Visual Neuroscience (2011), Page 1 of 11.

doi:10.1017/S0952523811000010

A novel rhodopsin-like gene expressed in zebrafish retina

JAMES M. MORROW,[1]* SAVO LAZIC,[2]* AND BELINDA S.W. CHANG[1,3,4]

[1]Department of Cell & Systems Biology, University of Toronto, Toronto, Ontario, Canada
[2]Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada
[3]Department of Ecology & Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada
[4]Centre for the Analysis of Genome Evolution and Function, University of Toronto, Toronto, Ontario, Canada

(RECEIVED October 03, 2010; ACCEPTED December 30, 2010)

Abstract

The visual pigment rhodopsin (rh1) constitutes the first step in the sensory transduction cascade in the rod photoreceptors of the vertebrate eye, forming the basis of vision at low light levels. In most vertebrates, rhodopsin is a single-copy gene whose function in rod photoreceptors is highly conserved. We found evidence for a second rhodopsin-like gene (*rh1-2*) in the zebrafish genome. This novel gene was not the product of a zebrafish-specific gene duplication event and contains a number of unique amino acid substitutions. Despite these differences, expression of *rh1-2 in vitro* yielded a protein that not only bound chromophore, producing an absorption spectrum in the visible range ($\lambda_{max} \approx 500$ nm), but also activated in response to light. Unlike *rh1*, *rh1-2* is not expressed during the first 4 days of embryonic development; it is expressed in the retina of adult fish but not the brain or muscle. Similar *rh1-2* sequences were found in two other *Danio* species, as well as a more distantly related cyprinid, *Epalzeorhynchos bicolor*. While sequences were only identified in cyprinid fish, phylogenetic analyses suggest an older origin for this gene family. Our study suggests that *rh1-2* is a functional opsin gene that is expressed in the retina later in development. The discovery of a new previously uncharacterized opsin gene in zebrafish retina is surprising given its status as a model system for studies of vertebrate vision and visual development.

Keywords: Visual pigment, Gene duplication, Teleost vision

Introduction

Opsins are members of the G protein-coupled receptor (GPCR) superfamily that is distinguished by a Schiff base linkage between a conserved lysine residue and a retinaldehyde chromophore (Ebrey & Koutalos, 2001). The photosensitive chromophore absorbs light to activate the opsin protein, initiating a signaling cascade in the photoreceptor cell (Burns & Baylor, 2001; Shichida & Morizumi, 2007). Gene duplication events throughout vertebrate evolution have given rise to several opsin families. There are five groups of visual opsins, including rhodopsin (*rh1*) and four cone opsin groups: rhodopsin-like (*rh2*), short-wavelength sensitive 1 (*sws1*), short-wavelength sensitive 2 (*sws2*), and long-wavelength sensitive (*lws*). Visual opsins are expressed in photoreceptor cells of the retina and, while being primarily responsible for initiating the visual transduction cascade (Menon et al., 2001), are also involved in nonvisual processes (Altimus et al., 2008; Lall et al., 2010). Nonvisual opsins, such as pinopsin, melanopsin, and exorhodopsin, are expressed in a wide variety of tissues and thought to only be involved in nonvisual light-dependant biological processes, such as the circadian system (Newman et al., 2003; Pierce et al., 2008; Peirson et al., 2009).

GPCRs comprise one of the largest known families of integral membrane proteins. Rhodopsin (rh1), a highly characterized GPCR, is the visual opsin that initiates dim-light vision in vertebrates (Okawa & Sampath, 2007). Because of its stability and abundance in rod photoreceptor outer segments, rhodopsin is amenable to detailed studies of structure and function, often through site-directed mutagenesis and *in vitro* expression (Imai et al., 2007; Knierim et al., 2007; Nickle & Robinson, 2007). Rhodopsin was also the first opsin with a high-resolution crystal structure of its dark state (Palczewski et al., 2000), with crystal structures of both its activated (Scheerer et al., 2008) and chromophore-free (Park et al., 2008) states also having been resolved. Due to high levels of sequence and structural conservation among GPCRs (Rosenbaum et al., 2009), data collected and methods developed on rhodopsin have also facilitated further studies on other visual and nonvisual opsin families (Teller et al., 2003; Ramon et al., 2009).

It is rare for a vertebrate to have more than one *rh1* gene. Despite two ancestral genome duplication events that are thought to predate the vertebrate radiation (Dehal & Boore, 2005), the majority of vertebrates maintain only a single copy of *rh1*. In contrast, gene numbers of some cone opsin groups can vary greatly in vertebrates. No mammalian *rh2* opsins have been identified (Trezise & Collin, 2005; Bowmaker, 2008), while many other species have up to four *rh2* genes (Chinen et al., 2003; Parry et al., 2005; Matsumoto et al., 2006). Derived primates have experienced *lws* opsin duplication (Dulai et al., 1999), as have teleost fish, where it is not uncommon to have at least four *lws* genes (Weadick & Chang, 2007; Owens et al., 2009). However, having multiple *rh1* genes is a very unique trait. Comprising 96% of living fish species due to an adaptive radiation after a genome duplication event 150 million years ago (Taylor et al.,

Address correspondence and reprint requests to: Dr. Belinda Chang, Departments of Ecology & Evolutionary/Cell & Systems Biology, University of Toronto, 25 Harbord Street, Toronto, Ontario, M5S 3G5, Canada. E-mail: belinda.chang@utoronto.ca

*These authors contributed equally to this work.

2003), teleosts are the only known group of vertebrates containing species expressing multiple *rh1* genes, although most are still believed to have only one. One example is the eel, having multiple species that express a second *rh1* gene during maturation, coupled with its migration from freshwater to seawater (Beatty, 1975; Hope et al., 1998; Zhang et al., 2000, 2002). Another example is the short fin pearleye (*Scopelarchus analis*), a deep-sea teleost that expresses up to two *rh1* genes depending on its lifecycle (Pointer et al., 2007). Our investigation of the genome of zebrafish (*Danio rerio*), a well-studied teleost and model vertebrate, revealed an open reading frame for a previously uncharacterized *rh1*-like gene, suggesting yet another case of multiple *rh1* genes in a species of teleost fish.

With version Zv9 of the zebrafish genome project having recently been made available, researchers are increasingly identifying and characterizing zebrafish genes and gene families of interest, including opsins. So far, nine visual opsins have been identified in zebrafish, including *rh1*, four *rh2*, *sws2*, *sws1*, and two *lws* (Chinen et al., 2003), a large complement even among teleosts. Additionally, many nonvisual opsins have been discovered in zebrafish, including exorhodopsin (Mano et al., 1999), two melanopsins (Bellingham et al., 2002, 2006), teleost multiple tissue (tmt) opsin (Moutsaki et al., 2003), and two vertebrate ancient long (VAL) opsins (Kojima et al., 2008); a nonannotated sequence for encephalopsin has also been highlighted in the zebrafish genome. The advantage of studying opsin genes in the zebrafish is not only due to the availability of a genome sequence, but also to the considerable body of work assembled on the zebrafish visual system (Bilotta & Saszik, 2001; Neuhauss, 2003; Fleisch & Neuhauss, 2006; Fadool & Dowling, 2008), and to the variety of existing and emerging tools available to study genes of interest in zebrafish (Amsterdam & Becker, 2005; Amacher, 2008; Halpern et al., 2008).

In this study, we used zebrafish as a model to identify and characterize a novel *rh1* gene. Data mining of the zebrafish genome revealed an open reading frame encoding a novel rhodopsin gene, herein referred to as *rh1-2*. The amino acid sequence of *rh1-2* contains several unique amino acid substitutions compared to normally conserved *rh1* residues and some substitutions more characteristic of cone opsins. Despite these substitutions, the rhodopsin-like protein was successfully expressed *in vitro*, and not only bound chromophore, yielding an absorption spectra in the visible range ($\lambda_{max} \approx 500$ nm), but also activated in response to light indicative of a functional opsin. In contrast to *rh1*, *rh1-2* is not expressed during the first 4 days of embryonic development but is expressed by day 21 and is expressed in the adult retina but not in adult brain or muscle tissue. While *rh1-2* sequences have only been identified in cyprinid fish, phylogenetic analyses suggest a much older origin. The novel gene is also intronless, similar to other *rh1* sequences of teleosts. The discovery of a new previously uncharacterized opsin gene in zebrafish retina is surprising, given its status as a model system for studies of vertebrate vision and visual development.

Materials and methods

Opsin sequences

The zebrafish genome assembly, currently in version Zv9 (http://www.sanger.ac.uk/Projects/D_rerio/) was explored through a BLAST search, using the full-length sequence of zebrafish *rh1* (GenBank: AB087811) to identify putative *rh1* homologs; various BLAST homology algorithms were employed to ensure the best possible coverage, including blastn, blastp, and tblastn. Genomic DNA was extracted from fish tissues using the DNeasy Blood & Tissue Kit (QIAGEN). Primers were designed to amplify fragments of the coding region of the *rh1-2* gene from cyprinid fish, including zebrafish, using genomic DNA as a template (Table S1). RNA was extracted from fish eyes using the TRIzol reagent (Invitrogen), and complementary DNA (cDNA) libraries were generated using the SMART cDNA Library Construction Kit (BD Biosciences). Teleost *rh1* gene fragments were amplified from these cDNA libraries using either custom *rh1* primers (Table S1) or previously designed acanthomorph *rh1* primers (Chen et al., 2003). PCR was performed using PfuTurbo (Stratagene) or FastStartTaq (Roche), with resulting bands being cloned into the pJET1.2 cloning vector (Fermentas) and sequenced using a 3730 DNA Analyzer (Applied Biosystems).

Protein expression

Complete coding sequences of zebrafish *rh1* and *rh1-2* were cloned into the p1D4-hrGFP II expression vector (Morrow & Chang, 2010). This vector was used to transiently transfect cultured HEK293T cells using Lipofectamine 2000 (Invitrogen; 12 μg of DNA per 10-cm plate). Cells were harvested 48 h posttransfection and regenerated using 11-*cis*-retinal, generously provided by Dr. Rosalie Crouch (Medical University of South Carolina). The pigments were then solubilized in 1% dodecyl maltoside and immunoaffinity purified with the 1D4 monoclonal antibody (Molday & MacKenzie, 1983), as previously described (Chang et al., 2002; Morrow & Chang, 2010). The ultraviolet-visible absorption spectra of purified zebrafish *rh1* and *rh1-2* were recorded at 25°C using the Cary4000 double-beam spectrophotometer (Varian). For zebrafish *rh1-2*, a difference spectrum was calculated as the difference between dark- and light-bleached spectra after bleaching for 60 s.

Localizing expression of zebrafish rh1-2

RNA was extracted from zebrafish embryos (1, 2, 3, 4 dpf), juvenile heads (21 dpf), and adult tissues (retina, eye, brain, and muscle) using TRIzol Reagent (Invitrogen), and cDNA libraries were generated using the SMART cDNA Library Construction Kit (BD Biosciences), with identical amounts of RNA used as template in all reactions. Primers were designed to amplify ~300 bp fragments of zebrafish *rh1* and *rh1-2*; primers for *rh1-2* were designed specifically to avoid amplification of *rh1* (Table S1). Primers for β-actin, used as a positive control for RT-PCR experiments, were obtained from a previous study (Wang et al., 2003). PCR was performed using PfuTurbo (Stratagene) under standard cycling conditions, with resulting bands being cloned into the pJET1.2 cloning vector using the CloneJET PCR cloning kit (Fermentas) and sequenced using a 3730 DNA Analyzer (Applied Biosystems).

Phylogenetic analyses

All *rh1* and *rh1-2* nucleotide sequences isolated from zebrafish, *Danio roseus*, *Danio albolineatus*, and *E. bicolor* in this study were aligned with other teleost *rh1* sequences obtained from NCBI (Table S2) using ClustalW (Larkin et al., 2007). Teleost exorhodopsin sequences were included as outgroups, in order to root the tree. Due to the short length of some of the teleost *rh1* sequences obtained from NCBI, this aligned data set was trimmed to include only nucleotides 94–912 and subjected to phylogenetic analyses using both maximum likelihood and Bayesian methods (Guindon & Gascuel, 2003; Ronquist & Huelsenbeck, 2003). Maximum likelihood phylogenetic methods were implemented in the program PHYML 3.0 (Guindon & Gascuel, 2003; Guindon et al., 2005),

under the HKY+I+G model. For the likelihood analyses, bootstrapping methods were used to assess the degree of confidence in nodes of the phylogeny (Felsenstein, 1985). Bayesian inference was performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), under the HKY+I+G model. To identify the most appropriate models for the molecular analyses, we used MrModeltest 2.3 (Nylander, 2004) and selected the best models favored under the Akaike Information Criterion (AIC). Two independent analyses were performed, each composed of four Markov chains with default heating values. Markov chains were run for 10 million generations, sampling trees (and parameters) every 1000 generations. Convergence was assessed using a number of methods. The average S.D. of split frequencies, as calculated in MrBayes, were all well below 0.01 at stationarity. Also implemented in MrBayes, a convergence diagnostic for branch length posterior probabilities, the potential scale reduction factor, roughly approached 1 as the runs converged (Gelman & Rubin, 1992). Convergence to stationarity was also assessed by plotting log-likelihood scores and other parameter values in the program Tracer 1.4.1 to ensure that there were no trends in the data post burn-in (Rambaut & Drummond, 2007). Finally, adequacy of mixing was assessed by examining acceptance rates for parameters in MrBayes, and by calculating in Tracer the effective sample sizes, the number of independent samples from the marginal posterior distribution for each parameter; higher values being indicative of better sampling from the posterior distribution. These values were all well above 100. By these measures, convergence was achieved within the first 25% of trees sampled, which were discarded as burn in, and remaining trees were taken as representative of the posterior probability distribution.

Results

Sequence analysis of rh1-2

Zebrafish *rh1-2* shares 78% amino acid sequence similarity to zebrafish *rh1*, and, like other teleost *rh1* genes, is intronless (Fitzgibbon et al., 1995). In general, the novel opsin gene shares much greater amino acid sequence similarity with the other visual opsins of zebrafish (38–78%), as opposed to its nonvisual opsins (25–32%); the only exception is the nonvisual exorhodopsin, paralog to mammalian *Rh1* genes (Mano et al., 1999), which shares 70% sequence similarity. Zebrafish *rh1-2* is not a tandem duplicate of *rh1*, as the two genes are located on chromosomes 11 and 8, respectively. Full-length *rh1-2* coding sequences were amplified from two other species from the *Danio* genus: *D. roseus* and *D. albolineatus*, while partial coding sequence was amplified from the cyprinid *E. bicolor*; partial *rh1* coding sequences were also isolated for these three fish, with all *rh1* and *rh1-2* sequences amplified in this study being deposited in GenBank under accession numbers HQ286326–32.

The *rh1-2* gene family contains a series of amino acid substitutions that suggest it might have properties different from other rhodopsins. The most unique substitutions are sites C51, K145, L151, E199, and S316, all of which are conserved in all four *rh1-2* sequences and not found in any other opsin gene family. There is also a group of residues, L45, N65, S/T149, Q150, and L183, whose identities are usually found in cone opsins or nonvisual opsins but rarely in rhodopsins (Table 1). All of these residues group into one of three distinct opsin motifs: helix 1/helix 8, cytoplasmic loop 2, or extracellular loop 2.

In addition to unique and cone-like substitutions, *rh1-2* contains many of the sites and motifs required for visual opsin function. Both K296, the lysine residue that forms a Schiff base linkage with the chromophore (Zhukovsky et al., 1991), and E113, the glutamate counter-ion to the Schiff base linkage (Sakmar et al., 1989) are present. Both E134 and R135 of the D/ERY motif are present, being highly conserved in opsins and involved in light-dependant proton uptake (Arnis et al., 1994) and transducin activation (Fanelli & Dell'Orco, 2008). Cysteine residues, C110 and C187, required for the formation of a critical disulfide bond (Davidson et al., 1994; Hwa et al., 1999), are present as well as conserved glycosylation sites N2 and N15 (Kaushal et al., 1994; Murray et al., 2009) and palmitoylation sites C322 and C323 (Traxler & Dewey, 1994).

In vitro expression

Complete coding sequences of zebrafish *rh1* and *rh1-2* were cloned into the p1D4-hrGFP II expression vector. These genes were expressed in mammalian cell culture, regenerated with 11-*cis*-retinal, solubilized with dodecyl maltoside, and purified with the 1D4 monoclonal antibody, as previously described (Chang et al., 2002; Morrow & Chang, 2010). Zebrafish rh1 was expressed and purified with relative ease (Fig. 1A) and produced spectra similar to previous studies (Chinen et al., 2003). Zebrafish rh1-2 was more difficult to express but still bound 11-*cis*-retinal and produced a stable photopigment with an absorption maximum (λ_{max}) of approximately 500 nm (Fig. 1B), a typical λ_{max} value among fish rhodopsins (Johnson et al., 1993; Yokoyama et al., 1995; Chang et al., 2009). When bleached with light, the absorption peak of zebrafish rh1-2 shifted to approximately 380 nm, characteristic of the biologically active meta II state of visual opsins. Both expression level and absorbance ratio (A_{280}/A_{MAX}) of zebrafish rh1-2 were significantly lower than those of zebrafish rh1, suggesting that the former is less stable and more likely to misfold than the latter. These results imply that, while unique substitutions in zebrafish rh1-2 might reduce its stability, it can be expressed, has an appropriate λ_{max} for a teleost rhodopsin, and properly activates when bleached with light.

Zebrafish rh1-2 expression pattern

The temporal and spatial expression pattern of zebrafish *rh1-2* was investigated using RT-PCR. Expression of zebrafish *rh1-2* was not detected during the first 4 days of embryonic development but was detected in the heads of juveniles by 21 days postfertilization (dpf). Expression of *rh1-2* was also detected in the adult retina but not in the adult brain or muscle (Fig. 2). Zebrafish *rh1* expression was detected as early as 2 dpf and strictly in the adult eye and retina, as previously reported (Raymond et al., 1995; Takechi & Kawamura, 2005). These results suggest that zebrafish *rh1-2* is not expressed as early as *rh1* in zebrafish development.

Phylogenetic analyses

Maximum likelihood and Bayesian phylogenetic analyses were performed on a dataset of aligned *rh1* and *rh1-2* sequences (Fig. 3A) in order to investigate the origins of the *rh1-2* gene family and its relationship to *rh1* visual pigments. These analyses indicate that all four *rh1-2* sequences isolated from cypriniform fish form a well-supported monophyletic group and that this novel gene was the result of a duplication that most likely occurred within the *rh1* gene family during teleost evolution (Fig. 4). Although the *rh1-2* genes form a well-defined group, due to the low support for many of the early teleost *rh1* lineages, it remains difficult to pinpoint exactly when in teleost evolution this novel gene family may have emerged. With respect to teleost *rh1* sequences, this phylogeny recovers many

Table 1. *Dominant identities of key sites of rh1-2 in other opsin families*

Site	Motif	*rh1-2*	*rh1*	exorh	*rh2*	*sws2*	*sws1*	*lws*	valop	tmtop	melop
45	H1	L	F	L/I	F	F	F/G	I	F	G	L
51	H1	C	G	A	G	G	G	S	S	G	G
65	H1	N	H	H	H	Y	Y	F	F	Y	N/S
145	CL2	K	N	N	N/S	N	N	N	N	A	S/A
149	CL2	S/T	G	G	S	R/K	S/N	D	R	N	S
150	CL2	Q	E	E	S/A	G/T	S	A	G	Y	K
151	CL2	L	N	K	T/S	S/P	K	K	K	K	R
183	EL2	L	M	M	M	L	L	L	I	P	L
199	EL2	E	N	N/H	N/H	N	N/K	G	N/H	N	R
316	H8	S	C	C	C	C	C	C	C	C	A

The following opsin gene families were sampled: *rh1-2*, *rh1* (rhodopsin), exorh (exorhodopsin), *rh2* (rod-like opsin), *sws2* (short-wavelength sensitive 2), *sws1* (short-wavelength sensitive 1), *lws* (long-wavelength sensitive), valop (vertebrate ancient opsin), tmtop (teleost multiple tissue opsin), and melop (melanopsin).

of the commonly accepted systematic relationships among ray-finned fish (Forey et al., 1996; Hurley et al., 2007; Wang et al., 2007). This includes support for both acanthopterygian and ostariophysian groups of fishes, although positions of the dory, cod, and pearl eye *rh1a* sequences are somewhat unresolved. With respect to duplications within teleost *rh1* genes, it is interesting that our analyses do not show strong support for a grouping of the *rh1-2* sequences with the duplicate pearl eye and eels sequences. This might suggest multiple gene duplications within teleost *rh1* genes, although due to the low support of many of these divergences, at the moment, this is difficult to conclude with certainty. Interestingly, in our analyses the pearl eye *rh1b* sequence does appear to group, albeit with variable support, with the duplicate copies of the eel *rh1* sequences.

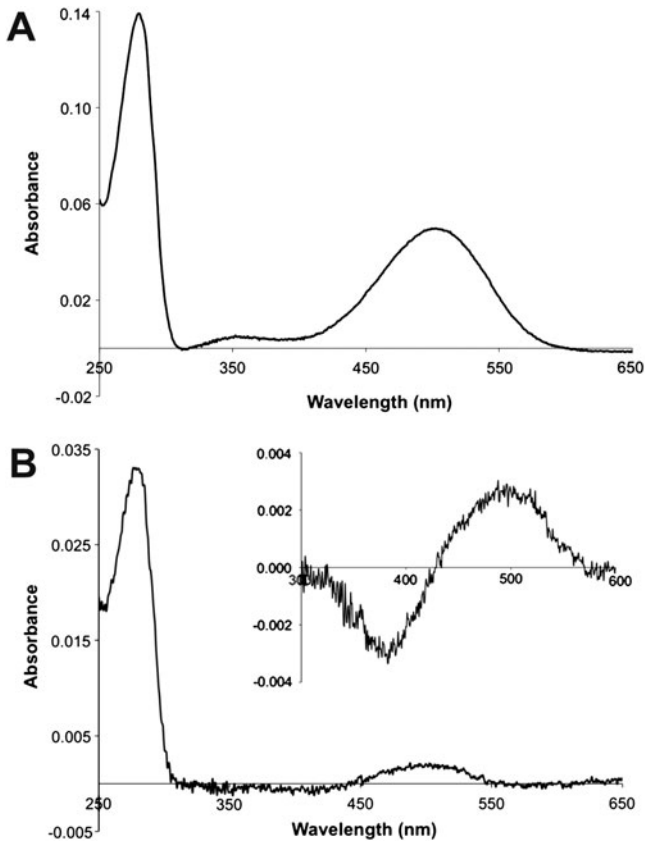


Fig. 1. Absorbance spectra of zebrafish rh1 and rh1-2 after *in vitro* expression and purification. (**A**) Dark spectrum of zebrafish rh1 comparable to previous expressions (Chinen et al., 2003). (**B**) Dark spectrum of zebrafish rh1-2, whose expression level and absorbance ratio (A_{280}/A_{MAX}) are significantly lower than zebrafish rh1. The absorption maximum of zebrafish rh1-2 is approximately 500 nm, which is highlighted in the dark-light difference spectrum (inset).

Discussion

In this study, we investigate *rh1-2*, a new family of opsin genes discovered in cyprinid fish. All four *rh1-2* sequences form a monophyletic group within the *rh1* clade of actinopterygian fish. While being most closely related to the *rh1* family of visual opsins, *rh1-2* has multiple substitutions that are either cone opsin like in nature or unique to the *rh1-2* gene family. Despite these unique substitutions at otherwise highly conserved sites, zebrafish *rh1-2* was successfully expressed *in vitro* and found to bind 11-*cis*-retinal with an absorption maximum around 500 nm; when bleached with light, it activated and changed conformation to its meta II intermediate. This activity coincides with the presence of conserved amino acids required for opsin function in the *rh1-2* gene sequence, including lysine at site 296 that forms a Schiff base linkage with 11-*cis*-retinal (Zhukovsky et al., 1991) and glutamate at site 113 that acts as a counterion to the protonated Schiff base linkage (Sakmar et al., 1989). Unlike *rh1*, *rh1-2* is not expressed during the first 4 days of embryonic development but is expressed by 21 days post fertilization. In the adult, *rh1-2* is expressed in the retina but not in the brain or muscle.

Although rare, zebrafish is not the only teleost to express a second *rh1* gene. The rod photoreceptors of eels start expressing an alternate *rh1* gene, during the transition from a freshwater habitat as a juvenile to a deep-sea habitat as an adult (Beatty, 1975; Archer et al., 1995; Zhang et al., 2000). This switch in *rh1* gene expression also results in a blueshift of λ_{max} of approximately 40 nm (Beatty, 1975; Hope et al., 1998). The short fin pearleye expresses an *rh1a* gene throughout its lifespan but only expresses the *rh1b* gene as an adult; the λ_{max} of *rh1b* is also blueshifted by 6 nm compared to *rh1a* (Pointer et al., 2007). Zebrafish *rh1* is expressed as early as 2 dpf and is expressed at high levels throughout adulthood (Raymond et al., 1995). Meanwhile, *rh1-2* expression initiates sometime between 4 and 21 dpf and also remains at more modest levels throughout adulthood. The λ_{max} values of zebrafish *rh1* (501 nm) (Chinen et al., 2003) and *rh1-2* (~500 nm) are similar. Ultimately, the *rh1* gene pair in zebrafish is similar to that of the pearleye, which has one gene expressed earlier in development at high levels, and a second gene

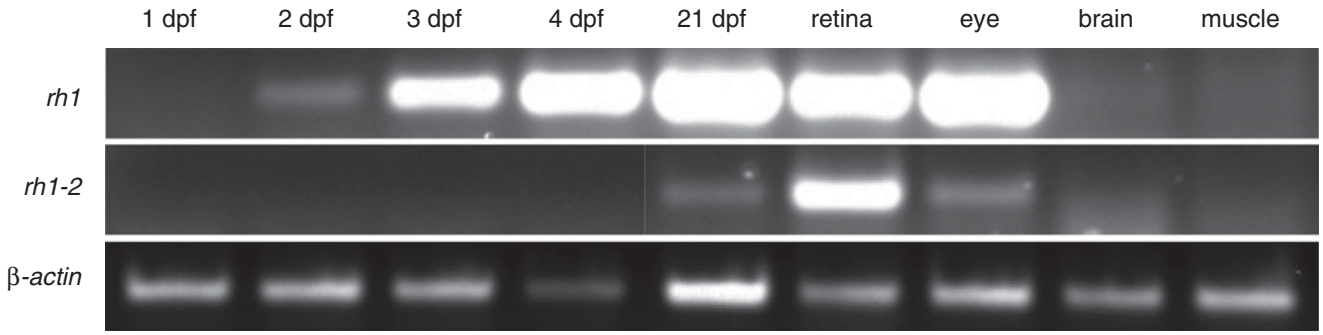


Fig. 2. RT-PCR analysis of zebrafish *rh1-2* expression. Zebrafish *rh1-2* is not expressed during the first 4 days of embryonic development but is expressed by the 21-day mark. In the adult, zebrafish *rh1-2* is expressed in the retina but not in brain or muscle tissue. Zebrafish *rh1* expression was similar to previous studies (Raymond et al., 1995; Takechi & Kawamura, 2005). β-actin serves as a positive control.

expressed later in development at lower levels. However, unlike the pearleye, the two *rh1* genes of zebrafish do not seem to differ significantly in their λ_{max} values.

Like most protein families, there are key residues and motifs present in opsins that are required for proper structure and function (Iannaccone et al., 2006). Alternatively, some sites in opsins act as determinants of *rh1* and cone opsin function (Imai et al., 1997; Carleton et al., 2005; Kuwayama et al., 2005) or differentiate between visual and nonvisual opsins (Bellingham et al., 2002; Murakami & Kouyama, 2008). The *rh1-2* gene family contains a number of substitutions at sites known to vary between rod, cone, and nonvisual opsins, as well as some that are entirely unique at otherwise highly conserved sites (Fig. 3B). Because opsins have been examined so extensively, many of these substitutions have been implicated in experimental opsin structure and function studies.

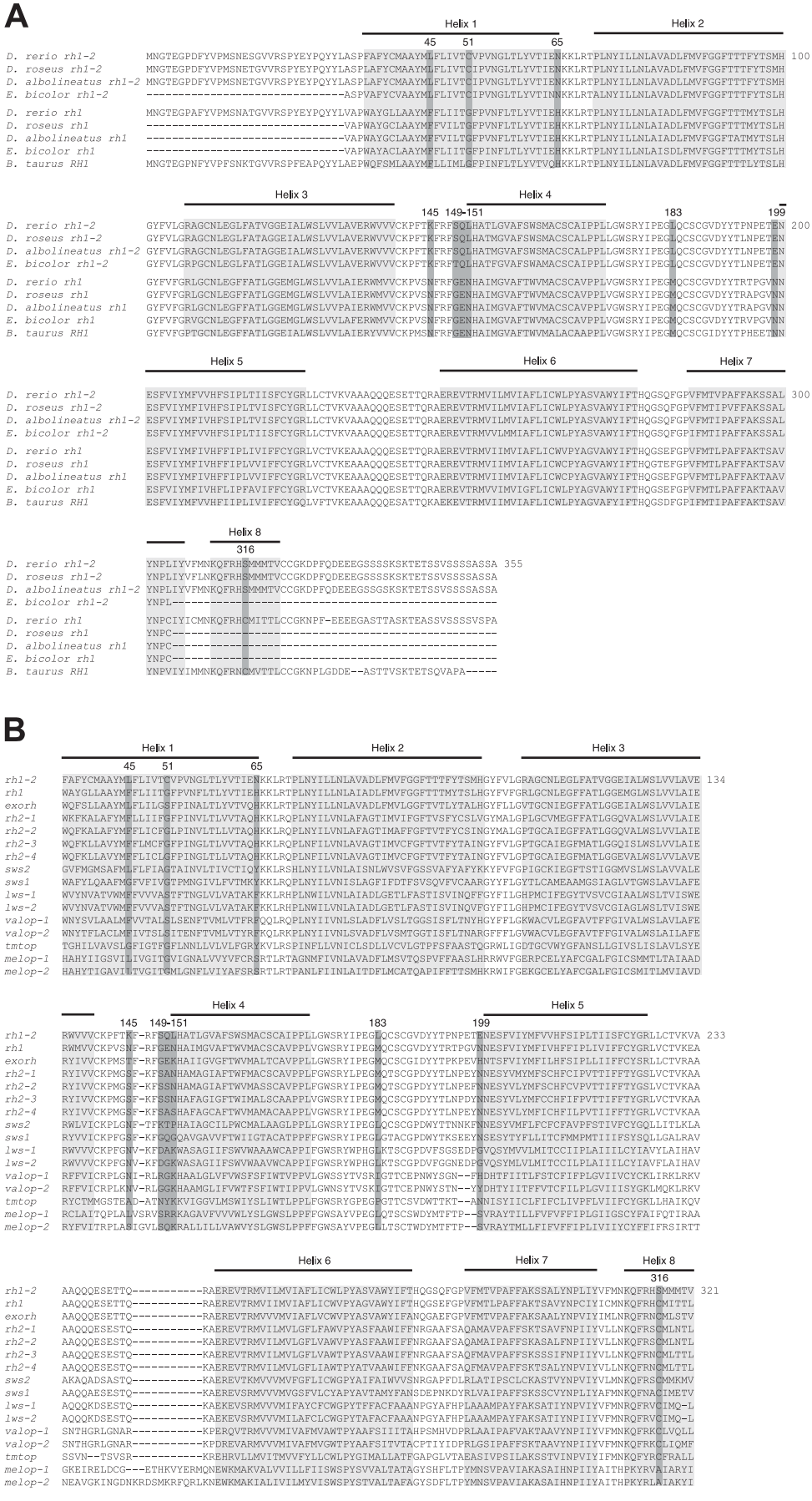
There are three motifs in rh1-2 that have substitutions suggesting altered function compared to rhodopsin: helix 1/helix 8, cytoplasmic loop 2, and extracellular loop 2 (Fig. 5). All three of these motifs are involved in meta II stability, transducin activation, or both, suggesting that rh1-2 does not behave as a traditional rhodopsin. Residues L45 and C51 are found in the first transmembrane helix of rh1-2. Helix 1 plays a role in assuring proper transmembrane packing of helices 2 and 7, with site 51 being of particular importance (Bosch et al., 2003). It is possible that interfering with helix 7 specifically could lead to disruption of helix 8, a key motif in both meta II stability and transducin activation (Ernst et al., 2000; Marin et al., 2000). Additionally, N65, a cone-like substitution at the cytoplasmic surface of helix 1, is in close proximity to S316, an rh1-2-specific substitution in helix 8, and a residue involved in the meta I/II equilibrium (Weitz & Nathans, 1992).

There are also a number of substitutions in the second cytoplasmic loop (K145, S/T149, Q150), as well as L151, the first residue of helix 4, which are either unique to rh1-2 or cone-like in nature. Cytoplasmic loop 2 is a key motif in transducin interaction and activation (König et al., 1989; Franke et al., 1992; Ernst et al., 1995; Natochin et al., 2003), with some residues of the loop also being implicated in phosphorylation and glycosylation patterning (Shi et al., 1995; Zhu et al., 2006). Finally, L183 and E199 are part of extracellular loop 2, which folds into two short β-strands that form a shield over the chromophore-binding site (Palczewski et al., 2000; Okada et al., 2004); the movement of this motif is key to *rh1* activation (Ahuja et al., 2009). Since other residues in extracellular loop 2 have been previously identified as key determinants of differences between rhodopsins and cone opsins, including meta II decay rate (Kuwayama et al., 2002), these substitutions could also be involved in altering rh1-2 function.

The λ_{max} of an opsin gene is a property often used as an indicator of biological function, with each group of visual opsins having a characteristic λ_{max} range: *rh1* ~500 nm, *rh2* = 470–510 nm, *sws2* = 440–460 nm, *sws1* = 360–430 nm, and *lws* = 510–560 nm (Trezise & Collin, 2005). The λ_{max} values of nonvisual opsins have not been analyzed to the same extent as those from visual opsins, often because they are more difficult to express. The only nonvisual opsins in fish that have been successfully expressed *in vitro* are the two isoforms of zebrafish VAL-opsin, both of which absorb maximally around 500 nm (Kojima et al., 2008). Melanopsin, a nonvisual opsin, has been investigated in mammals and reported to have a λ_{max} around either 480 nm (Dacey et al., 2005; Panda et al., 2005; Torii et al., 2007; Terakita et al., 2008) or 420 nm (Newman et al., 2003; Melyan et al., 2005). The λ_{max} of zebrafish rh1-2, at approximately 500 nm, falls within the range of both rh1 and rh2 visual opsin groups, but is also similar to some nonvisual opsins, such as VAL-opsin. While the λ_{max} value of rh1-2 might not help in distinguishing its functional role, the fact that it is much more difficult to express relative to zebrafish rh1 is indicative of a protein less stable than rhodopsin.

The spatial and temporal expression pattern of an opsin is an integral functional feature. The onset of expression of the visual opsin repertoire in zebrafish varies from 2 dpf for *rh1* and *lws-2* to 7 dpf for *rh2-2* (Takechi & Kawamura, 2005). Meanwhile, the expression of both isoforms of VAL-opsin can be seen as early as 1 dpf (Kojima et al., 2008). In adult vertebrates, visual opsins are expressed in the outer segments of photoreceptor cells found in the retina (Lamb et al., 2007) and, in rare circumstances, in the brain (Koyanagi et al., 2004). Nonvisual opsins can be expressed in a wide variety of tissues, often simultaneously, including the retina (Hannibal et al., 2002; Grone et al., 2007), brain (Mano et al., 1999; Philp et al., 2000), and even the testis (Tarttelin et al., 2003) and ovaries (Kojima et al., 2008). Zebrafish *rh1-2* expression initiates between 4 and 21 dpf and is localized in the retina. These results suggest that *rh1-2* is more likely to serve as a visual opsin, as few nonvisual opsins are expressed strictly in the retina. Furthermore, since the development of photoreceptors in vertebrates is dependent on rh1 expression (Lem et al., 1999), *rh1-2* likely lacks a similar role in photoreceptor development.

The evolutionary origins of the *rh1-2* gene family were investigated via phylogenetic analyses of *rh1* and *rh1-2* sequences. Both maximum likelihood and Bayesian methods recovered a phylogeny


A
Helix 1
Helix 2
45
51
65
D. rerio rhl-2
D. roseus rhl-2
D. albolineatus rhl-2
E. bicolor rhl-2
D. rerio rhl
D. roseus rhl
D. albolineatus rhl
E. bicolor rhl
B. taurus RH1
Helix 3
Helix 4
145
149-151
183
199
Helix 5
Helix 6
Helix 7
Helix 8
316
B
Helix 1
Helix 2
Helix 3
Helix 4
Helix 5
Helix 6
Helix 7
Helix 8
rhl-2
rhl
exorh
rh2-1
rh2-2
rh2-3
rh2-4
sws2
sws1
lws-1
lws-2
valop-1
valop-2
tmtop
melop-1
melop-2

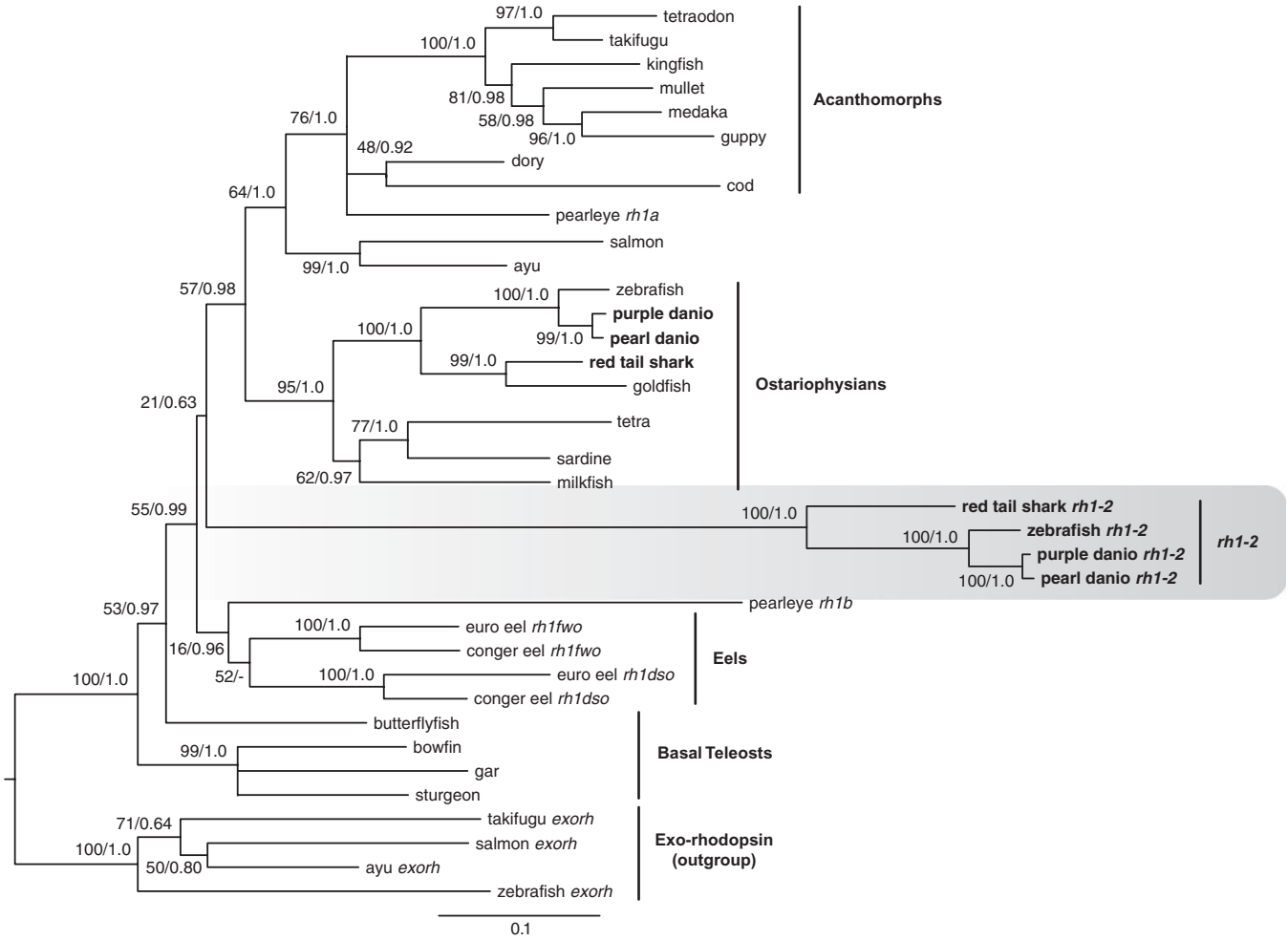


Fig. 4. Phylogeny of teleost *rh1* nucleotide sequences, including the *rh1-2* gene family, generated using both maximum likelihood and Bayesian methods; *rh1-2* sequences form a monophyletic group within the *rh1* gene family. Sequences amplified in this study are indicated in bold. Numbers above nodes indicate ML bootstraps (100 replications) followed by posterior probabilities. Branches are proportional to ML estimated branch lengths under HKY+I+G model, the best model as determined by Modeltest AIC. Sequences obtained from GenBank are listed in Table S2.

in which all *rh1-2* sequences formed a well-supported monophyletic group within the *rh1* gene family. So far, we have only isolated *rh1-2* sequences from cyprinids, a family of fish including zebrafish, goldfish, and barbs that originated between 32 and 39 million years ago (Zardoya & Doadrio, 1999; Wang et al., 2007). However, our phylogenetic analyses suggest a much older origin for this novel gene. As with *rh1* genes of all actinopterygians, *rh1-2* does not contain introns (Fitzgibbon et al., 1995), suggesting that the duplication event leading to the birth of the *rh1-2* family likely occurred after the retrotransposition event that split *rh1* and exorhodopsin in this group of fish (Bellingham et al., 2003). Although the exact time of actinopterygian divergence is a contentious issue, this would mean that the origin of the *rh1-2* gene family is certainly no older than 350 million years ago (Hurley et al., 2007). According to our analyses, there is some evidence that the origins of the *rh1-2* group might have occurred around the divergence of the eel *rh1* sequences. The divergence of the elopomorphs (eels) from more derived teleosts occurred approximately 140 million years ago (Forey et al., 1996).

Such origins of the *rh1-2* gene family suggest that its distribution among teleost fish may be much wider than our current experiments would indicate, particularly as we have not yet attempted to find *rh1-2* genes outside of cyprinid fishes. This raises the possibility that *rh1* gene duplicates in the pearl eye and eels might in fact be part of the same gene duplication that gave rise to the *rh1-2* gene family. This would be rather surprising, as these *rh1* duplicates are not generally thought to be widespread among fish. However, this possibility cannot be ruled out, as our analyses show

Fig. 3. Amino acid alignments of zebrafish *rh1-2*. (**A**) The *rh1-2* gene family aligned with corresponding *rh1* sequences and bovine rhodopsin. The seven transmembrane helices of rhodopsin, along with the eighth cytoplasmic helix, are labeled and shaded in light gray. Unique substitutions of the *rh1-2* gene family are numbered and shaded in dark gray. Sequences obtained from GenBank are listed in Table S2. (**B**) Zebrafish *rh1-2* aligned with other visual and nonvisual opsin genes from zebrafish. N- and C-termini were excluded as they were too variable to align.

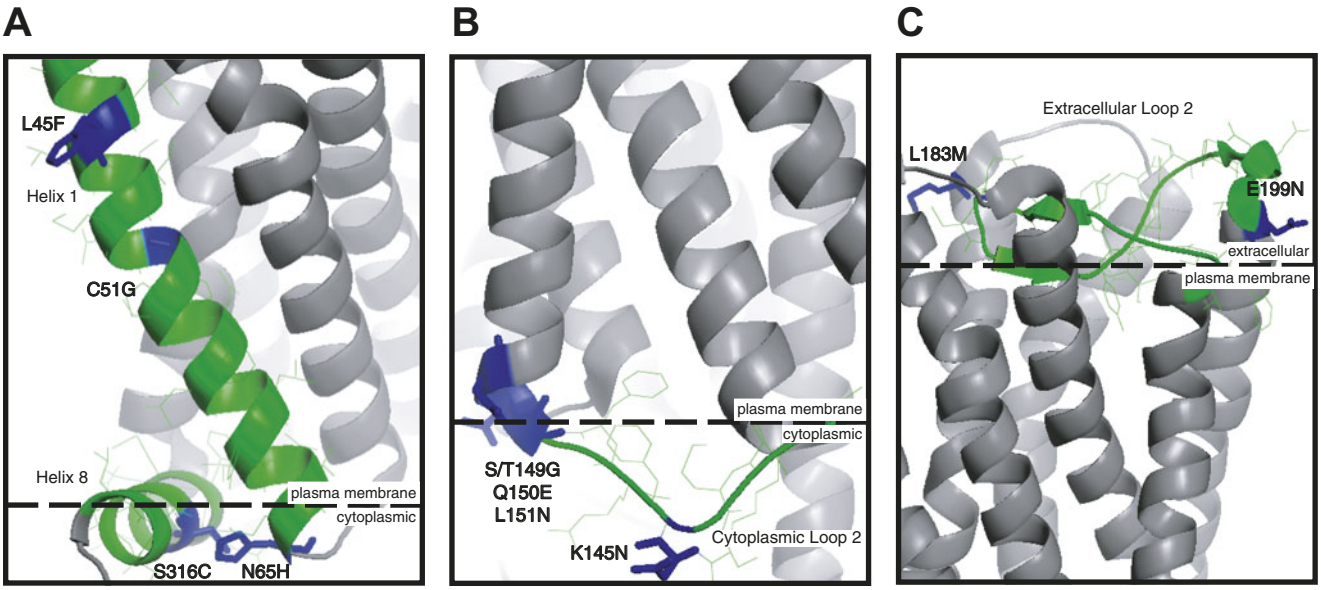


Fig. 5. Structures of three key motifs of *rh1* containing cone-like and unique substitutions in the *rh1-2* gene family. (**A**) The interaction between helix 1 and 8. (**B**) The second cytoplasmic loop. (**C**) The second extracellular loop. Unique substitutions of the *rh1-2* gene family are highlighted in blue, with key motifs being colored green, and other residues colored gray. Approximate plasma membrane boundaries with either the extracellular or cytoplasmic environment are emphasized with a dashed line. The crystal structure of dark state bovine rhodopsin (1U19) and MacPyMOL (DeLano, 2008) were used for all images.

low support at key divergences deep within the teleost *rh1* phylogeny. Better sampling of basal teleost *rh1* sequences, as well as a wider sampling of *rh1-2* sequences would be needed to resolve this issue.

While *rh1-2* is most closely related to the *rh1* visual opsin group, this alone cannot classify it as a visual opsin, as the same can be said about the nonvisual opsin, exorhodopsin (Mano et al., 1999). Despite expressing zebrafish *rh1-2* in cell culture and characterizing its expression pattern via RT-PCR, it is still difficult to ascertain its function, whether within the visual system or elsewhere. Regardless, the discovery of a new previously uncharacterized opsin gene in the zebrafish retina is an important and surprising discovery, given its status as a model system for studying vertebrate vision and visual development.

Additional insights into the function of this novel gene could help to further elucidate how the vertebrate visual system functions. Localizing the specific cell types within the retina that express *rh1-2* would help to determine its functional role. This could be done through extensive *in situ* hybridizations of adult zebrafish retina or by generating a transgenic zebrafish with expression of a GFP reporter driven by elements found upstream of the zebrafish *rh1-2* ORF. Additional biochemical characterization of *rh1-2* expressed *in vitro* would also help to determine the functional repercussions of its many unique amino acid substitutions. Finally, obtaining additional *rh1-2* nucleotide sequences from other teleosts would help to resolve the evolutionary origins of this novel gene family.

Acknowledgments

We would like to acknowledge Ashley Bruce, Ian Scott, and Vince Tropepe for advice, suggestions, and zebrafish tissue samples. This research was supported by an NSERC Discovery Grant (B.S.W.C.), an Early Researcher Award (B.S.W.C.), and a University Health Network (University of Toronto) Vision Science Research Program fellowship (J.M.M.).

References

Ahuja, S., Hornak, V., Yan, E.C., Syrett, N., Goncalves, J.A., Hirshfeld, A., Ziliox, M., Sakmar, T.P., Sheves, M., Reeves, P.J., Smith, S.O. & Eilers, M. (2009). Helix movement is coupled to displacement of the second extracellular loop in rhodopsin activation. *Nature Structural & Molecular Biology* **16**, 168–175.

Altimus, C.M., Güler, A.D., Villa, K.L., McNeill, D.S., Legates, T.A. & Hattar, S. (2008). Rods-cones and melanopsin detect light and dark to modulate sleep independent of image formation. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 19998–20003.

Amacher, S.L. (2008). Emerging gene knockout technology in zebrafish: Zinc-finger nucleases. *Briefings in Functional Genomics & Proteomics* **7**, 460–464.

Amsterdam, A. & Becker, T.S. (2005). Transgenes as screening tools to probe and manipulate the zebrafish genome. *Developmental Dynamics* **234**, 255–268.

Archer, S., Hope, A. & Partridge, J.C. (1995). The molecular basis for the green-blue sensitivity shift in the rod visual pigments of the European eel. *Proceedings. Biological Sciences / The Royal Society* **262**, 289–295.

Arnis, S., Fahmy, K., Hofmann, K.P. & Sakmar, T.P. (1994). A conserved carboxylic acid group mediates light-dependent proton uptake and signaling by rhodopsin. *The Journal of Biological Chemistry* **269**, 23879–23881.

Beatty, D.D. (1975). Visual pigments of the American eel Anguilla rostrata. *Vision Research* **15**, 771–776.

Bellingham, J., Chaurasia, S.S., Melyan, Z., Liu, C., Cameron, M.A., Tarttelin, E.E., Luvone, P.M., Hankins, M.W., Tosini, G. & Lucas, R.J. (2006). Evolution of melanopsin photoreceptors: Discovery and characterization of a new melanopsin in nonmammalian vertebrates. *PLoS Biology* **4**, e254.

Bellingham, J., Tarttelin, E.E., Foster, R.G. & Wells, D.J. (2003). Structure and evolution of the teleost extraretinal rod-like opsin (errlo) and ocular rod opsin (rho) genes: Is teleost rho a retrogene? *Journal of Experimental Zoology. Part B, Molecular & Developmental Evolution* **297**, 1–10.

Bellingham, J., Whitmore, D., Philp, A.R., Wells, D.J. & Foster, R.G. (2002). Zebrafish melanopsin: Isolation, tissue localisation and phylogenetic position. *Brain Research. Molecular Brain Research* **107**, 128–136.

Bilotta, J. & Saszik, S. (2001). The zebrafish as a model visual system. *International Journal of Developmental Neuroscience* **19**, 621–629.

Bosch, L., Ramon, E., del Valle, L.J. & Garriga, P. (2003). Structural and functional role of helices I and II in rhodopsin: A novel interplay evidenced by mutations at GLY-51 and GLY-89 in the transmembrane domain. *The Journal of Biological Chemistry* **278**, 20203–20209.

Bowmaker, J.K. (2008). Evolution of vertebrate visual pigments. *Vision Research* **48**, 2022–2041.

Burns, M.E. & Baylor, D.A. (2001). Activation, deactivation, and adaptation in vertebrate photoreceptor cells. *Annual Review of Neuroscience* **24**, 779–805.

Carleton, K.L., Spady, T.C. & Cote, R.H. (2005). Rod and cone opsin families differ in spectral tuning domains but not signal transducing domains as judged by saturated evolutionary trace analysis. *Journal of Molecular Evolution* **61**, 75–89.

Chang, C.H., Chiao, C.C. & Yan, H.Y. (2009). Ontogenetic changes in color vision in the milkfish (*Chanos chanos Forsskal*, 1775). *Zoological Science* **26**, 349–355.

Chang, B.S.W., Jönsson, K., Kazmi, M.A., Donoghue, M.J. & Sakmar, T.P. (2002). Recreating a functional ancestral archosaur visual pigment. *Molecular Biology & Evolution* **19**, 1483–1489.

Chen, W.J., Bonillo, C. & Lecointre, G. (2003). Repeatability of clades as a criterion of reliability: A case study for molecular phylogeny of Acanthomorpha (Teleostei) with larger number of taxa. *Molecular Phylogenetics & Evolution* **26**, 262–288.

Chinen, A., Hamaoka, T., Yamada, Y. & Kawamura, S. (2003). Gene duplication and spectral diversification of cone visual pigments of zebrafish. *Genetics* **163**, 663–675.

Dacey, D.M., Liao, H.W., Peterson, B.B., Robinson, F.R., Smith, V.C., Pokorny, J., Yau, K.W. & Gamlin, P.D. (2005). Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. *Nature* **433**, 749–754.

Davidson, F.F., Loewen, P.C. & Khorana, H.G. (1994). Structure and function in rhodopsin: Replacement by alanine of cysteine residues 110 and 187, components of a conserved disulfide bond in rhodopsin, affects the light-activated metarhodopsin II state. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 4029–4033.

Dehal, P. & Boore, J.L. (2005). Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biology* **3**, e314.

DeLano, W.L. (2008). *The PyMOL Molecular Graphics System*. Palo Alto, CA: DeLano Scientific LLC. http//www.pymol.org

Dulai, K.S., von Dornum, M., Mollon, J.D. & Hunt, D.M. (1999). The evolution of trichromatic color vision by opsin gene duplication in New World and Old World primates. *Genome Research* **9**, 629–638.

Ebrey, T. & Koutalos, Y. (2001). Vertebrate photoreceptors. *Progress in Retinal & Eye Research* **20**, 49–94.

Ernst, O.P., Hofmann, K.P. & Sakmar, T.P. (1995). Characterization of rhodopsin mutants that bind transducin but fail to induce GTP nucleotide uptake. *The Journal of Biological Chemistry* **270**, 10580–10586.

Ernst, O.P., Meyer, C.K., Marin, E.P., Henklein, P., Fu, W.Y., Sakmar, T.P. & Hofmann, K.P. (2000). Mutation of the fourth cytoplasmic loop of rhodopsin affects binding of transducin and peptides derived from the carboxyl-terminal sequences of transducin alpha and gamma subunits. *The Journal of Biological Chemistry* **275**, 1937–1943.

Fadool, J.M. & Dowling, J.E. (2008). Zebrafish: A model system for the study of eye genetics. *Progress in Retinal & Eye Research* **27**, 89–110.

Fanelli, F. & Dell'Orco, D. (2008). Dark and photoactivated rhodopsin share common binding modes to transducin. *FEBS Letters* **582**, 991–996.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783–791.

Fitzgibbon, J., Hope, A., Slobodyanyuk, S.J., Bellingham, J., Bowmaker, J.K. & Hunt, D.M. (1995). The rhodopsin-encoding gene of bony fish lacks introns. *Gene* **164**, 273–277.

Fleisch, V.C. & Neuhauss, S.C. (2006). Visual behavior in zebrafish. *Zebrafish* **3**, 191–201.

Forey, P.L., Littlewood, D.T.J., Ritchie, P. & Meyer, A. (1996). Relationships of elopomorphs. In *Interrelationships of Fishes*, ed. Greenwood, P.H., Miles, R.S. & Patterson, C., pp. 351–368. London: Academic Press.

Franke, R.R., Sakmar, T.P., Graham, R.M. & Khorana, H.G. (1992). Structure and function in rhodopsin. Studies of the interaction between the rhodopsin cytoplasmic domain and transducin. *The Journal of Biological Chemistry* **267**, 14767–14774.

Gelman, A. & Rubin, D.B. (1992). Inference from iterative simulation using multiple sequences. *Statistical Science* **7**, 457–511.

Grone, B.P., Zhao, S., Chen, C. & Fernald, R.D. (2007). Localization and diurnal expression of melanopsin, vertebrate ancient opsin, and pituitary adenylate cyclase-activating peptide mRNA in a teleost retina. *Journal of Biological Rhythms* **22**, 558–561.

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696–704.

Guindon, S., Lethiec, F., Duroux, P. & Gascuel, O. (2005). PHYML Online–a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Research* **33**, W557–W559.

Halpern, M.E., Rhee, J., Goll, M.G., Akitake, C.M., Parsons, M. & Leach, S.D. (2008). Gal4/UAS transgenic tools and their application to zebrafish. *Zebrafish* **5**, 97–110.

Hannibal, J., Hindersson, P., Nevo, E. & Fahrenkrug, J. (2002). The circadian photopigment melanopsin is expressed in the blind subterranean mole rat, Spalax. *Neuroreport* **13**, 1411–1414.

Hope, A.J., Partridge, J.C. & Hayes, P.K. (1998). Switch in rod opsin gene expression in the European eel, Anguilla anguilla (L.). *Proceedings. Biological Sciences / The Royal Society* **265**, 869–874.

Hurley, I.A., Mueller, R.L., Dunn, K.A., Schmidt, E.J., Friedman, M., Ho, R.K., Prince, V.E., Yang, Z., Thomas, M.G. & Coates, M.I. (2007). A new time-scale for ray-finned fish evolution. *Proceedings. Biological Sciences / The Royal Society* **274**, 489–498.

Hwa, J., Reeves, P.J., Klein-Seetharaman, J., Davidson, F. & Khorana, H.G. (1999). Structure and function in rhodopsin: Further elucidation of the role of the intradiscal cysteins, Cys-110, -185, and -187, in rhodopsin folding and function. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 1932–1935.

Iannaccone, A., Man, D., Waseem, N., Jennings, B.J., Ganapathiraju, M., Gallaher, K., Reese, E., Bhattacharya, S.S, & Klein-Seetharaman, J. (2006). *Retinitis pigmentosa* associated with rhodopsin mutations: Correlation between phenotypic variability and molecular effects. *Vision Research* **46**, 4556–4567.

Imai, H., Kefalov, V., Sakurai, K., Chisaka, O., Ueda, Y., Onishi, A., Morizumi, T., Fu, Y., Ichikawa, K., Nakatani, K., Honda, Y., Chen, J., Yau, K.W. & Shichida, Y. (2007). Molecular properties of rhodopsin and rod function. *The Journal of Biological Chemistry* **282**, 6677–6684.

Imai, H., Kojima, D., Oura, T., Tachibanaki, S., Terakita, A. & Shichida, Y. (1997). Single amino acid residue as a functional determinant of rod and cone visual pigments. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 2322–2326.

Johnson, R.L., Grant, K.B., Zankel, T.C., Boehm, M.F., Merbs, S.L., Nathans, J. & Nakanishi, K. (1993). Cloning and expression of goldfish opsin sequences. *Biochemistry* **32**, 208–214.

Kaushal, S., Ridge, K.D. & Khorana, H.G. (1994). Structure and function in rhodopsin: The role of asparagine-linked glycosylation. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 4024–4028.

Knierim, B., Hofmann, K.P., Ernst, O.P. & Hubbell, W.L. (2007). Sequence of late molecular events in the activation of rhodopsin. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 20290–20295.

Kojima, D., Torii, M., Fukada, Y. & Dowling, J.E. (2008). Differential expression of duplicated VAL-opsin genes in the developing zebrafish. *Journal of Neurochemistry* **104**, 1364–1371.

König, B., Arendt, A., McDowell, J.H., Kahlert, M., Hargrave, P.A. & Hofmann, K.P. (1989). Three cytoplasmic loops of rhodopsin interact with transducin. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 6878–6882.

Koyanagi, M., Kawano, E., Kinugawa, Y., Oishi, T., Shichida, Y., Tamotsu, S. & Terakita, A. (2004). Bistable UV pigment in the lamprey pineal. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 6687–6691.

Kuwayama, S., Imai, H., Hirano, T., Terakita, A. & Shichida, Y. (2002). Conserved proline residue at position 189 in cone visual pigments as a determinant of molecular properties different from rhodopsin. *Biochemistry* **41**, 15245–15252.

Kuwayama, S., Imai, H., Morizumi, T. & Shichida, Y. (2005). Amino acid residues responsible for the meta-III decay rates in rod and cone visual pigments. *Biochemistry* **44**, 2208–2215.

Lall, G.S., Revell, V.L., Momiji, H., Al Enezi, J., Altimus, C.M., Güler, A.D., Cameron, M.A., Allender, S., Hankins, M.W. & Lucas, R.J. (2010). Distinct contributions of rod, cone, and melanopsin photoreceptors to encoding irradiance. *Neuron* **66**, 417–428.

Lamb, T.D., Collin, S.P. & Pugh, E.N. Jr. (2007). Evolution of the vertebrate eye: Opsins, photoreceptors, retina and eye cup. *Nature Reviews. Neuroscience* **8**, 960–976.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. & Higgins, D.G. (2007). ClustalW and ClustalX version 2. *Bioinformatics* **23**, 2947–2948.

Lem, J., Krasnoperova, N.V., Calvert, P.D., Kosaras, B., Cameron, D. A., Nicolò, M., Makino, C.L. & Sidman, R.L. (1999). Morphological, physiological, and biochemical changes in rhodopsin knockout mice. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 736–741.

Mano, H., Kojima, D. & Fukada, Y. (1999). Exo-rhodopsin: A novel rhodopsin expressed in the zebrafish pineal gland. *Brain Research. Molecular Brain Research* **73**, 110–118.

Marin, E.P., Krishna, A.G., Zvyaga, T.A., Isele, J., Siebert, F. & Sakmar, T.P. (2000). The amino terminus of the fourth cytoplasmic loop of rhodopsin modulates rhodopsin-transducin interaction. *The Journal of Biological Chemistry* **275**, 1930–1936.

Matsumoto, Y., Fukamachi, S., Mitani, H. & Kawamura, S. (2006). Functional characterization of visual opsin repertoire in Medaka (*Oryzias latipes*). *Gene* **371**, 268–278.

Melyan, Z., Tarttelin, E.E., Bellingham, J., Lucas, R.J. & Hankins, M.W. (2005). Addition of human melanopsin renders mammalian cells photoresponsive. *Nature* **433**, 741–745.

Menon, S.T., Han, M. & Sakmar, T.P. (2001). Rhodopsin: Structural basis of molecular physiology. *Physiological Reviews* **81**, 1659–1688.

Molday, R.S. & MacKenzie, D. (1983). Monoclonal antibodies to rhodopsin: Characterization, cross-reactivity, and application as structural probes. *Biochemistry* **22**, 653–660.

Morrow, J.M. & Chang, B.S.W. (2010). The p1D4-hrGFP II expression vector: A tool for expressing and purifying visual pigments and other G protein-coupled receptors. *Plasmid* **64**, 162–169.

Moutsaki, P., Whitmore, D., Bellingham, J., Sakamoto, K., David-Gray, Z.K. & Foster, R.G. (2003). Teleost multiple tissue (tmt) opsin: A candidate photopigment regulating the peripheral clocks of zebrafish? *Brain Research. Molecular Brain Research* **112**, 135–145.

Murakami, M. & Kouyama, T. (2008). Crystal structure of squid rhodopsin. *Nature* **453**, 363–367.

Murray, A.R., Fliesler, S.J. & Al-Ubaidi, M.R. (2009). Rhodopsin: The functional significance of asn-linked glycosylation and other post-translational modification. *Ophthalmic Genetics* **30**, 109–120.

Natochin, M., Gasimov, K.G., Moussalf, M. & Artemyev, N.O. (2003). Rhodopsin determinants for transducin activation: A gain-of-function approach. *The Journal of Biological Chemistry* **278**, 37574–37581.

Neuhauss, S.C. (2003). Behavioral genetic approaches to visual system development and function in zebrafish. *Journal of Neurobiology* **54**, 148–160.

Newman, L.A., Walker, M.T., Brown, R.L., Cronin, T.W. & Robinson, P.R. (2003). Melanopsin forms a functional short-wavelength photopigment. *Biochemistry* **42**, 12734–12738.

Nickle, B. & Robinson, P.R. (2007). The opsins of the vertebrate retina: Insights from structural, biochemical, and evolutionary studies. *Cellular & Molecular Life Sciences* **64**, 2917–2932.

Nylander, J.A.A. (2004). *MrModeltest v2. Program distributed by the author*. Evolutionary Biology Centre, Uppsala University.

Okada, T., Sugihara, M., Bondar, A.N., Elstner, M., Entel, P. & Buss, V. (2004). The retinal conformation and its environment in rhodopsin in light of a new 2.2 A crystal structure. *Journal of Molecular Biology* **342**, 571–583.

Okawa, H. & Sampath, A.P. (2007). Optimization of single-photon response transmission at the rod-to-rod bipolar synapse. *Physiology* **22**, 279–286.

Owens, G.L., Windsor, D.J., Mui, J. & Taylor, J.S. (2009). A fish eye out of water: Ten visual opsins in the four-eyed fish, Anableps anableps. *PLoS One* **4**, e5970.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T., Stenkamp, R.E., Yamamoto, M. & Miyano M. (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**, 739–745.

Panda, S., Nayak, S.K., Campo, B., Walker, J.R., Hogenesch, J.B. & Jegla, T. (2005). Illumination of the melanopsin signaling pathway. *Science* **307**, 600–604.

Park, J.H., Scheerer, P., Hofmann, K.P., Choe, H.W. & Ernst, O.P. (2008). Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* **454**, 183–187.

Parry, J.W., Carleton, K.L., Spady, T., Carboo, A., Hunt, D.M. & Bowmaker, J.K. (2005). Mix and match color vision: Tuning spectral sensitivity by differential opsin gene expression in Lake Malawi cichlids. *Current Biology* **15**, 1734–1739.

Peirson, S.N., Halford, S. & Foster, R.G. (2009). The evolution of irradiance detection: Melanopsin and the non-visual opsins. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **364**, 2849–2865.

Philp, A.R., Bellingham, J., Garcia-Fernandez, J. & Foster, R.G. (2000). A novel rod-like opsin isolated from the extra-retinal photoreceptors of teleost fish. *FEBS Letters* **468**, 181–188.

Pierce, L.X., Noche, R.R., Ponomareva, O., Chang, C. & Liang, J.O. (2008). Novel functions for Period 3 and Exo-rhodopsin in rhythmic transcription and melatonin biosynthesis within the zebrafish pineal organ. *Brain Research* **1223**, 11–24.

Pointer, M.A., Carvalho, L.S., Cowing, J.A., Bowmaker, J.K. & Hunt, D.M. (2007). The visual pigments of a deep-sea teleost, the pearl eye Scopelarchus analis. *The Journal of Experimental Biology* **210**, 2829–2835.

Rambaut, A. & Drummond, A.J. (2007). Tracer v1.4. Available from http://beast.bio.ed.ac.uk/Tracer

Ramon, E., Mao, X. & Ridge, K.D. (2009). Studies on the stability of the human cone visual pigments. *Photochemistry & Photobiology* **85**, 509–516.

Raymond, P.A., Barthel, L.K. & Curran, G.A. (1995). Developmental patterning of rod and cone photoreceptors in embryonic zebrafish. *The Journal of Comparative Neurology* **359**, 537–550.

Ronquist, F. & Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Rosenbaum, D.M., Rasmussen, S.G. & Kobilka, B.K. (2009). The structure and function of G-protein-coupled receptors. *Nature* **459**, 356–363.

Sakmar, T.P., Franke, R.R. & Khorana, H.G. (1989). Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 8309–8313.

Scheerer, P., Park, J.H., Hildebrand, P.W., Kim, Y.J., Krauss, N., Choe, H.W., Hofmann, K.P. & Ernst, O.P. (2008). Crystal structure of opsin in its G-protein-interacting conformation. *Nature* **455**, 497–502.

Shi, W., Osawa, S., Dickerson, C.D. & Weiss, E.R. (1995). Rhodopsin mutants discriminate sites important for the activation of rhodopsin kinase and G_t. *The Journal of Biological Chemistry* **270**, 2112–2119.

Shichida, Y. & Morizumi, T. (2007). Mechanism of G-protein activation by rhodopsin. *Photochemistry & Photobiology* **83**, 70–75.

Takechi, M. & Kawamura, S. (2005). Temporal and spatial changes in the expression pattern of multiple red and green subtype opsin genes during zebrafish development. *The Journal of Experimental Biology* **208**, 1337–1345.

Tarttelin, E.E., Bellingham, J., Hankins, M.W., Foster, R.G. & Lucas, R.J. (2003). Neuropsin (Opn5): A novel opsin identified in mammalian neural tissue. *FEBS Letters* **554**, 410–416.

Taylor, J.S., Braasch, I., Frickey, T., Meyer, A. & Van de Peer, Y. (2003). Genome duplication, a trait shared by 22,000 species of ray-finned fish. *Genome Research* **13**, 382–390.

Teller, D.C., Stenkamp, R.E. & Palczewski, K. (2003). Evolutionary analysis of rhodopsin and cone pigments: Connecting the three-dimensional structure with spectral tuning and signal transfer. *FEBS Letters* **555**, 151–159.

Terakita, A., Tsukamoto, H., Koyanagi, M., Sugahara, M., Yamashita, T. & Shichida, Y. (2008). Expression and comparative characterization of Gq-coupled invertebrate visual pigments and melanopsin. *Journal of Neurochemistry* **105**, 883–890.

The Danio rerio sequencing project: Zv9. (in press). Cambridge, UK: Wellcome Trust Sanger Institute. http://www.sanger.ac.uk/Projects/D_rerio/

Torii, M., Kojima, D., Okano, T., Nakamura, A., Terakita, A., Shichida, Y., Wada, A. & Fukada, Y. (2007). Two isoforms of chicken melanopsins show blue light sensitivity. *FEBS Letters* **581**, 5327–5331.

Traxler, K.W. & Dewey, T.G. (1994). Effects of depalmitorylation on physicochemical properties of rhodopsin. *Biochemistry* **33**, 1718–1723.

Trezise, A.E. & Collin, S.P. (2005). Opsins: Evolution in waiting. *Current Biology* **15**, R794–R796.

Wang, X., Li, J. & He, S. (2007). Molecular evidence for the monophyly of East Asian groups of Cyprinidae (Teleostei: Cypriniformes) derived from the nuclear recombination activating gene 2 sequences. *Molecular Phylogenetics & Evolution* **42**, 157–170.

Wang, Y., Wong, A.O.L. & Ge, W. (2003). Cloning, regulation of messenger ribonucleic acid expression, and function of a new isoform of pituitary adenylate cyclase-activating polypeptide in the zebrafish ovary. *Endocrinology* **144**, 4799–4810.

Weadick, C.J. & Chang, B.S.W. (2007). Long-wavelength sensitive visual pigments of the guppy (*Poecilia reticulata*): Six opsins expressed in a single individual. *BMC Evolutionary Biology* **7**, S11.

Weitz, C.J. & Nathans, J. (1992). Histidine residues regulate the transition of photoexcited rhodopsin to its active conformation, metarhodopsin II. *Neuron* **8**, 465–472.

Yokoyama, R., Knox, B.E. & Yokoyama, S. (1995). Rhodopsin from the fish, Astyanax: Role of tyrosine 261 in the red shift. *Investigative Ophthalmology & Visual Science* **36**, 939–945.

Zardoya, R. & Doadrio, I. (1999). Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. *Journal of Molecular Evolution* **49**, 227–237.

Zhang, H., Futami, K., Horie, N., Okamura, A., Utoh, T., Mikawa, N., Yamada, Y., Tanaka, S. & Okamoto, N. (2000). Molecular cloning of fresh water and deep-sea rod opsin genes from Japanese eel *Anguilla japonica* and expressional analyses during sexual maturation. *FEBS Letters* **469**, 39–43.

Zhang, H., Futami, K., Yamada, Y., Horie, N., Okamura, A., Utoh, T., Mikawa, N., Tanaka, S., Okamoto, N. & Oka, H.P. (2002). Isolation of freshwater and deep-sea type opsin genes from the common Japanese conger. *Journal of Fish Biology* **61**, 313–324.

Zhu, L., Imanishi, Y., Filipek, S., Alekseev, A., Jastrzebska, B., Sun, W., Saperstein, D.A. & Palczewski, K. (2006). Autosomal recessive *retinitis pigmentosa* and E150K mutation in the opsin gene. *The Journal of Biological Chemistry* **281**, 22289–22298.

Zhukovsky, E.A., Robinson, P.R. & Oprian, D.D. (1991). Transducin activation by rhodopsin without a covalent bond to the 11- *cis*-retinal chromophore. *Science* **251**, 558–560.

Supplementary Data

Supplemental materials can be viewed in this issue of VNS by visiting journals.cambridge.org/VNS.

Table S1. List of primer sequences used in this study.

Table S2. List of opsin sequences from GenBank used for amino acid alignments or phylogenetic analyses.