spectral sensitivities of rod visual pigments shift to around 480 nm in order to match with the wavelength of the downwelling light (Partridge et al., 1988; Hunt et al., 2001). In Lake Baikal, located in Russia, the deepest lake in the world, cottoid fishes alter their spectral sensitivities of cone and rod photoreceptors to match with the shorter wavelength of ambient, deepwater light (Hunt et al., 1996; Cowing et al., 2002a). Additional examples of visual adaptation can be observed in some Antarctica fishes where notothenioid fishes lost red-sensitive photoreceptors and narrowed their sensitivity range of green-sensitive photoreceptors to adapt to the extreme light environment (Pointer et al., 2005). These visual adaptations are due to the changes of opsin pigments expressed in photoreceptor cells.

1.1 Opsin genes of vertebrates

Vision of vertebrates is modulated by five paralogous pigments expressed in rod and cone photoreceptor cells. These five pigments are encoded by rhodopsin (Rh1), short wavelength-sensitive type 1 (SWS1), short wavelength-sensitive type 2 (SWS2), rhodopsin-like (RH2), and middle and long wavelength-sensitive (M/LWS) opsin genes, respectively (Yokoyama & Yokoyama, 1996; Yokoyama, 2000; Ebrey & Koutalos, 2001). The Rh1 gene expresses in rod cell, and works in dim-light environments. The other opsin genes express in cone cells, and provide the color information in daylight. These visual pigments are composed of a light-sensitive

chromophore, either 11-cis-retinal or 3-dehydroretinal binding to the opsin proteins. Photoisomerization of the chromophore initiates the phototransduction pathway in a series of neural responses. The wavelength of maximum absorbance, defined as the λmax, of a visual pigment is determined by the interactions between the chromophore and the amino acid residues of the opsin protein (Sakmar et al., 1989; Zhukovsky & Oprian, 1989; Nathans, 1990a, b; Sakmar et al., 2002; Yokoyama, 2002).

1.2 The mechanisms of spectral tuning of photoreceptors

Several mechanisms have been demonstrated to be responsible for the tuning of the spectral sensitivity of photoreceptors in vertebrates. First, the spectral sensitivity can be modulated by differential expression of opsin genes (Carleton & Kocher, 2001; Parry et al., 2005). For instance, cichlid fishes have five cone opsin genes, SWS1, SWS2A, SWS2B, Rh2 and LWS. In order to adapt to various habitats, individual cichlid species acquires distinct visual ability by expressing different cone opsin genes. For example, the planktivore, *Metriaclima zebra*, expresses SWS1, SWS2 and Rh2, while the piscivore, *Dimidiochromis compressiceps*, expresses a different subset of opsins, SWS2A, Rh2 and LWS (Carleton & Kocher, 2001). Such differences in opsin gene expression lead to variations of color vision ability among cichlid fishes.

Second, the λmax of the visual pigment alters depending on whether the chromophore is derived from either vitamin A1 or A2. The visual pigment binding to A1-derived chromophore (11-cis-retinal) shows relatively shorter λmax than the pigment binding to A2-derived chromophore (3-dehydroretinal) (Yokoyama & Yokoyama, 1996). Fishes that live in turbid environments usually utilize A2 or A1-A2 mixed chromophore in their visual pigment (Bowmaker, 1995). For salmons (Salmonidae) and eel (*Anguilla rostrata*), different chromophores are used according

to the environment and the age of the species (Yokoyama & Yokoyama, 1996; Kusmic & Gualtieri, 2000). In cyprinids, A2-derived chromophore is predominant in goldfish (*Carassius auratus*), while A1 is used in zebrafish (*Danio rerio*) (Nawrocki et al., 1985; Palacios et al., 1998). These differences correspond well to the environmental differences of their habitats (Carleton et al., 2005).

Finally, amino acid substitutions of opsin genes can result in the spectral shift of visual pigments (Yokoyama, 2002; Takahashi & Ebrey, 2003; Takahashi & Yokoyama, 2005; Takenaka & Yokoyama, 2007). Based on the analysis of the three-dimensional crystal structure of bovine rhodopsin, there are 27 amino acids located within 4.5Å from 11-cis-retinal forming the chromophore binding pocket (Palczewski et al., 2000). Up to now, it is known that amino acid changes at 26 sites are involved in the spectral tuning of visual pigments in vertebrates (Yokoyama et al., 2007). Adaptive evolution will change the expression pattern of opsin genes, chromorphore usages or amino acid usages of the spectral tuning sites through natural or sexual selection.

1.3 Adaptive evolution of opsin genes

Many studies have shown that adaptive evolution of opsin genes is correlated with the photic environment inhabited by fishes. For instance, glutamic acid/glutamine substitution at amino acid site 122 (E122Q) introduced a 20nm spectral shift in Rh1 and Rh2 opsin genes of coelacanth to match the dwelling light (Yokoyama et al., 1999). In cottoid fish, several amino acid replacements were identified to shorten the λmax of SWS2 gene corresponding to the depth of their habitats (Cowing et al., 2002a). Moreover, adaptive molecular evolution, which was found to correlate well with the turbidity of the environment, was identified in LWS genes of cichlid fishes (Spady et al., 2005).

Sexual selection was reported to play a role in the evolution of visual genes of fishes as well, such as guppy (*Poecilia reticulata*) and cichlids (Cichlidae). In guppy, the close correlation between the high sequence variability of LWS gene and the male color polymorphism indicates that opsin genes co-evolved with the male ornaments through sexual selection (Hoffmann et al., 2007). In cichlids, differentiations of color patterns in conjunction with the visual system, corresponding to adaptation to different habitats, were proposed as the essential components of speciation (Parry et al., 2005). These cases indicate that differentiation of visual system may play an important role in the evolution of nuptial coloration through sexual selection.

1.4 Studies of opsin genes in cyprinids

Cyprinidae is the largest fish family with the majority of species inhabiting freshwater environments, and only a few exceptions residing in some salty inland lakes (Nelson, 1994). The habitats of cyprinids are highly divergent, ranging from pond to lake, tributary to mainstream, and therefore its photo-environments ranging from pristine and clear to highly turbid. In addition, the body colors of cyprinids are diverse. Most cyprinid species lack colorful patterns on their body, but some species of specific genera and subfamilies, such as members of genus *Opsariichthys* and subfamily Acheilognathinae, have adult males that possess brilliant nuptial coloration. Despite such high species diversity, wide distribution and variant color patterns, studies on visual genes of cyprinids are rather limited, with only three species (i.e., zebrafish, goldfish and common carp (*Cyprinus carpio*)) being characterized so far (Johnson et al., 1993; Tsai et al., 1994; Chinen et al., 2003). The molecular spectral tuning and the vision-environment relationship have been reported only for zebrafish and goldfish (Chinen et al., 2005b). In this study, three colorful cyprinids, which exhibited unique nuptial coloration, were selected to illustrate the relationships

between body coloration and visual system in cyprinids. Furthermore, four dull cyprinids, which had different habits and inhabited variant environments, were chosen to figure out the adaptive evolution of opsin genes, which in theory should be correlated with the photic environment of habitat.

1.5 Colorful cyprinids inhabited similar environments

The Opsariichthine, including genera *Candidia, Opsariichthys, Parazacco,* and *Zacco*, are a group of cyprinids with sexual dimorphism (Chen, 1982; Wang et al 2007). Among them, *Opsariichthys evolans, O. pachycephalus* and *Candidia barbatus* are three freshwater cyprinids exhibiting colorful nuptial coloration during mating season can be found here in Taiwan. The *O. pachycephalus and C. barbatus* cyprinids are distributed sympatrically in western Taiwan, while *O. evolans* and *O. pachycephalus* share the same habitats in northwest area of Taiwan (Tzeng, 1986). *O. evolans* and *O. pachycephalus* are endemic cyprinids inhabiting middle and lower reaches of the rivers in Taiwan. During breeding season, mature males of these two species express marked sexual dimorphism and brilliant nuptial coloration with bluish green cross stripes on the body and pinkish red coloring in pectoral- and ventral-fins, as well as in lower cheeks (Shen et al., 1993; Chen & Chang, 2005). On the other hand, *C. barbatus* is a widely distributed cyprinid that can be found in wide range of habitats ranging from high altitude to lowland waters. During breeding season, the adult males exhibit orange-red coloration on their pectoral fins and lower cheeks (Shen et al., 1993; Chen & Chang, 2005). All these three species are predators that feed on large invertebrates and small fishes, and live in very similar environments, i.e., clear streams with high oxygen content and pristine water quality. The three cyprinids, which use similar ecological habits, yet exhibit unique nuptial colorations, are suitable subjects to investigate the relationship between the evolution of color

vision and nuptial coloration.

1.6 Dull cyprinids inhabited variant photic environments

Four dull-colored cyprinids inhabited distinct environments were selected to examine the adaptive evolution of opsin genes of cyprinids. They were *Hemibarbus labeo*, *Pseudorasbora parva*, *Onychostoma barbatula* and *Acrossocheilus paradoxus*. Their biological habits were described as follows (Chen & Chang, 2005):

H. labeo, a regionally distributed riverine fish, principally dwells in lower water layer near bottom and feed almost exclusively on benthic macro-invertebrates such as insect larvae, mulluscs or crustaceans. The adult individuals usually cluster and cruise near the gravel substratum in low-light environment.

P. parva is a small fish that can be found ubiquitously in a wide array of habitats, from lowland rivers, streams, ditches to reservoir, lakes, pond, even marshes. It feeds mainly on benthic invertebrates, any sizable organic fragments and debris of dead fishes or other animals. Body is grayish sliver in dorsal and sliver white in ventral side.

A. paradoxus, a robust and highly rheophilic fish, usually inhabits riffles with torrents and feed mainly on benthic invertebrates, predominately insect larva or small crustaceans in the crevices of streambed rocks. Body coloration is grayish and belly shiny sliver. All fins are grayish.

O. barbatula is chiefly found in the upper reaches of streams, especially in those with lower average temperature, higher water quality and high transparency. This specie feed mainly on epilithic periphyton, scraps algae with its razor-like lower jaw margin, leaving patchy traces on the rock. Body coloration is grayish silver and belly shiny silver. All fins are grayish.

O. barbatula only lives in clear water, yet *H. labeo* and *P. parva* are found in

muddy water. Meanwhile, *A. paradoxus* inhabits from clear to turbid water. In addition, all these cyprinids live in mid-layer to low-layer water, except for *H. labeo* being benthic. These four cyprinids, which inhabit variant environments, yet exhibit plain colorations, are suitable study subjects to illustrate the relationship between the evolution of color vision and adaptation of the photic environment.

1.7 The purposes of this study

In essence, there are three main purposes of this study. First is to test the hypothesis that whether a relationship between differences in nuptial coloration and its opsin genes sequences among the three colorful cyprinids exists. Second is to figure out the adaptive evolution of opsin gene, which is affected by adjusting to the different photic environments. Finally, the evolutionary history of opsin genes of the Cyprinidae could be investigated by comparing the visual system of these selected cyprinids.

In order to achieve these purposes, a three-prong approach was used. First, we used a reflective photometer to measure and quantify the color spectra emitted by the male fish body with nuptial color. Second, we used a microspectrophotometer (MSP) to measure the λmax of photoreceptors of the seven cyprinids. Thirdly, we cloned and sequenced the visual pigment genes from these cyprinids. With this integrated approach, it allows us to understand the evolutionary processes involved in the adaptive evolution of visual genes of Cyprinidae through natural and sexual selection.

2. Materials and Methods

2.1 Sample collection

Individuals of the cyprinids listed in Table 1 were collected in Dabau and Houlong Stream located at the northwest area of Taiwan, and the fishes were shipped to a holding facility at the National Tsing-Hua University. Three adult individuals of each species were used for opsin genes cDNA cloning and sequencing, and 4 to 11 individuals were used for microspectrophotometry and reflectance spectra measurements.

2.2 Characterization of body coloration

The reflectance spectra (wavelength range: 380-700nm) of the studied subjects were measured using a USB 2000 spectrometer attached to a PC running OODBase32 software (Ocean Optics, Dunedin, Florida, USA). The spectrometer was calibrated against a diffuse reflectance standard WS-1 (Ocean Optics) before reflection measurement. A sensor connected to fiber optic probe, which was attached to the USB 2000 spectrometer, was placed close to the fish at a 45° angle so that the sensor sampled reflected light from the specific colored region and the data were collected and stored in the computer via spectrometer. Each measurement was an average of 3 to 10 samples of the same colored zone on the fish. The spectrum of body color can be described ether as step-like shapes (e.g., brown red, curve 1, Fig. 1B) or peak-like shapes (e.g., orange, curve 3, Fig 1B, (Chittka & Menzel, 1992)). The wavelength of a step-shaped color can be expressed as the wavelength corresponding to the 50% reflection point (R50) in the step (the half-way point from top to bottom of the step). This is similar to the 50% transmission (T50) used to quantify ocular media (Douglas & McGuigan, 1989; Siebeck & Marshall, 2001; Losey, 2003). Peaks are quantified

simply as the wavelength of highest reflection.

2.3 Microspectrophotometry

Test subjects were dark-adapted for at least 2hrs before the eyes were enucleated under dim red light. The retinas were removed with the aid of a night vision goggle, and immersed in chilled phosphate buffered saline containing 6% sucrose (Sigma, USA). The retina was cut into small pieces and placed on a cover glass and then sandwiched by a small glass cover slip sealed with silicone grease. The preparation was placed on the microspectrophotometer (MSP) stage. Absorption spectra of individual photoreceptors were determined by the computer-controlled modified single-beam MSP which had been previously described (Loew, 1994). Selected absorbance curves and the λmax of a photoreceptor 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 MacNichil (1986). The A1 and A2 templates used follow those of Lipetz and Cronin (1988). A decision as to which fitted best was made by visual examination. The best visual fit was usually the template fit with the lowest standard deviation (SD). If the SD of λmax was smaller than 7.5 nm, then the spectrum was considered valid and collected and stored into the computer (Sillman et al., 1999; Sillman et al., 2001). This process was repeated for each photoreceptor examined by the MSP. After the λmax values of each photoreceptor were averaged, a final estimate of mean λmax ± SD was obtained. T-test was used to verify the difference among the spectral sensitivities of photoreceptors of cyprinids tested.

2.4 cDNA synthesis and PCR amplification of opsin genes

Total RNA was extracted from freshly dissected retina using a QIAGEN RNeasy

Mini kit (Valencia, USA). Single stranded cDNA was synthesized using an oligo-d(T) primer and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, California, USA).

The complete open reading frames (ORFs) of one rhodopsin and four cone opsins were amplified from the retinal cDNA. Primer pairs were designed with conserved regions of the opsin genes of zebrafish, goldfish, and common carp were applied in the amplification of opsin genes of the cyprinids (Table 2). There were 10 primer sets in total, and the primer usages in amplification of opsin genes were listed in Table 3. The PCR reaction solution contained 1μl cDNA, 2μl 5X buffer A (5mM Mg^{2+}), 8μl 5X buffer B (10mM Mg^{2+}), 0.5μl (5mM) of each primer, 1μl Elongase (Invitrogen) add to 50 μl of ddH_2O. Reactions were conducted with a Takara PCR Thermal Cycler (Otsu, Shia, Japan) with denaturation at 94°C for 40sec, annealing at 55°C for 40sec, extension at 68°C for 1min 30sec for 35 cycles, and final extension at 68°C for 10min.

2.5 Cloning and sequencing

PCR products of five cone opsins were cloned individually into T-vectors using yT&A cloning kit (Yeasten Biotech, Taipei, Taiwan) and then sequenced respectively. The primers used for sequencing reaction were listed in Table 2. Sequencing reactions were carried out by dye terminator cycle sequencing method following the manufacturer's protocol (Applied Biosystems, Foster City, USA). All sequences were determined in both directions by an ABI-3100 automatic DNA sequencer (Applied Biosystems). Five to fifteen clones of each opsin gene were sequenced to rule out the artificial errors.

2.6 Sequence Analysis.

Alignments of the Rh1, LWS, RH2, SWS2 and SWS1 genes were carried out using their predicted amino acid sequences with CLUSTAL W (Thompson et al., 1994) computer software, and their nucleotide sequences were aligned in accordance with the amino acid alignments. The best-fit model of nucleotide evolution was determined by hierarchical likelihood ratio tests (LRT) implemented in Model Test (Posada & Crandall, 1998). The PAUP 4.0* (Swofford, 2000) was used to construct neighbor joining phylogenetic trees (Saitou & Nei, 1987) applying ML distances from the best-fit model. Given the tree topology reconstructed (Fig4~8), the ancestral amino acid sequences of opsin genes at every node in the tree were inferred by using the PAML computer program with a likelihood-based Bayesian method (Yang et al., 1995; Yang, 1997) (abacus.gene.ucl.ac.uk/software/paml.html). The evolutionary changes of the possible tuning sites of opsin genes that occurred in the phylogenetic clade derived from the ancestor could be estimated. The nucleotide sequences of the Rh1, LWS, RH2, SWS2 and SWS1 genes were retrieved from GenBank database, and the accession numbers were shown in Table 1. Substitution sites identified from amino acid alignments of opsin genes were numbered according to bovine rhodopsin (Palczewski et al., 2000).

3. Results

3.1 Differences in body coloration among cyprinids

The spectral reflectance of the studied cyprinids in body side, fins, and cheeks were measured using a USB 2000 spectrometer. For colorful cyprinids, the reflective spectra of bluish green stripes on body side, R50 of pinkish red pectoral fins, and cheeks are 495, 550 and 570nm in *O. pachycephalus*, respectively (Fig 1B). Similarly, the reflective spectra of bluish green stripes on body side, R50 of pinkish red pectoral fins, and cheeks are 400, 555 and 570nm in *O. evolans*, respectively (Fig 1D). Conversely, only the orange-red pectoral fin with R50 in 584nm was measured in *C. barbatus* (Fig 1F). The reflective spectrum, 400 and 495nm, could only be found on the bluish green stripes of their body side of *O. evolans* and *O. pachycephalus*. In contract to the colorful cyprinids, no peak- or step-like shapes were found in the reflectance spectra of the dull cyprinids (Fig 2), which indicated that no apparent color patterns could be observed on the body of plain colored cyprinids.

3.2 **MSP**

The microspectrophotometry measurement was carried out on the outer segments of photoreceptor from the retinas of the studied subjects. All species possess a single class of rod cells and four classes of cone cells (UV-, blue-, green- and red-cone cells).

3.2.1 λmax measurements of rod cells of cyprinids

The λmax of the rod cells measured are 499~507nm for the clear water-dwelling cyprinids; conversely, the λmax of the rod cells are 518~523nm for the turbid

water-dwelling species. Interestingly, the λmax of rod cells of *A. paradoxus* living in clear to turbid water is 499nm, similar with the species living in clear waters (Table 4).

3.2.2 λmax measurements of cone cells of colorful cyprinids

The λmax measurements are 376, 413, 486 and 567nm in *O. pachycephalus*, and 375, 416, 499 and 564nm in *O. evolans*. Similarly, for *C. barbatus*, the measurements are 374, 423, 500 and 564nm (Table 4). These four classes of single cones are UV-, blue-, green- and red-sensitive and correspond to the expression of SWS1, SWS2, Rh2, and LWS genes, respectively. No double cones could be found in these three species. Examples of mean absorbance spectra of the rod and cone pigments and the distribution of λmax of individual cells are shown in Fig 3.

Several significant features are observed in cyprinid spectral sensitivities among the three species (Table 4). First, the SWS2 pigment of *O. pachycephalus and O. evolans* shows a 10 and 7 nm blue shift compared to that of *C. barbatus*. Second, a 14nm spectral shift is observed between the λmax of the Rh2 pigments of *O. pachycephalus* and *C. barbatus*. Similarly, a 13nm spectral shift is identified among the λmax of the Rh2 pigments of *Opsariichthys* species. The T-test shows that the differences among the blue- and green- spectral sensitivities of colorful cyprinids are significant (Table 5). Finally, there are no significant differences in spectral sensitivities of the UV- and red-sensitive cones among the three colorful cyprinids.

3.2.3 λmax measurements of cone cells of plain-color cyprinids

Table 4 also shows the λmax measurements of cone cells of plain-color cyprinids. These measurements could be grouped into two categories: clear and turbid water-dwelling cyprinids. The cyprinid lives in clear water (*O. barbatula*) uses

retinal photoreceptors with shorter λmax. On the contrary, the λmax of retinal photoreceptors of the turbid water-dwelling cyprinids (*H. labeo, P. parva and A. paradoxus*) are significantly longer in blue-, green- and red-sensitive cones. This observation appears to be correlated with the adaptation of distinct photic environments. The T-test shows that the differences between the spectral sensitivities of the cyprinids inhabited clear and turbid environments are significant (Table 6). Green/red double cones were found in these plain-color cyprinids, except for *H. labeo*.

3.3 Nucleotide and amino acid sequences of cyprinids tested

Opsin cDNAs were isolated and sequenced from the tested cyprinids. Seven *O. pachycephalus* opsin genes (denoted as Opa- Rh1, SWS1, SWS2, Rh2A, Rh2B, Rh2C and LWS), seven *O. evolans* opsin genes (denoted as Oev- Rh1, SWS1, SWS2, Rh2A, Rh2B, Rh2C and LWS), six *C. barbatus* genes (denoted as Cba- Rh1, SWS1, SWS2, Rh2A, Rh2B and LWS) and five opsin genes for each plain-color cyprinid (denoted as Ppa-, Hla-, Oba-, Apa- Rh1, SWS1, SW2, Rh2 and LWS, respectively) were identified (Table 1). The sequences of opsin genes of three cyprinids (zebra fish, goldfish, and common carp which were not used directly in this study) were downloaded from the NCBI database for comparison. The neighbor-joining tree of cyprinid opsin genes was constructed using *Oncorhynchus keta* as an out-group species. The detailed amino acid sequence alignments of opsin genes of cyprinids were shown in Appendix 1~5.

3.3.1 Rh1 gene

Only one Rh1 gene is identified in each cyprinid. The amino acid sequences

show 89.3~98.8% shared identity. The size of amplified fragment of Rh1 gene is 996Bp in length.

Amino acids 83, 122, 211, 261, 265, 292 and 295 are important for spectral tuning of Rh1 gene (Yokoyama et al., 2007). All these sites are conserved across cyprinids, except for sites 261 (Table 7). Amino acid substitution from phenyalanine to tyrosine at site 261 (denoted as F261Y) was found in Opsariichthine species and *H. labeo*. Except for these 7 sites, the other 19 tuning sites (Yokoyama et al., 2007) are also conserved in Rh1 genes of cyprinids (the deep gray columns in Appendix 1). Comparison of the amino acid sequences of Rh1 genes has shown that no amino acid substitutions in the transmembrane domain could be correlated with the adaptation of different photic environments (Appendix 1).

Fig 4 is the phylogenetic tree of the cyprinid Rh1 genes. The tree topology of Rh1 gene is similar with the phylogenetic tree of Cyprinidae based on nuclear recombination activating gene (Wang et al., 2007). For example, zebrafish is located close to the common ancestor, and the Opsariichthine species were clustered with *H. labeo* and *P. parva* in SWS1 gene tree. Moreover, *A. paradoxus* and *O. barbatula*, form the sister group of goldfish and carp.

3.3.2 SWS1 gene

SWS1 gene was cloned and sequenced from the cyprinids, and only one locus was identified in each species. The size of SWS1 gene is 1011Bp in length. The amino acid sequences of the colorful cyprinids show 97.5~98.8% of shared identity. On the contrary, the dull-color cyprinids possessed higher variation of their amino acid sequences (85.9~95.7% similarity).

Amino acids 52, 86, 97,114 and 118 of SWS1 gene, which have been shown to play an important role for spectral tuning of SWS1 gene (Yokoyama et al., 2007;

Takahashi & Yokoyama, 2005), could also be involved in spectral tuning in cyprinids. In fact, amino acid 86 is conserved across cyprinids. Amino acid 86 is critical for the perception of UV- or violet- sensitive visual ability. Phenyalanine (Phe) was found present at this site (Yokoyama, 2000) upon comparing all teleost, amphibian, reptilian and mammalian UVS pigment sequences. All cyprinids in Table 7 possess a UVS SWS1 gene since Phe86 was present. This observation was consistent with the results of MSP study.

Instead of the conservation at amino acid site 86 of SWS1 gene, amino acid substitutions are observed at the other four possible tuning sites. At site 52, T52V substitution occurs in *O. barbatula* and *A. paradoxus*. Furthermore, amino acid substitution from Ser to Ala and Cys could be identified at site 97. Finally, A114S, S118T and S118A were observed among the cyprinids. Takahashi & Yokoyama (2005) have shown that red-shift of SWS1 resulting from amino acid substitutions, A114S and S118T, was identified in common carp. Comparison of the amino acid sequences and the evolutionary changes of these possible tuning sites of SWS1 genes (Fig 5A) indicate that the S97A and S118A may also cause red-shift of SWS1 in cyprinids, but T52V and S97C could not affect the λmax of SWS1 opsin (Table 7). Excluding these 5 sites, the other tuning sites (Yokoyama et al., 2007) are conserved in SWS1 genes of cyprinids (deep gray columns in Appendix 2).

Fig 5B shows the phylogenetic tree of cyprinid SWS1 genes. The tree topology of SWS1 gene is similar to the gene tree of Rh1 in that the zebrafish Rh1 genes is placed near the root of the tree while *H. labeo* and *P. parva* are clustered with Opsariichthine species.

3.3.3 SWS2 gene

A single copy of SWS2 gene, 1074Bp in length, is identified in each cyprinid

examined. The amino acid sequences of the colorful cyprinids show 95.4~99.1% of shared identity. On the contrary, the dull cyprinids exhibited higher variation of their amino acid sequences (80~98.5% similarity).

In colorful cyprinids, four of the 16 substitutions, namely A60V, S118T, L155I and A272S, are located in transmembrane domain (Appendix 3). Only one of these sites, amino acid 118, has been reported to alter the λmax of opsin gene (Wilkie et al., 2000; Janz & Farrens, 2001; Cowing et al., 2002b; Nagata et al., 2002).

Amino acids 94, 116, 117,118 and 295 of SWS2 gene, which have been shown to play an important role for spectral tuning of SWS2 gene (Yokoyama et al., 2007; Chinen et al., 2005b), could also be involved in spectral tuning in cyprinids (Table 7). Comparison of the amino acid sequences and the evolutionary changes of these possible tuning sites of SWS2 genes indicate that 5 amino acid sites could alter the λmax of SWS2 genes of cyprinids (Fig 6A and Table 7). It indicates that amino acid substitutions, A94S, T116A, A116L, S118T, A272S and C295S, could cause red shift in SWS2 genes in cyprinids.

Fig 6B shows the phylogenetic tree of cyprinid SWS2 genes. The tree topology of SWS2 gene is different from the phylogenetic tree of Cyprinidae based on nuclear recombination activating gene (Wang et al., 2007). Instead of clustering with Opsariichthine species, *H. labeo* and *P. paradoxus* were clustered with other dull cyprinids.

3.3.4 Rh2 gene

Two and three Rh2 genes are identified and designated as Cba-Rh2A/2B, Oev-Rh2A/2B/2C and Opa-Rh2A/2B/2C in *C. barbatus, O. evolans* and *O. pachycephalus*, respectively. In contrast, only one locus of Rh2 gene was identified in each plain-color cyprinid.

Glutamine/glutamic acid substitution at site 122 (Q122E) was reported to cause 15nm red-shift in Rh2 gene of zebrafish (Chinen et al., 2005a). In addition, four other amino acid replacements, T97A, E122Q, M207L, and A292S, caused significant λmax-shift of Rh2 gene (Takenaka & Yokoyama, 2007). In *O. evolans* and *O. pachycephalus*, Q122 is identified in Rh2A and Rh2C, while E122 is found in Rh2B. In *C. barbatus*, both Rh2 genes (Rh2A and Rh2B) contain E122, whereas Q122 can only be found in zebrafish and *O. pachycephalus* in cyprinids (Table 7). Conversely, E122 was conserved across all other dull-color cyprinids. Furthermore, C97 is found in Oev-Rh2C, Opa-Rh2C and the Rh2-1/-2 genes of zebrafish, while A97 is found in the Rh2 genes of the other species (Table 7). Finally, sites 207 (Met) and 292 (Ala) are conserved in all cyprinids studied.

Fig 7 shows the phylogenetic tree of cyprinids Rh2 genes. The tree can be divided into two major groups: Group I, (DreRh21 (OpaRh2C,OevRh2C), DreRh22))), and Group II, (((((CbaRh2A,CbaRh2B),((OevRh2A,OpaRh2A),(OevRh2B, OpaRh2B))),(HlaRh2,PpaRn2)),(((ObaRh2,ApaRh2),(CauRh21,CcaRh21)),(CauRh2 2,CcaRh22))),(DreRh23,DreRh24). There are two major notable events. First, gene duplication events of Rh2 occurred three times in cyprinids. Second, the E122Q substitution occurred repeatedly in cyprinids.

3.3.5 LWS gene

Only one LWS gene (1074Bp) is identified in each cyprinid. The amino acid sequences show 89.3~98.8% shared identity, which is higher than that observed for other cyprinid cone opsin genes.

It has been shown that amino acids 164, 181, 261, 269 and 392 of LWS, which corresponded to sites 180, 197, 277, 285 and 308 of human red- and green opsin, are important for spectral tuning (known as the “Five Site Rule”; Yokoyama &

Radlwimmer, 1998). The five sites are conserved across cyprinids, except at sites 180 and 197 in zebrafish (Table 7). Except for these 5 sites, the other 21 tuning sites (Yokoyama et al., 2007) appear high conservation in LWS genes of cyprinids (the deep gray columns in Appendix 5), only three substitutions at two sites observed. Comparison of the LWS genes of cyprinids has shown that no amino acid substitutions in the transmembrane domain could be correlated to the inference of adapting to photic environments (Appendix 5).

Fig 8 is the phylogenetic tree of the cyprinid LWS genes. The tree topology is similar to that of SWS1 in that the two zebrafish LWS genes are placed near to the root of the tree, and *H. labeo* and *P. parva* form the sister group of Opsariichthine.

4. Discussion

4.1 The relationship between differences in nuptial coloration and its opsin gene sequences among the colorful cyprinids

4.1.1 Positive correlation between spectral sensitivity and nuptial coloration

Maan et al. (2006) have shown that the sibling cichlids exhibited distinct visual abilities, male colors, and female mating preferences differed in behavioral responses to color stimuli outside the mate choice context. This behavioral difference may play an important role in evolution of male nuptial coloration. Wang et al. (1995) have shown that the adult males of *O. pachycephalus* displayed their color patterns to threaten competitors, dashing aggressively and fighting with each other. Only the winner followed the female and mated. According to our observations in the field, *O. evolans* and *C. barbatus* also showed similar mating behaviors during their breeding season. The mating behaviors indicated that the nuptial coloration of the colorful cyprinids could be affected by sexual selection.

Upon the comparison of these colorful cyprinids, two important findings were made. First, the MSP and reflectance spectra data in this study show that the visual sensitivity correlates closely to color patterns between *Opsariichthys* species and *C. barbatus*. The reflective spectra, 400 and 495nm, could only be measured on the bluish green pattern of *O. evolans* and *O. pachycephalus* individually, which corresponds to the λmax of blue- and green-sensitive photoreceptors. The 10 and 14nm shift in λmax of blue- and green-sensitive photoreceptors were found between *O. pachycephalus* and *C. barbatus*, and 7nm shift of blue-sensitive photoreceptors between *O. evolans* and *C. barbatus*. Meanwhile, Carleton et al. (2005) have shown that small shifts (4nm) in λmax could cause significant differences in photoreceptor

responses and play an important role in visual perception and mate choice. Therefore, the spectral shifts between *Opsariichthys* species and *C. barbatus* could lead to conspicuous differences in photoreceptor responses resulted from their unique nuptial coloration.

Second, the MSP and reflectance spectra data were also different between the two *Opsariichthys* species. Their reflectance spectra of the bluish green stripe were dramatically different with nearly 100nm spectral shift, that is, 495nm for *O. pachycephalus* and 400nm for *O. evolans* (Fig 1B and 1D). Moreover, the 13nm spectral shifts of their cone opsin sensitivities of green light were also observed (Table 4). Therefore, the variant abilities of light sensitivities of the two *Opsariichthys* species could also cause conspicuous differences in photoreceptor responses resulting from their divergent nuptial coloration.

The aforementioned arguments point to the possibility that differentiation of spectral sensitivities and unique nuptial coloration of these vivid cyprinids might have coevolved through sexual selection.

4.1.2 Molecular mechanisms of the spectral shift among the colorful cyprinids

Significant spectral shifts of SWS2 and Rh2 were observed among the three colorful cyprinids. On the contrary, the spectral shifts of Rh1, SWS1 and LWS were not significant since they were smaller than stander error (Table 4). The spectral shift observed among the colorful cyprinids could result from the replacements of amino acids or differential expression of opsin genes. Other than mutation in the retinal-binding pocket, accumulation of the complex interactive substitutions locating distantly from the pocket could also change the λmax of opsin genes (Chinen et al., 2005a, b; Takenaka & Yokoyama, 2007).

In SWS2 gene, two amino acid substitutions, site 118 and 272, could alter the

λmax among the colorful cyprinids (Fig 6A). T118S and S272A could induce the blue shifts between *Ospariichthys* species and *C. barbatus*. T118S has been reported to cause 13nm blue shift in Rh1 gene (Nagata et al., 2002). It could also induce blue shift in SWS2 genes of the colorful cyprinids. The site 272 was located in the sixth transmembrane domain and faced to the retinal-binding pocket (Palczewski et al., 2000). Substitution in this site could also affect the spectral sensitivity of SWS2 gene of colorful cyprinids.

In Rh2 genes, the difference of the gene expression may dominate the spectral shifts among the vivid cyprinids in this study. There were two and three clones identified from *C. barbatus* and *Opsariichthys* species, respectively. In previous study, Chinen et al. (2005a) has shown that there were four duplicated Rh2 genes in zebrafish, and the λmax of these duplicates, 467, 476, 488, and 505nm, were measured *in vitro* using 11-cis-retinal. The λmax 480nm measured by MSP was found corresponding to the expression of Rh2-2 in zebrafish. The three duplicated Rh2 genes of *O. pachycephalus*, Opa-Rh2A, -2B and -2C, shared sequence similarity at tuning sites to Rh2-3, Rh2-4 and Rh2-2 gene of zebrafish, respectively. Likewise, the three duplicated Rh2 genes of *O. evolans*, Oev-Rh2A, -2B and -2C, also showed the similarity at tuning sites to Rh2-3, Rh2-4 and Rh2-2 gene of zebrafish. Like zebrafish, λmax of these duplicates of the two *Opsariichthys* species may differ. The λmax 486nm of photoreceptor of *O. pachycephalus* might result from the expression of Rh2C gene, which used glutamine at site 122. However, the λmax 499nm of photoreceptor of *O. evolans* might result from the expression of Rh2B gene, which used glutamic acid at site 122. On the other hand, both Rh2 duplicates of *C. barbatus*, which possessed glutamic acid at site 122, were homogeneous at tuning sites to Rh2-4 gene of zebrafish and Rh2 genes of goldfish. Like goldfish (Johnson et al., 1993), the λmax of the Rh2 genes of *C. barbatus* could be different. The

λmax 500nm of Rh2 genes of *C. barbatus* could have achieved by the expression of one of the Rh2 duplicates.

As discussed above, the spectral-shifts among the green-sensitive photoreceptors of the three vivid cyprinids were consistent with the effect of the Q122E substitution of Rh2 gene in coelacanth and zebrafish (Yokoyama et al., 1999; Chinen et al., 2005a). The difference in green-light sensitivities could be induced by expressing different Rh2 gene with either Q122 exhibition or not.

4.1.3 Opsin gene polymorphism of the colorful cyprinids

Opsin gene polymorphism within and between populations are observed in guppy and cichlids, and could be a factor for sexual selection in them (Parry et al., 2005; Hoffmann et al., 2007). In this study, two and three clones of Rh2 genes were identified from retinal cDNA of *C. barbatus*, *Opsariichthys* species, respectively. Sequence polymorphism of these duplicates was found within species. An eleven amino acid substitution between Cba-Rh2A and -Rh2B, 22 amino acid substitutions between allele Opa-Rh2A and -2B, 61 amino acid substitutions between Opa-Rh2A and -2C, 21 amino acid substitutions between allele Oev-Rh2A and -2B, and 33 amino acid substitutions between Oev-Rh2A and -2C, were identified. In *O. pachycephalus*, Opa-Rh2A allele from one of the three specimens exhibited a divergent sequence when compared to the other two specimens (denoted as Opa2Rh2A in Fig 3), while Opa-Rh2B and -C alleles shared sequence similarity in all specimens. The existence of an alternative Opa-Rh2A allele indicates that more Rh2 alleles could be discovered and the allelic polymorphism of Rh2A may exist within *O. pachycephalus* populations. In *O. evolans*, Oev-Rh2C allele was only isolated from one out of the three specimens; conversely, Oeva-Rh2A/B were found in all specimens. Like *O. evolans*, Cba-Rh2B allele couldn't be isolated from the PCR

product for two out of the three specimens in *C. barbatus*, while Cba-Rh2A was found in all specimens. The failure in isolating Oev-Rh2C and Cba-Rh2B could result from the differentiation of opsin gene expression or the allelic divergence within population. Therefore, we propose that polymorphisms of Rh2 alleles could exist within and among the Opsariichthine species, while *O. pachycephalus* shows a higher divergence. Since only three specimens were examined in this study, a survey of Rh2 alleles at population level should be proceeded in the future to test this hypothesis.

4.2 Adaptive evolution of opsin genes results from adjusting to the distinct photic environments

4.2.1 Positive correlation between spectral sensitivity and photic environments

Rod and cone photoreceptors are two different systems to take charge of visual sensitivities in dim-light and daylight environments, respectively (Yokoyama, 2000; Bowmaker, 1995). Kusmic and Gualtier (2000) summarized many MSP data of freshwater fishes, and four types of freshwater fishes were classified. They were described as follows: type I is consisted of diurnal species, which lived just below the water surface or in very shallow water along the shores of lakes and rivers, usually possessed rods with absorbance peaking at ca 500nm. Type II consists of the generalized diurnal midwater species, and used a rhodopsin with λmax from 500 to 530nm. Type III and IV, composed by crepuscular and nocturnal species, owned rods with λmax at ca 520nm. According to this classification, the cyprinids tested in this study belonged to type II and their λmax of rod photoreceptors were divergent.

H. labeo was benthic and crepuscular, and *P. parva* was midwater-dwelling. Both species inhabited turbid water. Hence, they possessed rods with longer λmax at

ca 520nm. In contrast, *O. barbatula* and Opsariichthine species inhabited clear mid-layer to low-layer water, and owned rods with λmax at ca 500nm. *A. paradoxus* lived in clear to turbid water and used the rod with shorter wavelength. The λmax of rod photoreceptors of cyprinids showed close correlation with their photic environment. In all, the positive correlation between rhodopsin and photic environments were observed in the studied cyprinids.

For the cone opsins, significant differences in λmax of blue-, green- and red-sensitive photoreceptors were found between the clear and the turbid habitat-dwelling cyprinids, which included all species tested in this study and the species of previous studies (Table 4). These differences could result from adapting to the distinct photic environments in which they reside. The clear habitat-dwelling cyprinids inhabit the environment which is dominated by short-wavelength background spectra (Chen & Chang, 2005) and their photoreceptors possessed shorter λmax. The turbid habitat-dwelling cyprinids live in muddy environment, where the short-wavelength light was absorbed easily by particulates (Roesler, 1998), and therefore they possess the photoreceptors with longer wavelengths. In summary, the positive correlation was also observed between cone opsins and photic environments in the studied cyprinids.

Except for the difference induced by different photic environments, spectral shifts were also characterized between vivid and dull cyprinids lived in the clear water (Table 4). The colorful species used blue-sensitive photoreceptors with shorter wavelength. It seems that the vivid clear-water dwelling cyprinids may push their photoreceptor to absorb blue and green light with shorter wavelength than dull ones. Another possible explanation is that the difference of λmax between vivid and dull cyprinids in clear water can be result from their different biological habits, such as being predators or algae eaters.

4.2.2 Molecular mechanisms of the spectral shift resulting from adapting different photic environments in Cyprinidae

The mechanisms of the spectral shift between the clear and turbid habitat-dwelling cyprinids could be caused by the differences of chromophore usage and the amino acid substitutions, which were shown by Chinen et al. (2005b) between the SWS2 genes of zebrafish and goldfish. It may be implied to all cyprinids in this study.

For rhodopsin and LWS, Table 4 shows that these two opsin genes of cyprinids share the high sequence similarity at the well-known tuning sites; moreover, the tuning sites summarized by Yokoyama et al. (2007) are also conserved in these two opsin genes (Appendix 1 and 5). Furthermore, no amino acid sites in transmembrane domain of rhodopsin and LWS were characterized to correlate to the inference of adapting their habitats (Appendix 1 and 5). Two possible mechanisms could cause the spectral shift of Rh1 and LWS genes that were affected by different photic environments. First, Parry and Bowmaker (2000) showed that the distinct chromophore caused the dramatic spectral shift, 20 and 53nm, of rhodopsin and LWS in goldfish respectively. Hence, the possible mechanism to cause the spectral shift of rhodopsin and LWS between clear and turbid water-dwelling species may be the different chromophore usage. Rh1 and LWS opsin genes of clear water-dwelling cyprinids may predominately use A1-derived chromophore, but those of turbid water-dwelling cyprinids may principally have A2-derived chromophore. Second, the accumulation of the interactive effects of other substitutions could also cause the spectral shift of Rh1 and LWS genes. Chinen et al. (2005a) and Takenaka & Yokoyama (2007) have shown that accumulation of the complex interactive substitutions, which located distantly to the pocket, could affect the spectral sensitivities of opsin genes.

For SWS1, all cyprinids in this study possessed UV vision, and the critical amino

acid (F86) for UV vision were conserved. The different spectral sensitivities among cyprinids may not cause by the different chromophore usage, since 11-cis-retinal (A1-derived) usually appeared in UV opsin of vertebrates (Shi et al., 2001). Several amino acid substitutions known to alter the λmax of SWS1, such as A114S and S118T, were observed in SWS1 genes of cyprinids, and corresponding spectral shift were observed. According to the comparison of amino acid sequences and the reconstitution of ancestral amino acid sequences, two new amino acid substitutions, S97A and S118A, could induce red shift of SWS1 gene of cyprinids.

For SWS2, the amino acid 295 was indicated to reflect the influence of adapting the photic environment by comparing SWS2 genes of zebrafish and goldfish, while C295 kept the λmax of SWS2 at ca 400nm (Chinen et al., 2005b). It is consistent with the findings in this study. The cyprinids live in clear water use C295 in their SWS2 genes, except for *O. barbatula* (Table 7). Moreover, amino acid substitutions may cause red shift of SWS2 genes in turbid water-dwelling cyprinids. Fig 6A has shown that C295S, T116A, A116L and A94S may shift the λmax of SWS2 genes from 430 to 458nm. C295 and T116A may shift the λmax to 453nm *(P. parva* and *H. labeo*), then A94S and A116L may push the λmax to 458nm (goldfish, common carp and *A. paradoxus*). According to the comparison of SWS2 sequences, the λmax of SWS2 gene of *O. barbatus* should be similar with *A. paradoxus*. In fact, the MSP data has shown that there is a 20nm spectral shift between the blue cone photoreceptors of these two cyprinids. The possible mechanisms could be amino acid substitutions or different chromophore usage. There are four amino acid substitutions, G45A, P102S, L169I and L230V (Appendix 2), were found between *O. barbatula* and *A. paradoxus*, and none of them was located around the retinal-binding pocket. The accumulation of interactive effects (Chinen et al., 2005a, b) of these four substitutions could induce the spectral shift. Furthermore, the different

chromophore usage could also cause the spectral shift. Parry and Bowmaker (2000) showed that the different chromphore usage (A1/A2) caused a 10 nm spectral shift of SWS2 gene of goldfish. It was similar with the spectral shift between blue cone photoreceptors of *O. barbatula* and *A. paradoxus*. In all, amino acid substitutions and different chromophore usage could work together to adjust the spectral sensitivities of blue-sensitive photoreceptors of cyprinids.

For Rh2 genes, unlike the Opsariichthine species tuning their Rh2 gene through differential expression pattern, the different spectral sensitivities of Rh2 genes between clear and turbid water-dwelling cyprinids may result from different mechanism. Table 7 shows that two of the tuning sites (Yokoyama, 2007) showed their conservation (M207 and A292) among the cyprinids, and the substitutions (T97C and E122Q) of the other two tuning sites were only found in the duplicates of *Opsariichthys* species and zebrafish. Moreover, no substitutions in Rh2 genes were observed to be correlated to the photic environments (Appendix 4). All well known tuning sites are failed to explain the spectral shift between Rh2 genes of the clear and turbid water-dwelling cyprinids. There are two possible mechanisms to induce the spectral shift of Rh2 genes. First, the chromophore usage may play a role to induce this shift. Johnson et al. (1993) showed that the λmax of the two duplicates of goldfish were 511 and 505 nm individually, when A1-drived retinal was used as their chromophore. Conversely, goldfish used A2-drived retinal dominantly in nature, and the λmax of green-sensitive photoreceptor was 537nm. This shift induced by different chromopore usage is consistent with the shift caused by adaptation of the photic environments in cyprinids. Second, the accumulation of the interactive effects of other substitutions could also cause the spectral shift of Rh2 genes. Chinen et al. (2005a) has shown that accumulation of the complex interactive substitutions, which located distantly to the pocket, could affect the spectral

sensitivities of the Rh2 genes of zebrafish.

In conclusion, the photic environments of habitats the cyprinids lived can indeed affect their visual abilities in blue-, green-, and red-sensitive photoreceptors. The cyprinids inhabited clear water use photoreceptors with shorter wave-length, yet the species live in turbid water possess photoreceptors with longer wave-length. The accumulation of the complex interactive substitutions and the chromophore usage may dominate the obvious spectral differences of rhodopsin, Rh2, and LWS between the cyprinids inhabit two distinct photic environments, and amino acid substitutions may rule SWS1 and SWS2.

4.3 Evolution of opsin gene in Cyprinidae

Opsin gene duplication is quite common in teleost, such as SWS2 and Rh2 in cichlids, Rh2 in pufferfish (*Takifugu rubripes*), SWS2, LWS, and Rh2 in medaka (*Oryzias latipes*), SWS1 and Rh2 in ayu (*Plecoglossus altivelis*), Rh2 in goldfish and carp, and LWS and Rh2 in zebrafish (Johnson et al., 1993; Chinen et al., 2003; Minamoto & Shimizu, 2005; Neafsey & Hartl, 2005; Parry et al., 2005; Matsumoto et al., 2006).

Rh2 gene duplication took place independently several times and was shown to have evolved independently in cyprinids and cichlids. In cichlids, two major groups, Acanthoptrygii-Rh2A and B, existed and Acanthoptrygii-Rh2A could be divided into group 2Aα and 2Aβ (Parry et al., 2005). The Rh2 gene of these three groups of cichlids possessed different λmax values, and the gene duplication events occurred before the appearance of the genera of cichlids in group Acanthoptrygii-Rh2A.

In cyprinids, gene duplication occurred before the appearance of cyprinids and can be divided into group I and II (Fig 7). The Rh2 duplicates of group I could be lost in *C. barbatus* and other dull cyprinids during the evolution of cyprinids. On the

contrary, the Rh2 duplicates of group II were kept in all cyprinids. Losing Rh2 duplicate of group I in *C. barbatus* may be a factor that enables the ancestors of Opsariichthys species and *C. barbatus* to gain different visual ability and resulted in the division of genus *Opsariichthys* and *Candidia*. In group II, gene duplication events happened before the appearance of this group (Fig 7). *The A. paradoxus, O. barbatula, H. labor* and *P. parva* may loss one Rh2 duplicate during the evolution. E122Q substitution happened several time in the evolutionary history of Rh2 genes of cyprinids, and it could be only observed in the lineage where the species with bluish green color pattern could be found. It appears that E122Q in Rh2 gene may be correlated with the appearance of the bluish green pattern of cyprinids.

In this study, the adaptive evolution of opsin genes of cyprinids was illustrated through sexual and natural selection. First, the relationship between differences in nuptial coloration and its visual system among *O. evolans, O. pachycephalus,* and *C. barbatus* was investigated through comparing with their opsin genes, λmax of cone photoreceptors and body colors. Differentiation of spectral sensitivities and unique nuptial coloration of these colorful cyprinids may have evolved through sexual selection. The spectral shift among these colorful cyprinids may result from differential expression of opsin genes and the amino acid substitutions. In the future, quantitative PCR and Southern blotting within populations should be performed to test the hypothesis that the allelic polymorphism of opsin genes and differential opsin gene expression exists in Opsariichthine. Visual systems from additional species shall be examined to reconstruct the evolutionary histories of visual genes in Opsariichthine. The experiments of mating behavior of *O. evolans*, *C. barbatus* and the other species shall be conducted to investigate the relationship between male coloration and the sexual selection in Opsariichthine. Through this integrated approach, the mechanism of evolution among visual system, male coloration, and

sexual selection in cyprinids could be deciphered.

Second, the photic environments of habitats the cyprinids lived can affect their visual abilities in rod, blue, green and red cone photoreceptors. The cyprinids inhabited clear water use photoreceptors with shorter wavelength opsins, yet the species lived in turbid water possess photoreceptors with longer wavelength opsins. The obvious differences of the visual abilities of rhodopsin, Rh2, and LWS between clear and turbid water-dwelling cyprinids may be dominated by the distinct chromophore usage and the accumulation of the complex interactive substitutions. On the contrary, the amino acid substitutions at the tuning sites could control the visual abilities of SWS1 of cyprinids. In the future, opsin reconstruction with different chromophores and mutagenesis of opsin genes should be processed to test the mechanisms, which we hypothesized, of the spectral shift resulting from adapting different photic environments. The experiments of biological habits of *A. paradoxus* shall be processed to figure out that why its rod cells perform like the clear water-dwelling species, but cone cells perform like the turbid species. In order to reconstruct the evolutionary histories of visual genes of cyprinids, the visual system of other cyprinids live in different habitats shall be examined. Through this integrated approach, more details in the mechanism of adaptive evolution of visual system in cyprinids can be unveiled.

5. Conclusion

In summary, the results of this study showed that the spectral sensitivities of cyprinids were affected by different photic environments. The clear water-dwelling species used photoreceptors with shorter wavelength opsins, yet the turbid water-dwelling species used longer ones. Furthermore, positive correlation between spectral sensitivity and nuptial coloration was observed in the colorful cyprinids. The species with bluish green cross stripes changed their spectral sensitivities of blue and green cone photoreceptors. The mechanisms of the spectral shifts in cyprinids could be the different chromophore, differential opsin genes expression and amino acid substitutions.

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Table 1. Species and visual genes analyzed in this study.

Species	Opsin gene	Symbol	Source
Opsariichthys pachycephalus	Rh1	OpaRh1	θ
	SWS1	OpaS1	EU410468θ
	SWS2	OpaS2	EU410467θ
	RH2	OpaRh2A	EU410463θ
		OpaRh2B	EU410464θ
		OpaRh2C	EU410465θ
	LWS	OpaL	EU410466θ
Opsariichthys evolans	Rh1	OevRh1	θ
	SWS1	OevS1	θ
	SWS2	OevS2	θ
	RH2	OevRh2A	θ
		OevRh2B	θ
		OevRh2C	θ
	LWS	OplL	θ
Candidia barbatus	Rh1	CbaRh1	θ
	SWS1	CbaS1	EU410458θ
	SWS2	CbaS2	EU410459θ
	RH2	CbaRh2A	EU410460θ
		CbaRh2B	EU410461θ
	LWS	CbaL	EU410457θ
Hemibarbus labeo	Rh1	HlaRh1	θ
	SWS1	HlaS1	θ
	SWS2	HlaS2	θ
	RH2	HlaRh2	θ
	LWS	HlaL	θ
Pseudorasbora parva	Rh1	PpaRh1	θ
	SWS1	PpaS1	θ
	SWS2	PpaS2	θ
	RH2	PpaRh2	θ
	LWS	PpaL	θ
Onychostoma barbatula	Rh1	ObaRh1	θ
	SWS1	ObaS1	θ
	SWS2	ObaS2	θ
	RH2	ObaRh2	θ
	LWS	ObsL	θ

Table 1. Species and visual genes analyzed in this study (continued).

Species	Opsin gene	Symbol	Source
Acrossocheilus paradoxus	Rh1	ApaRh1	θ
	SWS1	ApaS1	θ
	SWS2	ApaS2	θ
	RH2	ApaRh2	θ
	LWS	ApaL	θ
Carassius auratus	Rh1	CauRh1	L11863*
	SWS1	CauS1	D85863*
	SWS2	CauS2	L11864*
	RH2	CauRh21	L11865*
		CauRh22	L11866*
	LWS	CauL	L11867*
Cyprinus carpio	Rh1	CcaRh1	U02475*
	SWS1	CcaS1	AB113669*
	SWS2	CcaS2	AB113668*
	RH2	CcaRh21	AB110602*
		CcaRh22	AB110603*
	LWS	CcaL	AB055656*
Danio rerio	Rh1	DreRh1	AB087811*
	SWS1	DreS1	AB087810*
	SWS2	DreS2	AB087809*
	RH2	DreRh21	AB087805*
		DreRh22	AB087806*
		DreRh23	AB087807*
		DreRh24	AB087808*
	LWS	DreL1	AB087803*
		DreL2	AB087804*
Oncorhynchus keta	Rh1	OkeRh1	AY214141*
	SWS1	OkeS1	AY214143*
	SWS2	OkeS2	AY214144*
	RH2	OkeRh2	AY214142*
	LWS	OkeL	AY214140*

θ and * indicate that the sequences are either obtained in this study (θ) or downloaded (*) from the GenBank database, respectively.

Table 2. Primer sequences used in the visual genes amplification and sequencing reactions.

Opsin	PCR primer (5' to 3')
O_SWS1_F	ATGGACGCGTGGGCCGTTCAGTTCG
D_SWS1_F	AGAAsAAGGsCTCyAACGGyrCAACC
O_SWS1_R	CAGAGAAGTTGTAAATGTGGTGTGG
D_SWS1_R	CrGGATTTGAACAATCAGGGG
O_SWS2_F	GGTGTTwCAGCATTCTCGGTGG
D_SWS2_F	ATGCTrCAGTAATyTGCArArGAAAT
O_SWS2_R	TTCACTGCCAGCAGAGTGGTTCTGTC
D_SWS2_R	GTTCAGCAAGCCAArAymAAGTTAC
O_Rh2_F	GGCACTGAGGGAAACAACTTCTACATC
D_RH2_F	TCTGGATCACTAGyrGGCArAGA
O_Rh2_R	GAACATAATCyGTGArAGkTTGACAAG
D_RH2_R	CAGATGCyCrsATTGsTCArTvACTAA
Rh2-1_F	GGCAGAACCATGGmArTTyAAGGC
Rh2-1_R	CACCTCTGTTTTGCTTGTTGAmACTGAG
O_LWS_F	GGGCTATACAACAACCCCAAAAATG
D_LWS_F	TTTACGACAGCyArGTrACTACAGG
O_LWS_R	CCTGGCTCAGGATCCTTGCTCTGAG
D_LWS_R	CACTCCAAGGGAAATATCTGGAAGC
Rh1_F	ACCGCAACAATGAACGGTACAGAGGG
Rh1_R	CGGTCTTGGAkGCrGTrGTGGAGGCkCC
	Sequencing primer (5' to 3')
O_SWS1_F2	GGAGCCGTGGCGTTCACCTGGG
O_SWS1_R2	CTGCAACGGCACGCAGGGCTC
O_SWS2_F2	GGGGTGACAGCATGTAAAATTGAGGG
O_SWS2_R2	GAATCTGCTTGAGCTTTGGCTGC
O_Rh2_F2	GGGCTTCATGGCCACACTTGGAGG
O_Rh2_R2	GCAGCAACAGTGGCATAAGGGG
O_LWS_F2	GGGAGAGATGGGTGGTCGTCTGC
O_LWS_R2	CCCCAGCAAACACAGTAGGCCAGG
TA-F*	CAAGGCGATTAAGTTGGGTA
TA-R*	GGAATTGTGAGCGGATAACA

* TA-F, TA-R are the primers of T-vector (Yeasten Biotech, Taipei, Taiwan).

Table 3. Primer Pairs used for PCR reaction.

	Rh1	SWS1	SWS2	Rh2	LWS
O. pachycephalus	Rh1_F x Rh1_R	O_SWS1_F x O_SWS1_R	O_SWS2_F x O_SWS2_R	O_Rh2_F x O_Rh2_R Rh2-1_F x Rh2-1_R	O_LWS_F x O_LWS_R
O. evolans	Rh1_F x Rh1_R	O_SWS1_F x O_SWS1_R	O_SWS2_F x O_SWS2_R	O_Rh2_F x O_Rh2_R Rh2-1_F x Rh2-1_R	O_LWS_F x O_LWS_R
C. barbatus	Rh1_F x Rh1_R	O_SWS1_F x O_SWS1_R	O_SWS2_F x O_SWS2_R	O_Rh2_F x O_Rh2_R	O_LWS_F x O_LWS_R
H. labeo	Rh1_F x Rh1_R	O_SWS1_F x O_SWS1_R	O_SWS2_F x O_SWS2_R	O_Rh2_F x O_Rh2_R	O_LWS_F x O_LWS_R
P. parva	Rh1_F x Rh1_R	D_SWS1_F x DO_SWS1_R	D_SWS2_F x O_SWS2_R	O_Rh2_F x D_Rh2_R	O_LWS_F x O_LWS_R
O. barbatula	Rh1_F x Rh1_R	D_SWS1_F x D_SWS1_R	D_SWS2_F x O_SWS2_R	O_Rh2_F x D_Rh2_R	D_LWS_F x D_LWS_R
A. paradoxus	Rh1_F x Rh1_R	O_SWS1_F x D_SWS1_R	D_SWS2_F x O_SWS2_R	O_Rh2_F x D_Rh2_R	D_LWS_F x D_LWS_R

Table 4. The λmax for rod and cone cells from cyprinids measured by the MSP. All values are expressed in nanometers (nm) and where appropriate with nm ± SD. Numbers in parentheses indicate the number of the photoreceptor cells measured. The grey and white rows indicate the cyprinid species that inhabit turbid and clear environments, respectively.

Species	Rod Rhodopsin	UV single cone SWS1	Blue single cone SWS2	Green single cone Rh2	Red single cone LWS
Opsariichthys evolans N=7*	507 ± 4.8(49)	375 (1)	416 ± 3.5(8)	499 ± 7.37(28)	564 ± 7.6(28)
Opsariichthys pachycephalus N= 4*	505 ± 7.3(41)	376 ± 6.8(3)	413 ± 5.5(12)	486 ± 9.9(21)	567 ± 8.3(19)
Candidia barbatus N= 5*	504 ± 8.4(31)	374 ± 7.6(5)	423 ± 5.1(11)	500 ± 8.7(16)	564 ± 4.2(8)
Hemibarbus labeo N= 6*	523 ± 8.5(44)	389 ± 3(3)	453 ± 8.8(14)	522 ± 8.1(18)	588 ± 10.9(13)
Pseudorasbora parva N=4*	518 ± 4.5(59)	390 ± 8.9(4)	453 ± 7.5(22)	523 ± 9.8(43)	603 ± 11.3(6)
Onychostoma barbatula N= 5*	500 ± 6.7(39)	380 ± 8.3(5)	438 ± 7.6(11)	504 ± 7.7(31)	569 ± 14.7(12)
Acrossocheilus paradoxus *N= 10**	499 ± 8.9(48)	383 ± 4.1(2)	458 ± 6(7)	522 ± 13(22)	595 ± 6.2(9)
Carassius auratus	523[e]	365[e]	458[e]	537[e]	618[e]
Cyprinus carpio	523[f]	377.5[d]	458[d]	532[d]	600[d]
Danio rerio	501[a]	362[b]	417[a]	480[a]	556[a]

* N indicates the number of the specimens examined.

[a] Measured by Nawrocki *et al.*, (1985)

[b] Measured by Robinson *et al.*, (1993)

[c] Measured by Palacios *et al.*, (1998)

[d] Measured by Hawryshyn *et al.*, (1991)

[e] Measured by Parry & Bowmaker, (2000)

[f] Measured by Crescitelli *et al.*, (1954)

Table 5. T-test of spectral sensitivities among blue- and green-light sensitive photoreceptors of colorful cyprinids.

		Blue photoreceptors		
		O. pachycephalus	*O. evolans*	*C. barbatus*
Green photoreceptors	*O. pachycephalus*		NA	**
	O. evolans	**		*
	C. barbatus	**	**	

The upper rows are the results of T-test among blue-light photoreceptors of colorful cyprinids; the lower rows exhibit the results of green-light photoreceptors

* indicates P<0.05; ** indicates P<0.001

Table 6. T-test of spectral sensitivities between the cyprinids inhabited clear and turbid environments.

	Rod cells	Cone cells		
		Blue	Green	Red
Clear *vs.* turbid group	**	**	**	**
A. paradoxus vs. clear group	NA	**	**	**
A. paradoxus vs. turbid group	**	NA	NA	NA
O. barbatula vs. colorful cyprinids	NA	**	**	NA

The clear group included *O. pachycephalus, O. evolans, C. barbatus* and *O. barbatula*; the turbid group included *A. paradoxus, H. labeo* and *P. parva*.

* indicates P<0.05; ** indicates P<0.001

Table 7. Comparisons of opsin sequences of cyprinids

		Rhodopsin (Rh1)						
	MSP	A.A. Sites						
Species		83	122	211	261	265	292	295
Consensus		D	E	H	F	W	A	A
O. evolans	507	.	.	.	Y	.	.	.
O. pachycephalus	505	.	.	.	Y	.	.	.
C. barbatus	504	.	.	.	Y	.	.	.
H. labeo	524	.	.	.	Y	.	.	.
P. parva	518	.	.	.	.	.	.	.
O. barbatula	500	.	.	.	.	.	.	.
A. paradoxus	499	.	.	.	.	.	.	.
C. auratus	523	.	.	.	.	.	.	.
C. carpio	523	.	.	.	.	.	.	.
D. rerio	500	.	.	.	.	.	.	.

	UV · sensitive (SWS1)						Blue · sensitive (SWS2)					
	MSP	A.A. Sites					MSP	A.A. Sites				
Species	λmax	52	86	97	114	118	λmax	94	116	117	118	295
Consensus		T	F	S	A	S		A	T	S	T	C
O. evolans	375	.	.	.	.	A	419	.	.	.	S	.
O. pachycephalus	376	.	.	.	.	A	413	.	.	.	S	.
C. barbatus	373	.	.	.	.	A	423	.	.	.	.	.
H. labeo	389	.	.	C	S	T	453	.	A	.	.	S
P. parva	390	.	.	C	S	T	453	.	A	.	.	S
O. barbatula	380	V	.	A	.	.	438	S	L	A	.	S
A. paradoxus	383	V	.	.	S	.	458	S	L	A	.	S
C. auratus	365	.	.	.	.	.	458	S	L	A	.	S
C. carpio	377	.	.	.	S	.	458	S	L	A	.	S
D. rerio	362	.	.	C	.	.	417	.	.	.	.	.

Table 7. Comparisons of opsin sequences of cyprinids (continued)

	Red sensitive (LWS)							Green sensitive (Rh2)					
	MSP	A.A. Site						MSP	A.A. Site				
Species	λmax	Loci	164	181	261	269	292	λmax	Loci	97	122	207	292
Consensus			S	H	Y	T	A			T	E	M	A
O. evolans	564	L	.	.	.	.	.	499	Rh2A	.	Q	.	.
									Rh2B	.	.	.	.
									Rh2C	C	Q	.	.
O. pachycephalus	567	L	.	.	.	.	.	486	Rh2A	.	Q	.	.
									Rh2B	.	.	.	.
									Rh2C	C	Q	.	.
C. barbatus	564	L	.	.	.	.	.	500	Rh2A	.	.	.	.
									Rh2B	.	.	.	.
H. labeo	603	L	.	.	.	.	.	522	Rh2	.	.	.	.
P. parva	588	L	.	.	.	.	.	523	Rn2	.	.	.	.
O. barbatula	569	L	.	.	.	.	.	504	Rh2	.	.	.	.
A. paradoxus	595	L	A	.	.	.	.	522	Rh2	.	.	.	.
C. auratus	623	L	.	.	.	.	.	537	Rh21	.	.	.	.
									Rh22	.	.	.	.
C. carpio	600	L	.	.	.	.	.	532	Rh22	.	.	.	.
									Rh21	.	.	.	.
D. rerio	556	L1	A	.	.	.	.	467*	Rh21	C	Q	.	.
								476*	Rh22	C	Q	.	.
		L2	A	.	F	.	.	488*	Rh23	.	Q	.	.
								505*	Rh24	.	.	.	.

Sequences are compared to the consensus sequence with similar identity indicated by a dot. Sites are numbered according to bovine rhodopsin. λmax from MSP (in nm) are listed for those genes that are expressed in cyprinids. The light grey and white rows indicate the cyprinid species that inhabit turbid and clear environments, respectively. The λmax of Rh2 gene could result from the expression of one of the multi-loci, since multi-loci were found in Rh2 genes. * indicated the Rh2 genes were expressed and measured by *Chinen et al.* (2003).

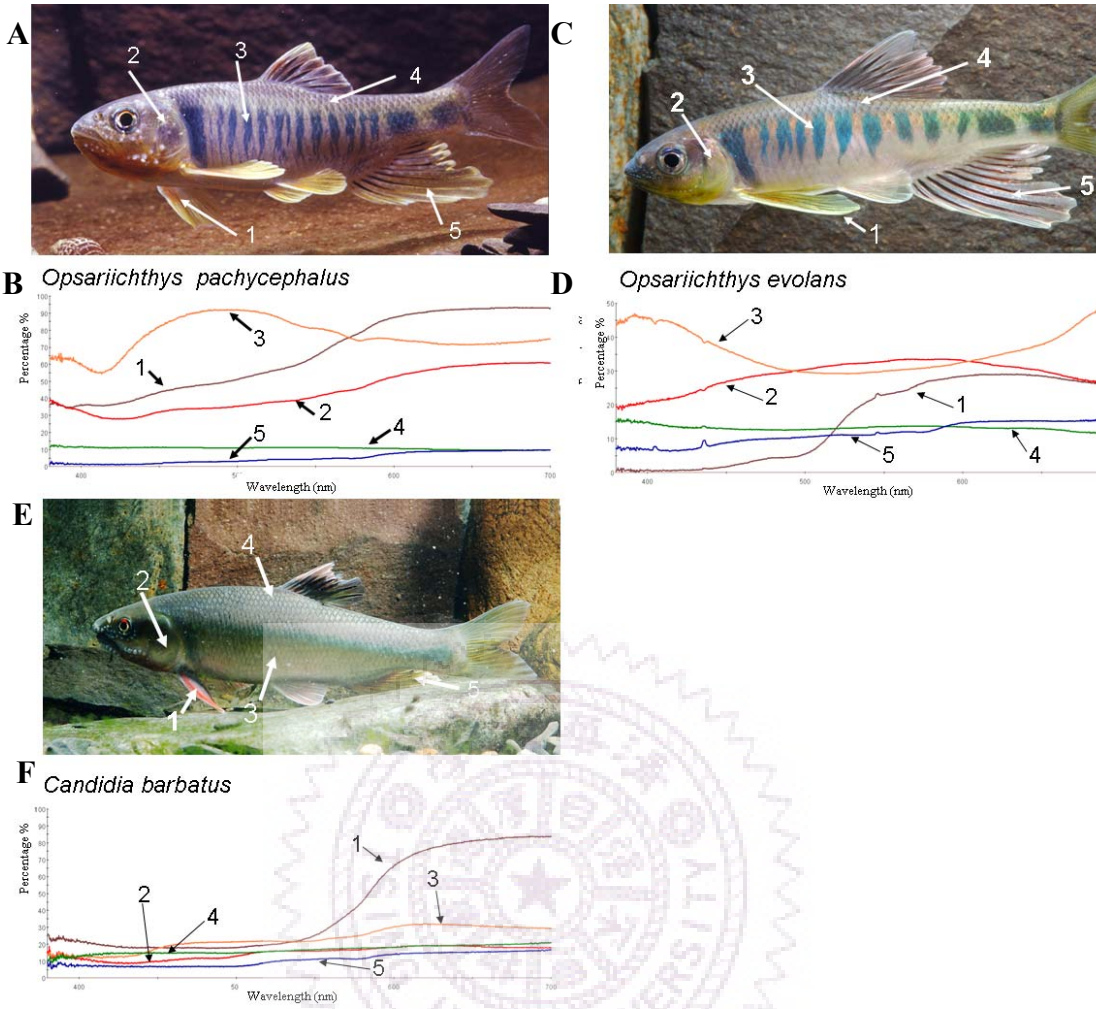


Fig 1. Fish body coloration plotted as percent reflectance spectra. The number in the photographs corresponds to the numbered reflection curves for the fish. (A-B) *O. pachycephalus*. (C-D) *O. evolans*. (E-F) *C. barbatus*. Curves 1 to 5 indicate the reflectance spectra of the pectoral-fin, cheek, body side, dorsal side of body and anal-fin of the colorful cyprinids, respectively. Low reflectance of the dorsal side, cheek and anal-fin are common characteristics of the three cyprinids. The R50 value of reflectance spectra of the pectoral-fin (curve 1) is similar between the two cyprinids (560nm for *O. evolans*, 550nm for *O. pachycephalus* and 584nm for *C. barbatus*). The significant differences between the reflectance spectra of body side (curve 3) are observed between the three cyprinids. The reflectance spectrum of bluish green stripes of *O. evolans* and *O. pachycephalus* on body side are 400nm and 495nm, but no colored stripes could be found in *C. barbatus*.

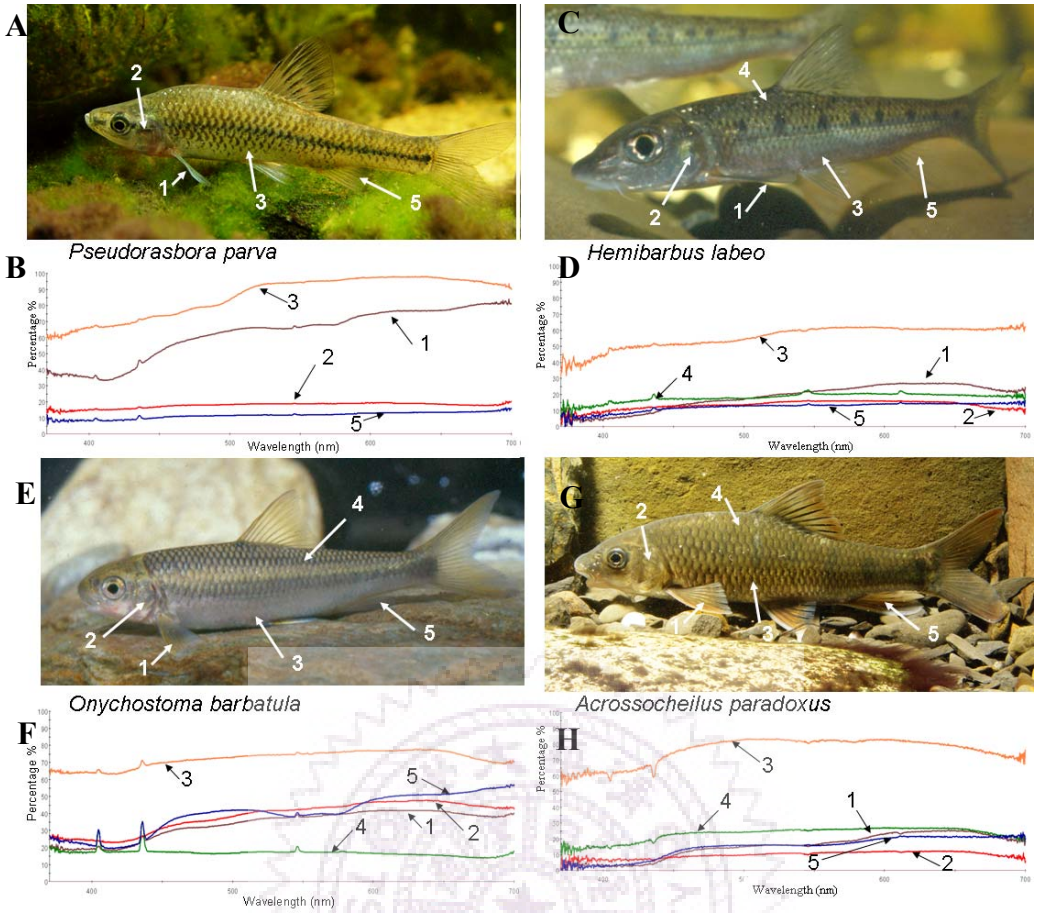


Fig 2. Fish body coloration plotted as percent reflectance spectra. The number in the photographs corresponds to the numbered reflection curves for the fish. (A-B) *P. parva*. (C-D) *H. labeo*. (E-F) *O. barbatula*. (G-F) *A. paradoxus*. Curves 1 to 5 indicate the reflectance spectra of the pectoral-fin, cheek, the ventral side, dorsal side of body and anal-fin of the dull cyprinids, respectively. Low reflectance is common characteristics of the three cyprinids, excepted for the ventral side of body. These dull cyprinids exhibit high reflectance on their dorsal side of bodies, but no significant step-like or peak-like shapes are found.

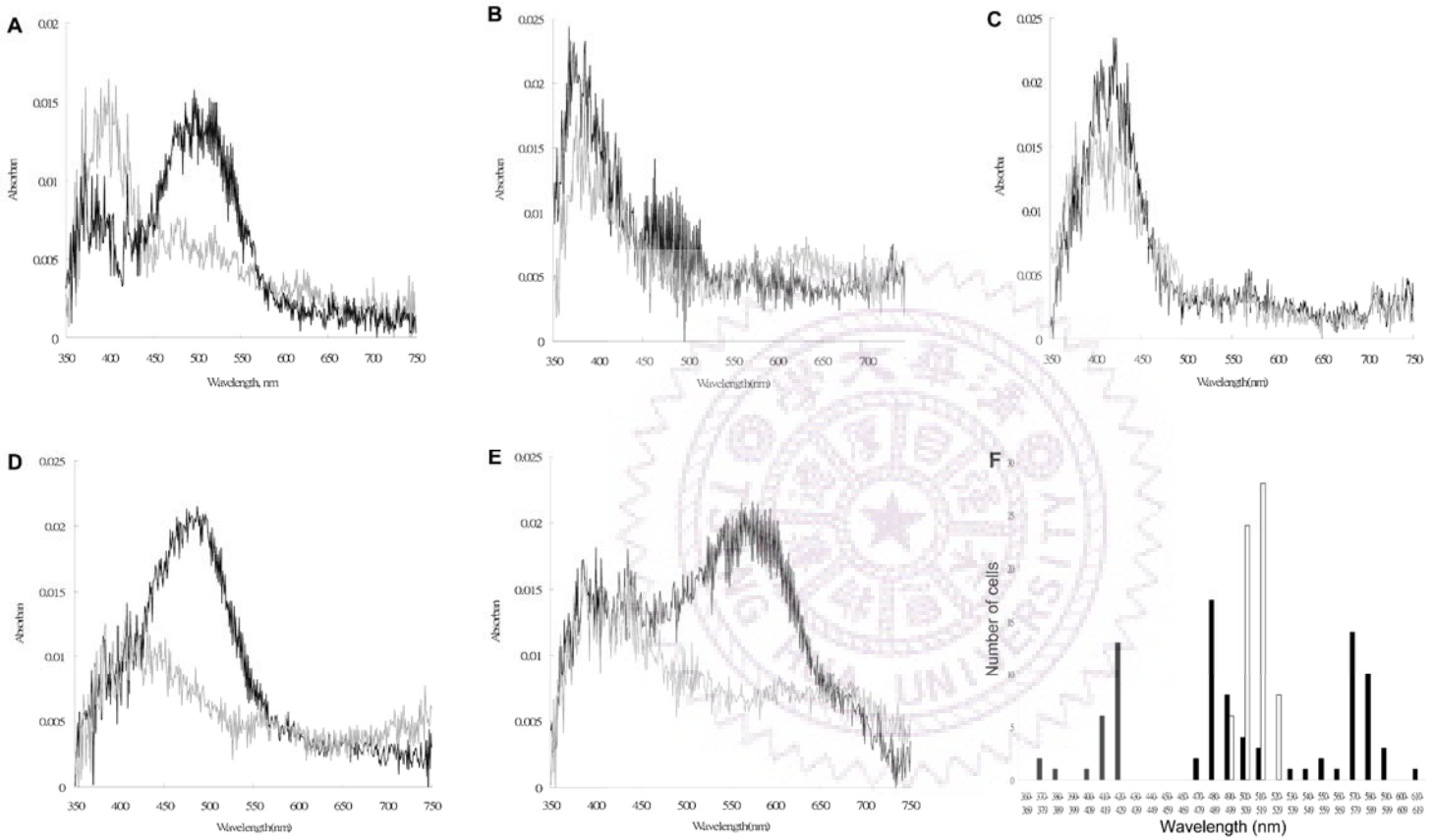


Fig 3. Mean absorbance spectra of rods and cones from *O. pachycephalus*. (A)Rods. (B) SWS1 cones. (C) SWS2 cones. (D) Rh2 cones. (E) LWS cones. Black lines: before bleaching; grey lines: after light-bleaching. The bleaching experiments were conducted as part of standard MSP protocol to ensure the cones or rods measured were functional. (F) Distribution histograms of λmax of individual photoreceptor cells. Filled bars are cone cell

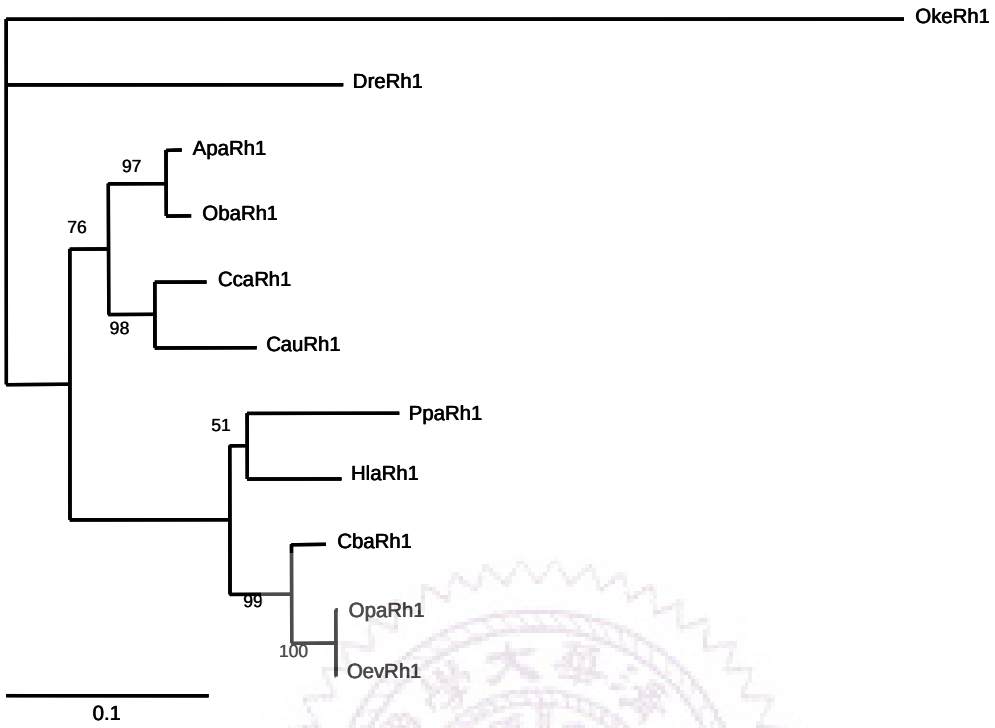
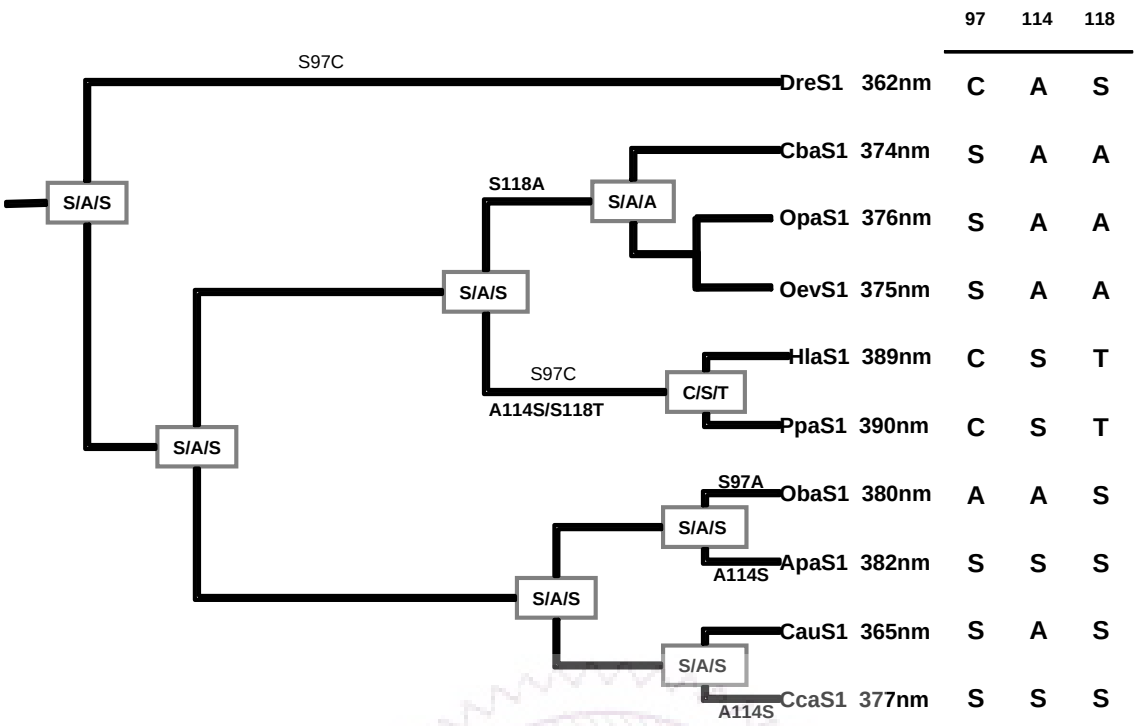


Fig 4. Neighbor joining trees of the Cyprinid Rh1 opsin gene based on the ML distances from model HKY+G (Hasegawa et al., 1985; Posada & Crandall, 1998), the best-fit model of modeltest.

A



B

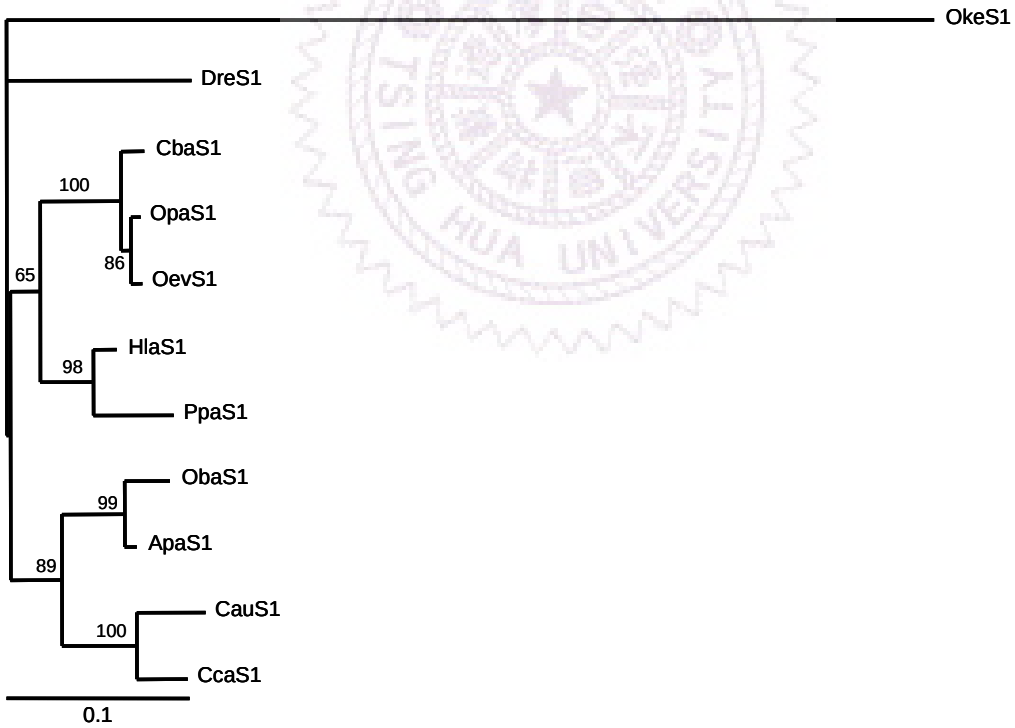


Fig 5. A. The evolutionary changes of the possible tuning sites of SWS1 genes that occurred in the phylogenetic clade derived from the ancestor. B. Neighbor joining trees of the Cyprinid SWS1 opsin gene based on the ML distances from model HKY+G (Hasegawa et al., 1985; Posada & Crandall, 1998), the best-fit model of modeltest.

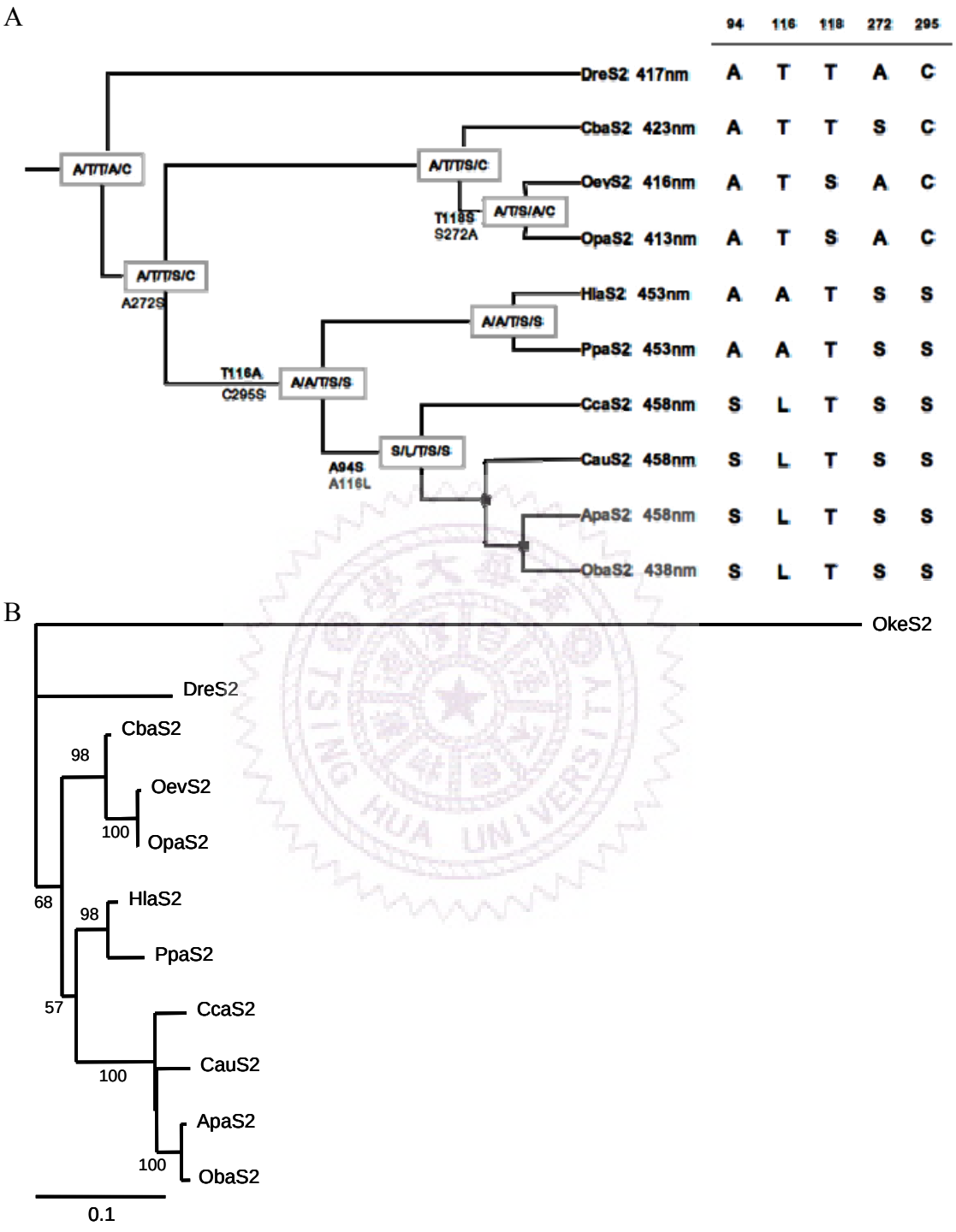


Fig 6. A. The evolutionary changes of the possible tuning sites of SWS2 genes that occurred in the phylogenetic clade derived from the ancestor. B. Neighbor joining trees of the Cyprinid SWS2 opsin gene based on the ML distances from model HKY+G (Hasegawa et al., 1985; Posada & Crandall, 1998), the best-fit model of modeltest.

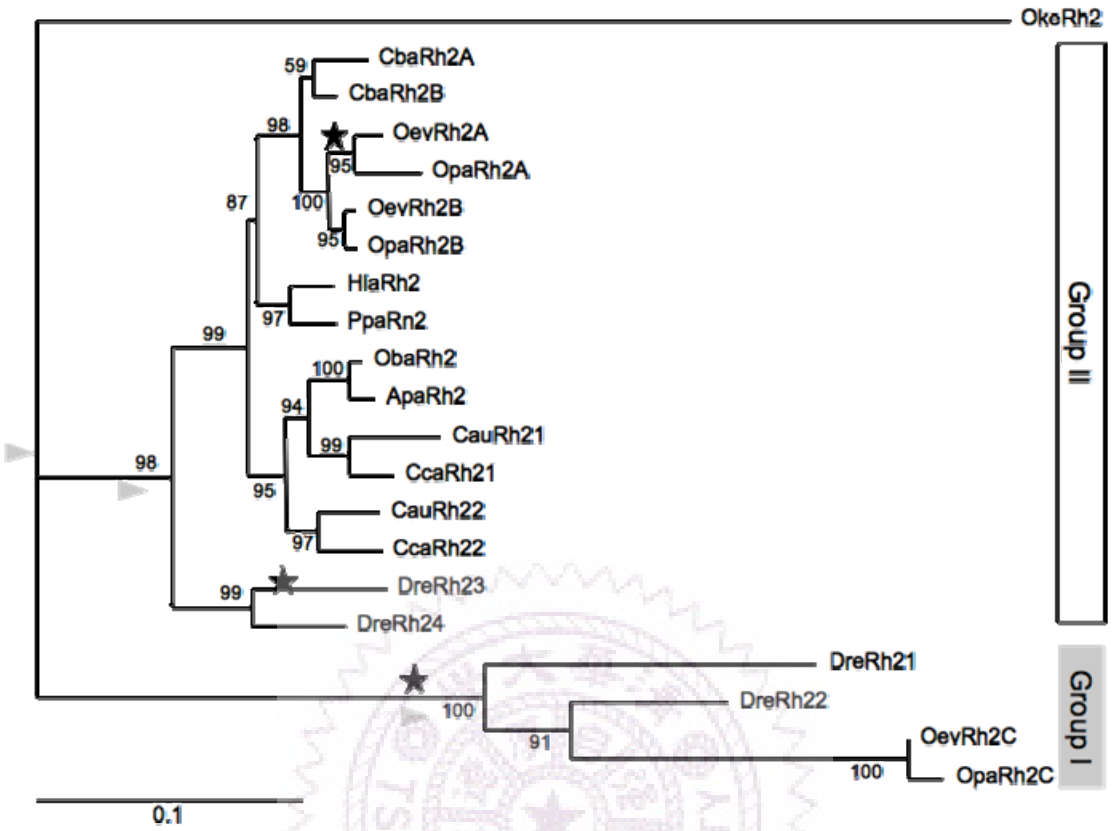


Fig 7. Neighbor joining trees of the Cyprinid Rh2 opsin gene based on the ML distances from model HKY+G (Hasegawa et al., 1985; Posada & Crandall, 1998), the best-fit model of modeltest. The black starts indicate E122Q substitution events. The grey triangles indicate gene duplication events occurred.

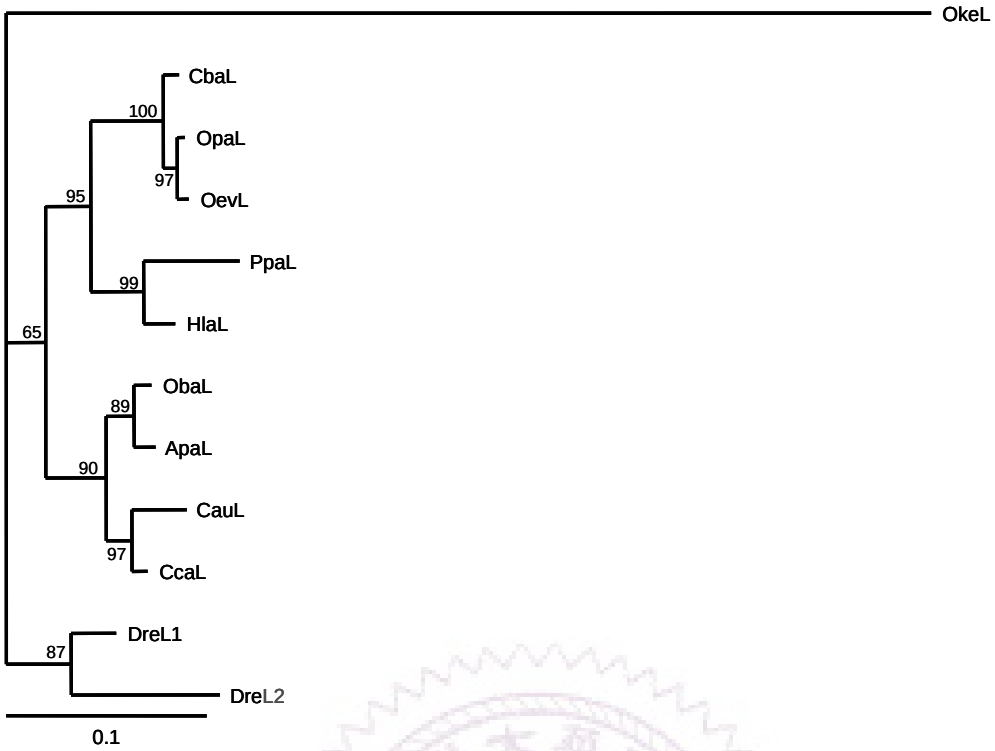


Fig 8. Neighbor joining trees of the Cyprinid LWS opsin gene based on the ML distances from model HKY+G (Hasegawa et al., 1985; Posada & Crandall, 1998), the best-fit model of modeltest.

Appendix 1. Amino acid sequence alignments of Rh1 genes of cyprnids.

	1	2	3	4	5	6	7	8	9	0	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
CbaCRh1	M	N	G	T	E	G	P	A	F	Y	V	P	M	S	N	E	T	G	V	V	R	S	P	Y	D	Y	P	Q	Y	Y	L	V	A	P	W	A	Y	G	C	L	A	A	Y	M	F	F	L	I	L	T
OpaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
OevCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
HlaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	E	.	.	.	F	A	.	.	.	.	.	.	.	.	.	.	I	.
PpaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	I	.
ObaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	I	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
ApaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
CcaRh1	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	A	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.
CauRh1	.	.	.	.	.	.	D	M	.	.	.	.	.	.	.	A	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	I	.
DreRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.	.	F	V	.	.	.	.	.	.	.	.	I	.

	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
CbaCRh1	G	F	P	V	N	F	L	T	L	Y	V	T	L	E	H	K	K	L	R	T	P	L	N	Y	S	L	L	N	L	A	V	A	D	L	F	M	V	F	G	G	F	T	T	T	M	Y	T	S	L	H
OpaCRh1	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
OevCRh1	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N
HlaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PpaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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CauRh1	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	I	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DreRh1	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
CbaCRh1	G	Y	F	V	F	G	R	L	G	C	N	L	E	G	F	F	A	T	L	G	G	E	M	A	L	W	S	I	V	T	L	A	I	E	R	W	L	V	V	C	K	P	I	S	N	F	R	F	G	E
OpaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
OevCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HlaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PpaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ObaCRh1	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	L	.	V	.	.	F	.	.	.	M	.	.	.	.	.	V	.	.	.	.	.	.	.
ApaCRh1	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	L	.	V	.	.	F	.	.	.	M	.	.	.	.	.	V	.	.	.	.	.	.	.
CcaRh1	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	L	.	V	.	.	F	.	.	.	M	.	.	.	.	.	V	.	.	.	.	.	.	.
CauRh1	.	.	.	.	.	.	.	V	.	.	.	P	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	L	.	V	.	.	F	.	.	.	M	.	.	.	.	.	V	.	.	.	.	.	.	.
DreRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	K	.	L	.	V	.	.	.	.	.	.	M	.	.	.	.	.	V	.	.	.	.	.	.	.

	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
CbaCRh1	N	H	A	I	I	G	V	V	F	T	W	V	M	A	C	S	C	A	V	P	P	L	V	G	W	S	R	Y	I	P	E	G	M	Q	C	S	C	G	V	D	Y	Y	T	R	A	E	G	Y	N	N
OpaCRh1	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
OevCRh1	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.
HlaCRh1	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PpaCRh1	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
CbaCRh1	E	S	F	V	I	Y	M	F	L	V	H	A	L	I	P	F	V	V	I	F	F	C	Y	G	R	L	V	C	A	V	K	D	A	A	A	Q	Q	Q	E	S	E	T	T	Q	R	A	E	R	E	V
OpaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
CbaCRh1	T	R	M	V	I	L	M	G	F	A	Y	L	V	C	W	L	P	Y	A	S	T	A	W	Y	I	F	T	H	K	G	T	S	F	G	P	V	F	M	T	I	P	A	F	F	A	K	T	S	A	V
OpaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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ApaCRh1	.	.	.	.	.	I	.	V	I	G	F	.	I	.	.	T	.	.	.	.	V	.	.	.	.	.	.	N	Q	.	S	E	.	.	.	L	.	.	.	L	.	.	.	.	.	.	.	A	.	.
CcaRh1	.	.	.	.	V	I	.	V	I	G	F	.	I	.	.	I	.	.	.	.	V	.	.	.	.	.	.	.	Q	.	S	E	.	.	.	.	.	.	.	V	.	.	.	.	.	.	S	A	.	.
CauRh1	.	.	.	.	V	I	.	V	I	G	F	.	I	.	.	I	.	.	.	.	V	.	.	.	.	.	.	.	Q	.	S	E	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	A	.	.
DreRh1	.	.	.	.	.	I	.	V	I	.	F	.	I	.	.	.	.	.	.	G	V	.	.	.	.	.	.	.	Q	.	S	E	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.

	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332
CbaCRh1	Y	N	P	V	I	Y	I	C	M	N	K	Q	F	R	H	C	M	I	T	T	L	C	C	G	K	N	P	F	E	E	E	E
OpaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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PpaCRh1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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DreRh1	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

The grey areas indicate the transmember domains of opsin gene, and the deep grey columns indicate the spectral tuning sites well known.

The site is numbered according to the sequences alignment directly.

Apx 2 Amino acid sequence alignments of SWS1 genes of cyprnids

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
OpaS1	M	D	A	W	A	V	Q	F	G	N	L	S	K	V	S	P	F	E	G	P	Q	Y	H	L	A	P	K	W	A	F	Y	L	Q	A	A	F	M	G	F	V	F	F	V	G	T	P	L	N	A	V
OevS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
OpaS1	V	L	F	V	T	M	K	Y	K	K	L	R	Q	P	L	N	Y	I	L	V	N	I	S	L	A	G	F	I	F	D	T	F	S	V	S	Q	V	F	V	S	A	L	R	G	Y	Y	F	L	S	P
OevS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
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	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
OpaS1	T	L	C	A	M	E	A	A	M	G	A	I	A	G	L	V	T	G	W	S	L	A	V	L	A	F	E	R	Y	V	V	I	C	K	P	F	G	S	F	K	F	G	Q	S	Q	A	M	G	A	V
OevS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
OpaS1	A	F	T	W	V	V	G	T	G	C	A	T	P	P	F	F	G	W	S	R	Y	I	P	E	G	L	G	T	A	C	G	P	D	W	Y	T	K	S	E	E	Y	N	S	E	S	Y	T	Y	F	L
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PpaS1	V	.	.	.	L	I	.	V	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ObaS1	V	.	.	.	I	.	.	V	.	.	.	.	.	.	.	W	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
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CauS1	.	L	.	.	I	I	.	I	.	.	.	.	.	.	.	W	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	T	.	.	.	.	.	.	.
CcaS1	.	L	.	.	.	I	.	I	.	.	.	.	.	.	.	W	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	N	.	.	.	.	C	.	.	.	.	.	.	.
DreS1	V	.	.	.	I	I	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
OpaS1	I	V	T	C	F	F	M	P	M	T	I	I	I	F	S	Y	S	Q	L	L	G	A	L	R	A	V	A	A	Q	Q	A	E	S	A	S	T	Q	K	A	E	K	E	V	S	R	M	V	I	V	M
OevS1	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.
CbaS1	T	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HlaS1	M	I	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	V	.	.
PpaS1	L	.	.	.	.	.	L	.	.	F	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	V	.	.
ObaS1	L	.	S	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
ApaS1	L	.	S	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
CauS1	L	.	S	.	.	M	.	.	I	M	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
CcaS1	M	.	.	.	.	I	L	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
DreS1	L	I	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	R	.	.	.	.	.	.	V	.	.

	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
OpaS1	V	G	S	F	V	L	C	Y	A	P	Y	A	A	T	A	M	W	F	A	N	A	E	E	S	N	K	D	Y	R	M	V	A	I	P	A	F	F	S	K	S	S	C	V	Y	N	P	L	I	Y	V
OevS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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HlaS1	.	.	.	Y	.	I	.	.	G	.	.	.	I	.	.	L	.	.	.	Y	.	D	D	.	.	.	.	.	.	L	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	A
PpaS1	.	.	.	Y	I	V	.	.	G	.	.	.	I	.	.	L	.	.	.	Y	.	D	D	.	H	.	.	F	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	A
ObaS1	.	.	.	.	I	V	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	S	L	.	.	.	.	.	.	.	.	.	.	.	.	.	A
ApaS1	.	.	.	.	I	V	.	.	G	.	.	.	I	.	.	L	.	.	.	Y	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	A
CauS1	.	.	.	.	.	V	.	.	G	.	.	.	I	.	.	L	Y	.	S	Y	.	.	D	.	.	.	.	.	.	L	.	.	.	S	L	.	.	.	.	.	.	.	.	.	.	.	.	.	A
CcaS1	.	.	.	Y	I	V	.	.	G	.	.	.	I	A	.	L	Y	.	G	Y	.	.	D	T	.	.	.	.	.	L	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	A
DreS1	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	Y	.	.	.	S	D	.	P	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A

	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336
OpaS1	F	M	N	K	Q	F	N	A	C	I	M	E	T	V	F	G	K	K	I	D	E	A	S	E	V	S	S	K	T	E	T	S	S	V	A	A
OevS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CbaS1	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
HlaS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PpaS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ObaS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ApaS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	S	.
CauS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	S	.
CcaS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	S	.
DreS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	S	.

The grey areas indicate the transmember domains of opsin gene, and the deep grey rows indicate the spectral tuning sites well known.

The site is numbered according to the sequences alignment directly.

Appendix 3. Amino acid sequence alignments of SWS2 genes of cyprnids

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
OevS2	M	K	-	-	-	A	I	P	-	-	-	E	F	Q	E	D	F	Y	I	P	I	A	L	D	T	N	N	I	S	A	F	S	P	F	N	V	P	Q	D	Y	L	G	H	H	G	V	F	I	A	M
OpaS2	.	.	-	-	-	.	.	.	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CbaS2	.	.	-	-	-	.	V	.	-	-	-	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	.	Y	.	.	.	T	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.
HlaS2	.	.	-	-	-	.	.	.	-	-	-	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.	.	.	Y	.	.	.	L	.	.	.	.	H	.	.	.	.	A	.	.	M	.	.
PpaS2	.	.	-	-	-	S	V	.	-	-	-	.	.	.	.	.	.	F	M	.	.	P	.	.	.	.	.	.	.	.	Y	.	.	.	L	.	.	.	.	H	.	.	.	.	A	I	Y	M	.	.
ObaS2	.	.	-	-	-	Q	V	.	-	-	-	.	.	H	.	.	.	.	.	.	.	P	.	.	F	.	.	L	.	.	Y	.	.	.	L	.	.	.	.	H	.	.	S	Q	.	M	.	M	V	.
ApaS2	.	.	-	-	-	Q	V	.	-	-	-	.	.	H	.	.	.	.	.	.	.	P	.	.	F	.	.	L	.	.	Y	.	.	.	L	.	.	.	.	H	.	.	S	Q	A	M	.	M	.	.
CauS2	.	.	-	-	-	Q	V	.	-	-	-	.	.	H	.	.	.	.	.	.	.	P	.	.	I	.	.	L	.	.	Y	.	.	.	L	.	.	.	.	H	.	.	N	Q	.	I	.	M	.	.
CcaS2	.	.	-	-	-	Q	.	.	-	-	-	.	.	H	.	.	.	.	.	.	.	P	.	.	I	.	.	L	.	.	Y	.	.	.	L	.	.	.	.	H	.	.	N	Q	.	.	.	M	.	.
DreS2	.	.	Q	Q	Q	Q	T	.	-	-	-	.	L	F	.	.	.	H	M	.	.	T	.	.	V	S	.	.	.	.	Y	.	.	.	L	.	.	.	.	H	.	.	.	S	.	.	.	M	G	.

	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
OevS2	A	A	F	M	F	F	L	F	V	A	G	T	A	I	N	V	L	T	I	A	C	T	I	Q	Y	K	K	L	R	S	H	L	N	Y	I	L	V	N	L	A	V	A	N	L	W	V	S	V	F	G
OpaS2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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HlaS2	.	.	.	T	.	.	.	.	I	.	.	.	.	.	.	I	.	.	.	V	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.
PpaS2	.	.	.	T	.	.	.	.	.	.	.	.	S	.	.	I	.	.	.	V	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.
ObaS2	.	V	.	.	.	V	I	.	.	G	.	A	S	.	.	I	.	.	.	V	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	I	.	.	.	F	.	A	I	.	.
ApaS2	.	V	.	.	.	V	I	.	.	G	.	A	S	.	.	I	.	.	.	V	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	I	.	.	.	F	.	A	I	.	.
CauS2	S	V	.	.	.	.	I	.	I	G	.	A	S	.	.	I	.	.	.	L	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	I	.	.	.	F	.	A	I	.	.
CcaS2	.	V	.	.	.	.	I	.	T	G	.	.	S	.	.	I	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	F	.	A	I	.	A
DreS2	S	.	.	.	L	.	.	.	I	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	S	.	.	.	.	.	.	.	.

	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
OevS2	S	S	I	A	F	Y	C	F	W	K	R	Y	F	I	L	G	V	T	A	C	K	I	E	G	F	T	S	S	I	G	G	M	V	S	L	W	S	L	A	V	V	A	L	E	R	W	L	V	I	C
OpaS2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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HlaS2	.	.	L	.	.	V	S	.	.	N	K	.	.	.	F	.	S	L	G	.	.	.	.	.	.	A	.	T	F	.	.	.	.	G	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.
PpaS2	.	.	L	.	.	V	S	.	.	N	K	.	.	.	F	.	S	L	G	.	.	.	.	.	.	A	.	T	F	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.
ObaS2	.	P	L	S	.	.	S	.	F	N	.	.	.	.	F	.	A	.	.	.	.	.	.	.	.	L	A	T	L	.	.	.	.	G	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.
ApaS2	.	.	L	S	.	.	S	.	F	N	.	.	.	.	F	.	A	.	.	.	.	.	.	.	.	L	A	T	L	.	.	.	.	G	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.
CauS2	.	P	L	S	.	.	S	.	F	N	.	.	.	.	F	.	A	.	.	.	.	.	.	.	.	L	A	T	L	.	.	.	.	G	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.
CcaS2	.	P	L	S	.	.	S	.	I	N	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	L	A	T	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.
DreS2	.	.	V	.	.	.	A	.	Y	.	K	.	.	V	F	.	P	I	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
OevS2	K	P	L	G	N	F	T	F	K	T	H	H	A	I	L	G	C	V	F	P	W	I	F	A	L	A	A	A	L	P	P	L	F	G	W	S	R	Y	I	P	E	G	L	Q	C	S	C	G	P	D
OpaS2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
OevS2	W	Y	T	T	N	N	K	Y	N	N	E	T	Y	V	M	F	L	F	C	F	C	F	A	V	P	F	S	T	I	V	I	C	Y	G	Q	L	L	I	T	L	K	L	A	A	K	A	Q	A	D	S
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OevS2	A	S	T	Q	K	A	E	R	E	V	T	K	M	V	V	V	M	V	F	G	F	L	L	C	W	G	P	Y	A	I	F	A	L	W	L	I	T	H	R	G	E	S	F	D	L	R	L	A	T	I
OpaS2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350
OevS2	P	S	C	L	C	K	A	S	T	V	Y	N	P	V	I	Y	V	V	M	N	K	Q	F	R	S	C	M	M	K	M	V	C	G	K	N	I	D	E	E	E	A	S	S	S	S	Q	V	T	Q	V
OpaS2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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	351	352	353	354	355	356	357
OevS2	S	S	V	A	P	E	K
OpaS2	.	.	.	.	.	.	.
CbaS2	.	.	.	.	.	.	.
HlaS2	.	.	.	.	.	.	.
PpaS2	.	.	.	.	.	.	.
ObaS2	.	.	.	.	.	.	.
ApaS2	.	.	.	.	.	.	.
CauS2	.	.	.	.	.	.	.
CcaS2	.	.	.	.	.	.	.
DreS2	.	.	.	.	.	.	.

The grey areas indicate the transmember domains of opsin gene, and the deep grey columns indicate the spectral tuning sites well known.

The site is numbered according to the sequences alignment directly.

Appendix 4. Amino acid sequence alignments of Rh2 genes of cyprnids.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
OevRh2A	-	-	-	G	T	E	G	N	N	F	Y	I	P	M	S	N	R	T	G	L	V	R	S	P	Y	E	Y	P	Q	Y	Y	L	A	E	P	W	Q	F	K	L	L	A	V	Y	M	F	L	L	I	C
OevRh2B	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.
OevRh2C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	.	G	F	.	.	.	.	.	.	.
OpaRh2A	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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OpaRh2C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	.	.	.	.	.	A	.	G	F	.	.	.	.	.	.	.
CbaRh2A	-	-	-	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	F	.	.	.
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CauRh21	M	-	N	.	.	.	.	K	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	L	.	L	.	F	.	M	S
CauRh22	M	-	N	.	.	.	.	.	.	.	.	V	.	L	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.
CcaRh22	M	-	N	.	.	.	.	.	.	.	.	V	.	L	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	K	.	.	V	.	.	.	.	I	.	F	.	.	.
CcaRh21	M	-	N	.	.	.	.	K	.	.	.	V	.	L	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	T	.	I	.	F	.	M	.
DreRh21	M	-	N	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	T	.	.	.	.	.	.	.	.	K	.	.	A	.	.	F	.	.	.	.	.	.	I
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OevRh2A	L	G	L	P	I	N	G	L	T	L	V	V	T	A	Q	H	K	K	L	R	Q	P	L	N	Y	I	L	V	N	L	A	V	A	G	T	I	M	V	C	F	G	F	T	V	T	F	Y	T	A	I
OevRh2B	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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OevRh2A	N	G	Y	F	V	L	G	P	I	G	C	A	V	E	G	F	M	A	T	L	G	G	Q	V	S	L	W	S	L	V	V	L	A	I	E	R	Y	I	V	V	C	K	P	M	G	S	F	K	F	S
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OevRh2A	N	T	H	A	L	A	G	I	A	F	T	W	I	M	A	M	A	C	A	A	P	P	L	V	G	W	S	R	Y	I	P	E	G	M	Q	C	S	C	G	P	D	Y	Y	T	L	N	P	D	Y	N
OevRh2B	S	S	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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OevRh2A	N	E	S	Y	V	I	Y	M	F	S	C	H	F	I	L	P	V	T	V	I	F	F	T	Y	G	R	L	V	C	T	V	K	A	A	A	A	Q	Q	Q	D	S	A	S	T	Q	K	A	E	R	E
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OevRh2A	V	T	K	M	V	I	L	M	V	L	G	F	L	V	A	W	T	P	Y	A	T	V	A	A	W	I	F	F	S	K	G	A	S	F	T	A	Q	S	M	A	V	P	A	F	F	S	K	T	S	A
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OevRh2A	L	Y	N	P	V	I	Y	V	L	L	N	K	Q	F	R	N	C	M	L	T	T	L	F	C	G	K	N	P	L	G	D	E	E	S	S	T	V	S	T	S	K	T	E	V	S	S	V	S	P	A
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The light grey areas indicate the transmember domains of opsin gene, and the deep grey columns indicate the spectral tuning sites well known.

The site is numbered according to the sequences alignment directly.

Appendix 5. Amino acid sequence alignments of LWS genes of cyprnids

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
OevL	M	A	E	Q	W	G	D	A	I	F	A	A	R	R	K	G	D	E	T	T	R	E	A	M	F	V	Y	T	N	S	N	N	T	K	D	P	F	E	G	P	N	Y	H	I	A	P	R	W	V	Y
OpaL	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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OevL	N	L	A	T	L	W	M	F	F	V	V	V	A	S	T	F	T	N	G	L	V	L	V	A	T	A	K	F	K	K	L	R	H	P	L	N	W	I	L	V	N	L	A	V	A	D	L	A	E	T
OpaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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OevL	L	L	A	S	T	I	S	V	T	N	Q	F	F	G	Y	F	I	L	G	H	P	M	C	V	F	E	G	Y	T	V	S	V	C	G	I	A	G	L	W	S	L	T	M	I	S	W	E	R	W	V
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	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
OevL	V	V	C	K	P	F	G	N	V	K	F	D	G	K	W	A	S	A	G	I	V	F	T	W	V	W	S	A	I	W	C	A	P	P	I	F	G	W	S	R	Y	W	P	H	G	L	K	T	S	C
OpaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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HlaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	I	.	C	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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ApaL	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	I	.	S	.	.	.	A	.	F	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.
CauL	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	I	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.
CcaL	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	I	.	S	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.
DreL1	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	I	.	S	.	.	.	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DreL2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	S	.	.	.	A	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
OevL	G	P	D	V	F	S	G	S	E	D	P	G	V	Q	S	Y	M	I	V	L	M	V	T	C	C	L	I	P	L	A	I	I	I	L	C	Y	L	A	V	F	M	A	I	H	A	V	A	Q	Q	Q
OpaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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PpaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	F	.	.	.	G	V	.	.	F	.	.	I	.	.	.	L	.	.	.	.	.	.	.	.	.
HlaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	L	.	.	.	.	.	.	.	.	.
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ApaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	I	L	.	.	.	V	.	.	.	.	.	I	.	.	W	L	.	.	R	.	.	.	.	.	.
CauL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	I	.	.	.	.	.	.	.	.	.	.	I	.	.	W	L	.	.	R	T	.	.	.	.	.
CcaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	I	.	.	.	.	.	.	.	.	.	.	I	.	.	W	L	.	.	R	.	.	.	.	.	.
DreL1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	I	.	.	.	I	.	.	.	.	.	.	.	.	.	.	I	.	.	Y	L	.	.	.	.	.	.	.	.	.
DreL2	.	.	.	.	.	G	.	N	.	.	.	.	.	.	.	.	.	L	.	.	.	I	.	.	.	I	L	.	.	.	.	.	.	.	.	.	I	.	.	.	L	.	.	.	.	.	.	.	.	.

	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
OevL	K	D	S	E	S	T	Q	K	A	E	K	E	V	S	R	M	V	V	V	M	I	L	A	Y	C	V	C	W	G	P	Y	T	F	F	A	C	F	A	A	A	N	P	G	Y	A	F	H	P	L	A
OpaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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PpaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	F	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HlaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	F	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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CauL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	F	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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DreL1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	.	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DreL2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	F	.	L	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350
OevL	A	A	M	P	A	Y	F	A	K	S	A	T	I	Y	N	P	V	I	Y	V	F	M	N	R	Q	F	R	V	C	I	L	Q	L	F	G	K	K	V	D	D	G	S	E	V	S	T	S	K	T	E
OpaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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HlaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ObaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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CcaL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DreL1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DreL2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

	351	352	353	354	355	356	357
OevL	V	S	S	V	A	P	A
OpaL	.	.	.	.	.	.	.
CbaL	.	.	.	.	.	.	.
PpaL	.	.	.	.	.	.	.
HlaL	.	.	.	.	.	.	.
ObaL	.	.	.	.	.	.	.
ApaL	.	.	.	.	.	.	.
CauL	.	.	.	.	.	.	.
CcaL	.	.	.	.	.	.	.
DreL1	.	.	.	.	.	.	.
DreL2	.	.	.	.	.	.	.

The grey areas indicate the transmember domains of opsin gene, and the deep grey columns indicate the spectral tuning sites well known. The site is numbered according to the sequences alignment directly.