Differentially-expressed *opsin* genes identified in *Sinocyclocheilus* cavefish endemic to China

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Abstract

Eye degeneration is a common troglomorphic character of cave-dwelling organisms. Comparing the morphology and molecular biology of cave species and their close surface relatives is a powerful tool for studying regressive eye evolution and other adaptive phenotypes. We compared two co-occurring and closely-related species of the fish genus *Sinocyclocheilus*, which is endemic to China and includes both surface- and cave-dwelling species. *Sinocyclocheilus tileihornes*, a cave species, had smaller eyes than *Sinocyclocheilus angustiporus*, a surface species. Histological and immunohistochemical analyses revealed that the cavefish had shorter cones and more disorderly rods than did the surface-dwelling species. Using quantitative PCR and *in situ* hybridization, we found that *rhodopsin* and a long-wavelength sensitive *opsin* had significantly lower expression levels in the cavefish. Furthermore, one of two short-wavelength-sensitive *opsins* was expressed at significantly higher levels in the cavefish. Changes in the expression of *opsin* genes may have played a role in the degeneration of cavefish eyes.

Keywords

*Sinocyclocheilus*; Cavefish; Eye; Retina; Opsin

Cave dwelling animals that live in perpetual darkness have evolved a number of striking troglomorphic characters. The most common phenotypes are the reduction or loss of eyesight and pigmentation (Jeffery, 2009; Poulson and White, 1969; Protas et al., 2006). Among vertebrates, most research has focused on the regressive evolution of the teleost *Astyanax mexicanus* in North America. The eye degeneration of cave dwelling *Astyanax* is induced by lens apoptosis caused by the over-expression of *sonic hedgehog* (*shh*) (Yamamoto et al., 2004). Researchers also found a mutation in the *oca2* gene that blocked development of melanin-containing pigment cells (Protas et al., 2006). However, the general importance of these phenomena and the genetic basis of eye degeneration remains unclear.

*Sinocyclocheilus* (Cypriniformes, Cyprinidae), a freshwater teleost genus, is endemic to China and includes many cave-dwellers among its 55 known species. This genus is only found in the central eastern Yungui Plateau and its surrounding region, Guangxi, in southwestern China (Xiao et al., 2005; Chen et al., 2009; Wu et al., 2010). *Sinocyclocheilus* is remarkable in its high species diversity and troglomorphic character variation within the genus. Thus, *Sinocyclocheilus* is an ideal model system for investigating the evolutionary biology of, and natural selection in, troglobionts.
Most vertebrate retinas consist of two types of photoreceptor cells, rod and cone cells, which contain different visual pigments (Swaroop et al., 2010). The primary components of visual pigments are opsins, a group of light-sensitive membrane-bound G-protein-coupled receptors that mediate the conversion of energy in photons into an electrochemical signal (Fain et al., 2010). Opsins include rhodopsin (Rho) and cone opsins, which are responsible for dim vision in rod cells and color vision in cone cells, respectively (Shichida and Matsuyama, 2009). The cone opsins contain four subgroups, which form primarily red-sensitive (long-wavelength), green-sensitive (middle-wavelength), blue-sensitive (shorter wavelength, SWS2), and UV/violet-sensitive (shorter wavelength, SWS1) pigments. (Chinen et al., 2003; Terakita, 2005).

Li and colleagues analyzed the molecular evolution of rhodopsin and two cone opsin genes in several Sinocyclocheilus cavefishes (Li and He, 2009; Li et al., 2009). They found that rhodopsin was subject to relaxed purifying selection, while LWS1 (long wavelength sensitive-1) had experienced strong positive selection that increased the substitution rate in the pocket-forming residues. No positive selection was observed in SWS2 (short wavelength sensitive-2). During eye degeneration in cavefish, morphological and functional changes may result from both divergence in coding DNA sequences and by differential levels of gene expression. While the expression of rhodopsin, SWS1, MWS (middle wavelength sensitive), and LWS are reduced in the eye of cave populations relative to surface populations of Astyanax mexicanus (Strickler and Jeffery, 2009; Tobler et al., 2010), it is unclear whether opsin gene expression is reduced in Sinocyclocheilus cavefishes. To address this, we detected morphological differences between a surface dwelling species S. angustiporus and a cavefish S. tileihornes using antibody markers to label rod and cone cells. In addition, we prepared eye cDNA samples from surface- and cave-dwelling species and identified differentially-expressed opsin genes in cave species relative to surface species.

1 Materials and Methods

1.1 Sample collection and eye size analysis

Both S. tileihornes cavefish and S. angustiporus surface fish were collected from the same sinkhole in Huangnihe River in Agang Town. The sinkhole connects underground to Huangnihe River, Luoping County, Yunnan Province, China (Fig. 1). The Huangnihe River feeds the Nanpanjiang River, which is the biggest tributary of the Xijiang River in the Pearl River basin. The cavefish studied here is only known to inhabit this sinkhole, whereas the surface species is widely distributed in Huangnihe River. Agang sinkhole has a shorter day length than the surface water but still receives natural light during the day (~ 3 h/day in summer).

After capture, individuals of both species were sacrificed and immediately measured (eye diameter and standard body length). After removing associated tissues, the left eye was placed in RNAlater (Ambion, Carlsbad, CA, USA) for RNA extraction and the right eye was fixed with 4% PFA (paraformaldehyde in phosphate buffer) for immunohistochemistry. Laboratory stocks of Sinocyclocheilus were exposed to a short light cycle (3 hours light/21 hours dark). To determine whether cavefish could respond to light stimulation, we observed behavior responds of both species when they were exposed to rapid changes in sunlight intensity or stimulated using a laser pointer.
1.2 Histological analysis and in situ hybridization (ISH)

After being fixed in 4% PFA, the eyes of cave and surface fish were stored in 30% sucrose at 4°C overnight prior to cryosectioning. The sections were stained with hematoxylin and eosin (H&E).

For immunohistochemistry, we labeled the cone and rod photoreceptors with the monoclonal antibodies Zpr1 and Zpr3 (Zebrafish International Resource Center, Eugene, OR, USA; gift from Jennifer Phillips) (Vihelic et al., 1999). Zpr1 labels an unknown surface epitope of red/green double cones whereas Zpr3 labels rhodopsin expressed in the rods in Zebrafish. These antibodies were used at 1:200 dilution. We used ToPro3 (Invitrogen, Carlsbad, CA, USA) to label DNA. Fluorescent images were taken using a Bio-Rad Radiance 2100MP confocal microscope system. We compared the photoreceptor length between surface fish and cavefish using a t-test. All data met the assumption of normality.

For ISH, RNA probes were generated from partial surface fish rhodopsin cDNA. The following primers were used to clone probein PCR reactions: rhodopsin-forward: 5’-CATCATGGGTGTTGCCCTT-3’ and rhodopsin-reverse: 5’-ACACGGAGCTGGAAGACAC-3’. ISH was performed using digoxygenin-labeled probes with the color visualized using NBT/BCIP. Slides were analyzed under a Nikon E600 microscope (Tokyo, Japan). The ISH experiment was repeated three times.

1.3 RNA isolation, reverse transcription, and Real-time PCR

We used real-time PCR to determine whether opsin gene expression levels were reduced in S. tileihornes relative to S. angustiporus. The eyes of surface and cavefish adults that had been preserved in RNAlater solution immediately after wild capture were removed from solution and total RNA was isolated with TRIzol reagent (Invitrogen). Purification of poly-A mRNA was performed using MicroPoly (A) Purist™ (Ambion) following the manufacturer’s protocol. Samples of cDNA samples were created using the Superscript III First-Strand Synthesis System (Invitrogen).

Real-time PCR was performed using SYBR Green (TaKaRa, Kyoto, Japan) chemistry. The primer sequences were as follows: β-actin: 5’-GAAGATCAAGATCATTGCC-3’, and 5’-ATGTCATCTTGTTCCGAGAGG-3’; rhodopsin: 5’-AACCCGTGACTACACTCTG-3’, and 5’-CTTTGTGGTCTCTGCGTGTCT-3’; SWS1: 5’-CCTGTGTAACATCCCTCCTG-3’, and 5’-CCATCCCGAAATGGAGGAGG-3’; SWS2: 5’-TCGGAGGGACTTCAATAC-3’, and 5’-AATTTTACATGCTGTCGCCC-3’; MWS4: 5’-GCTTGTTTGCAGCTCAAGG-3’, and 5’-ATTAAAGAGATCCACGCACGCC-3’; LWS1: 5’-GTGGGCCACAGCCAATTTAAGG-3’, and 5’-CCAGAGAGTAGCAATCAGTCAAAGG-3’. The expression of β-actin (actin, beta 1) was used as reference. The reference samples were used in each assay and the expression levels of target genes were quantified relative to the reference sample. Each PCR run included three separate cDNA samples for each species. Each reaction (samples and primers) was repeated at least three times. Statistical analysis of the data was performed using a two-tailed Student’s t-test using Microsoft Excel. * P<0.05, ** P<0.01.

2 Results

2.1 Eye degeneration in the cavefish S. tileihornes

Although both S. angustiporus and S. tileihornes were caught in the same sinkhole, they have distinct morphological differences. Typically, the surface fish S. angustiporus having normal eyes (Fig. 2A) are found outside caves or sinkholes (Fig. 1). S. tileihornes, a close relative of S. angustiporus, is a cave-dwelling species and lives only in a sinkhole in Agang.
Town (Fig. 1). It was named for its tile-like horn (Fig. 2B), the function of which is unknown. The cavefish *S. tileihornes* have retained small eyes on the body surface, but there is no obvious response to sudden sunlight and laser stimulations, unlike surface fish. The relative eye diameter (eye diameter/standard body length) of *S. tileihornes* (0.0298 ± 0.004, n = 5) was significantly smaller than that of *S. angustiporus* (P<0.01 (0.0616 ± 0.006, n =6)).

The results of H&E-staining suggested that the basic eye structures were formed in cavefish (Fig. 2C, D). However, the retina of the cavefish was thinner than that of the surface species (Fig. 3A–F). The immunohistochemistry revealed that cones and rods had developed in both species, but had aberrant morphology in the cavefish. The cones were 48.5% (P < 0.001) shorter in the cavefish (n=5) relative to the surface species (n=6) (Fig. 3A, B). There was no obvious difference in the length of the rods between surface and cave species. But, the rods in the cavefish (Fig. 3D) were extremely disorganized relative to those of surface fish (Fig. 3C). We found that the arrangement of cavefish rods was disorganized, and the positive signals of Zpr3 partially overlapped with the outer nuclear layer (ONL) (Fig. 3D). Using ISH, we noted that the expression of rhodopsin mRNA was strong and orderly in the surface fish retina (Fig. 3E). However, its expression was significantly reduced in the cavefish retina (Fig. 3F). As a response light stimulating, surface fish swim away rapidly, while cavefish's swimming is unaffected.

### 2.2 Differential opsin gene expression in surface fish and cavefish eyes

Although both eye size and retinal thickness were significantly reduced in *S. tileihornes*, the expression levels of the opsin genes were not always concordant with them. Using real-time PCR, we observed a significant reduction in the expression of rhodopsin (P < 0.001) and LWS1 (P < 0.01) in cavefish, whereas SWS1 was expressed at significantly higher levels in the cave species (P < 0.001). The other two opsins, SWS2 and MWS4, had similar expression levels in the surface and cave species (Fig. 4).

### 3 Discussion

We used the cavefish *S. tileihornes* and its close surface relative *S. angustiporus* as experimental animal models to study the evolution of regressive eye traits. To do this, we compared the morphological characters of the eye and opsin gene expression patterns between the two species. Our results show that the cones of cavefish were shorter and their rods more disorganized than in the surface species. We also noted that the visual function of *S. tileihornes* eyes was very weak or absent. In future, we propose to use electroretinography to measure electrical responses of cavefish retina to the light signal at different wavelengths.

Of the five opsin genes we detected in this study, three were differentially expressed between surface and cave species. Expression of the *LWS1* gene was significantly reduced in cavefish relative to surface fish (Fig. 4). A previous study concluded that the reduction of *LWS* expression in cave-dwelling Astyanax was caused by the attenuation of long-wavelength light in dim environments (Tobler et al., 2010). This logic may also explain the down-regulation of *LWS1* expression in *S. tileihornes*, which was caught in low-light conditions in a sinkhole. We also noted a significant reduction in the expression level of rhodopsin, a rod opsin, in the eyes of cavefish (Fig. 4). Rhodopsin protein is normally synthesized in the ER, modified in the Golgi, and finally transported to the outer segments of rod cells (Deretic, 2006), as seen in the surface fish (Fig. 3C). In contrast, rhodopsin was distributed more broadly in the cavefish rod cells. Part of the rhodopsin protein may have accumulated elsewhere in the cavefish rod cells out of the outer segment for unknown reasons (Fig. 3D). The results of in situ hybridization also confirm that rhodopsin mRNA transcription had decreased in the cavefish retina (Fig. 3E, F).
Rhodopsin also plays a role in photoreceptor development and differentiation. Homozygotes of *Rhodopsin*-gene knockout mice fail to develop retinal rod outer segments and lose their photoreceptors; heterozygotes exhibit some disorganization of their photoreceptors and a shortening of the outer segments with age (Humphries et al., 1997). Based on these data, we hypothesize that the down-regulation of *rhodopsin* occurring during evolutionary adaptation to diminished light in the sinkhole might play a role in eye degradation in cavefish.

Expression of rod and cone *opsins* can be regulated by a broad range of transcriptional factors, including *crx* (cone-rod homeobox), *rx* (retinal homeobox), *nrl* (neural retina leucine zipper), *thrb* (thyroid hormone receptor beta), *atax7* (ataxin 7-like 3), and *Ep300* (E1A binding protein p300) (Hennig et al., 2008; Peng and Chen, 2007). In future research, we propose to identify the changes on the promoter region of the *rhodopsin* gene and candidate genes that are responsible for the differential expression of *opsin* genes in the cavefish *S. tileihornes*.

A number of conclusions can be drawn from our results. First, the zebrafish monoclonal antibodies Zpr1 and Zpr3 can specifically recognize antigens of cones and rods, respectively, in *Sinocyclocheilus*. Second, the eyes of the cavefish *S. tileihornes* are smaller than that of the surface fish *S. angustiporus* and the rods and cones exhibit disorder or are short, respectively in cavefish. Third, based on real-time PCR and ISH, the expression of *rhodopsin* and *LWS1* is strongly downregulated in the cavefish eye. Taken together our results are consistent with occupancy of the low light environment in the sinkhole. Our research provides insight into the mechanisms of initial eye degeneration.

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Fig. 1. Collection sites for the surface-dwelling fish *Sinocyclocheilus angustiporus* and the cavefish *Sinocyclocheilus tileihornes*

The rectangle on the inset map of China indicates the location of the larger map. Black circles indicate the known distribution of *S. angustiporus*; *S. tileihornes* is endemic to Agang sinkhole (red circle), where both species were collected for this study.
Fig. 2. The eyes of *S. tileihornes* were significantly smaller than those of *S. angustiporus*
Surface fish *Sinocyclocheilus angustiporus* (A) and cavefish *Sinocyclocheilus tileihornes* (B). Sections of adult surface (C) and cavefish (D) eyes. The results of H&E-staining revealed that cavefish had normal eye structure. C: Cornea. I: Iris. NR: Neural retina. Scale bar in (A, B): 1 cm, (C, D): 1 mm.
Fig. 3. Retina degeneration in the cavefish *S. tileihornes*

Representative double-labeling in the retina of surface (A, C) and cavefish (B, D) eyes; cell nuclei are dye-labeled red; Zpr1 and 3 are indicated in green. The cones (Zpr1; B) of cavefish were shorter than those of surface fish (A). Compared with the rods of surface fish (Zpr3; C), the rods of cavefish were extremely disorganized (D). The arrangement of the rods was disorganized in the cavefish retina, and the positive signals of Zpr3 partially overlapped with the ONL region. We performed *in situ* hybridization using probes for surface fish *rhodopsin* on retinal sections from both species (E, F). The blue region indicates the expression of *rhodopsin* (black arrow). The *rhodopsin* was strongly expressed in the surface fish retina (E) but the expression was significantly reduced in the cavefish retina (F).
ONL: Outer nuclear layer. INL: Inner nuclear layer. GCL: Ganglion cell layer. RPE: Retina pigment epithelium. Scale bar (A–F): 50 µm.
Fig. 4. Relative expression levels of rod and cone opsins for surface and cave species
The expression levels of rhodopsin, opsin 1 short-wave-sensitive 1 (SWS1) and 2 (SWS2), opsin 1, medium-wave-sensitive 4 (MWS4), and opsin 1 long-wave-sensitive 1 (LWS1) were quantified and normalized to that of bactin. Relative expression values represent the mean ± SD of at least three independent experiments. * P < 0.01, ** P < 0.001.