Spatial Characterization of Visual Opsin Gene Expression in the Guppy (*Poecilia reticulata*)

by

Diana Jessie Rennison (Née Windsor)
Bachelor of Science, University of Victoria, 2008

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Supervisory Committee

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Abstract

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Guppies exhibit color based sexual dimorphism and females generally prefer the most colorful males. It has also recently been found that guppies possess a large opsin repertoire. As opsins are the receptors responsible for color vision, this ten gene repertoire might have contributed to the evolution of extravagant male coloration in this species. My study starts by characterizing the opsin repertoire of *Jenynsia onca*, a non-colorful relative of the guppy belonging to the family Anablepidae (sister group to Poeciliidae, of which the guppy is a member). A PCR based survey indicated that *J. onca* had a very similar opsin repertoire to the guppy; *J. onca* had nine genes including orthologs of all but one of the guppy opsins. To gain further insight into the origin of the guppy repertoire, a bioinformatics based survey of ray-finned fish opsins was undertaken. This revealed that large opsin repertoires are common in ray-finned fish and are the product of gene duplication events, spanning the age of the taxon Teleostei. Given that the large opsin repertoire of the guppy did not appear to be perfectly correlated with the evolution of color based sexual selection in this lineage, I turned to investigating the expression of this opsin repertoire. *In situ* hybridization was used to characterize the

pattern of opsin expression across the surface of the retina of adult male and female guppies. *In situ* hybridization demonstrated that most opsin genes had distinct expression profiles. These expression patterns also indicated that sensitivity and discrimination in the dorsal retina might differ from the ventral retina; the ventral retina appears to be tuned to middle-wavelength light (green), while the dorsal retina is predicted to have exceptional wavelength discriminatory ability and broad spectral sensitivity. This expression data was then used to evaluate models of sexual selection in the context of the predicted visual capacity of the guppy.

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List of Abbreviations

3’	Three prime
3R	Teleost whole genome duplication
5’	Five prime
7TMD	Seven transmembrane domain
Å	Ångström
α	Alpha
AP	Alkaline phosphatase
β	Beta
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BLAST	Basic local alignment search tool
bp	Base pairs
^{o}C	Degrees Celcius
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
γ	Gamma
GENECONV	Computer program using statistical tests to detect gene conversion
GPCR	G-protein coupled receptor
hr(s)	Hour(s)
LWS	Long-wavelength sensitive
MEGA	Molecular evolution genetics analysis
min(S)	Minute(s)
mg	Milligram
ML	Maximum likelihood
ml	Millilitre
mRNA	Messenger RNA
MS222	Tricaine methanesulfonate
MSP	Microspectrophotometry
MYA	Million years ago
MY	Million years
NBT	Nitro-blue-tetrazoleum
NJ	Neighbor joining

nm	Nanometer
PAUP	Phylogenetic analysis using parsimony and other methods
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RH1	Rhodopsin
RH2	Rhodopsin like
RNA	Ribonucleic acid
RT	Reverse transcription
s	Seconds
SWS	Short-wavelength sensitive
TEA	Triethanolamine
Tw	Tween
µl	Microlitre
µg	Microgram
µm	Micrometer
UV	Ultraviolet
w/v	Weight to volume
X	Times

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Chapter 1
Seeing a mate's true colors: vision based sexual selection in the guppy (*Poecilia reticulata*)

Morphology

Guppies (*Poecilia reticulata*) are small freshwater fish native to the streams of Trinidad, Venezuela, Guyana and Surinam (Haskins et al., 1961; Bailey, 1963; Endler, 1978). They are sexually dimorphic: males are smaller than females, but are very colorful. Male color consists of a combination of stripes and spots distributed across the body. The colors are a mix of melanin pigments (black), carotenoid pigments (red, yellow, orange) and structural colors (blue, green, purple and silver) (Endler, 1983; Houde & Endler, 1990). In contrast, female guppies are a uniform dull silver-brown color. This sexual dimorphism is one of the reasons the guppy garnered attention and has become a classic model for sexual selection.

Breeding experiments have indicated that size and location of carotenoid spots are heritable (Winge & Ditlevsen, 1947; Endler, 1983; Houde, 1992). Coloration is also influenced by diet; carotinoids found in algae and vascular plants influence the brightness of the red, yellow and orange spots but not the location, number or size of these spots (Fox, 1979, Kodric-Brown 1989). The intensity of carotenoid-based pigments is thought to reflect the overall condition of male fish (Endler, 1980; Nicoletto, 1991), as this trait is positively correlated with swimming performance and is reduced by parasitic infection (Houde & Torio, 1992). These observations support the hypothesis that carotenoid-derived coloration is an honest signal for sexual selection (i.e. a signal that genuinely reflects the quality of the individual) (Zahavi 1975; Grether et al., 1999).

Predation

Early in the study of guppies, it was found that males from low predation habitats are brighter and possess a greater diversity of color patterns than males from high predation regions (Haskins et al., 1961; Endler, 1983). Subsequent experiments also supported the hypothesis that predators could influence a diversity of guppy characteristics including: male coloration, time between broods, and body size (Reznick & Endler 1982; Endler 1983). Additionally, more colorful males have been found to be eaten more often than

those that are less colorful (Godin & McDonough, 2003). The association between variation in male color, and predation regime indicates that there may be a trade off between being conspicuous to attract mates and being cryptic to avoid predation (Endler, 1980).

A diversity of vertebrate and invertebrate species feed on guppies (Endler, 1978). The pike cichlid, *Crenicichla alta,* appears to be a guppy specialist, at least in the streams of the northern range mountains of Trinidad (Endler, 1978; Reznick & Endler, 1982). Other piscivores such as giant rivulus (*Rivulus hartii*), blue acara (*Aequidens pulcher*) and wolf fish (*Hoplias malabaricus*) also feed on guppies, but do so less preferentially (guppies constitute 10% or less of diet of their) (Endler, 1978; Reznick & Endler, 1982). There is also a freshwater prawn, *Macrobrachium crenulatum*, which is considered moderately dangerous to the guppy (Endler, 1978). Generally, guppy populations that live with these prawns are considered to be low-predation, yet the influence of the prawns on male coloration has been noted. Non-aquatic predators of guppies include *Noctilio leporinus*, a fish-eating bat, and three kingfishers, *Chloroceryle americana, C. amazona,* and *C. aenae* (Bloedel, 1955; Worth, 1967). However, despite the acknowledgement of their presence and observations of their effect on guppy behavior (Seghers, 1974; Templeton and Shriner, 2004), the terms low and high predation are used without consideration of bats and kingfishers.

Sexual selection

Guppy populations are typically large and therefore, each female usually has a large pool of potential mates to select from. In guppies, female mate choice is primarily based on the male secondary sexual trait of body coloration; in particular, females prefer the males with the greatest chroma (color saturation) and orange area (Endler, 1980; Endler, 1983; Houde. 1988; Kodric-Brown, 1989; Houde & Endler, 1990; Houde & Torio, 1992; Grether, 2000). Males are most colorful in low predation populations (Haskins et al., 1961; Endler, 1983), suggesting that there is a trade off between natural selection and sexual selection in male guppy color. Female mate preference for male color pattern, specifically orange spots, varies between populations with some showing strong preference for colorful males and others avoiding the most colorful males (Breden &

Stoner, 1987; Houde, 1988; Stoner & Breden 1988; Houde & Endler, 1990). This variation is maintained for several generations in the lab, even in the absence of predators, indicating that there are genes that influence female preference as well as male coloration. Given the data correlating variation among populations in male coloration, predation risk and female preference, guppies have become ideal subjects for testing the validity of different models that attempt to explain the evolution of a conspicuous male trait and female preference for that trait. In guppies the basis of female preference, the ability of a female to evaluate the male trait of color, is dependent on visual capacity. The role that vision might play in sexual selection was recognize a long time ago, as guppies were one of the first species to be studied with microspectrophotometry (MSP) (Levine & MacNichol, 1979), where the absorption properties of photoreceptor cells can be determined using a spectrophotometer. Here I study the genes responsible for light sensitivity in guppy photoreceptors.

Vision is mediated by opsins

At the molecular level vision starts with light absorption by proteins called opsins, which are expressed in the photoreceptor cells of the retina. Opsins belong to one group of genes among the thousands that are collectively called Seven Trans-membrane Domain (7TMD) G-protein Coupled Receptors (GPCRs). Opsin genes form a monophyletic group within the Rhodopsin-like GPCR family, which also includes olfactory receptors, neurotransmitter receptors and hormone receptors (Kolakowski, 1994). There are two major opsin lineages: the ciliary- or c-opsins are expressed in ciliary photoreceptor cells (including rod and cone photoreceptors) (See (Lamb, 2009) for a recent review). C-ospins also occur in invertebrates including cnidarians (Eakin & Westfall, 1962; Martin, 2002). The second opsin lineage consists of the rhabdomeric- or r-opsins.

Opsins are ~350 amino acids in length and bind a vitamin A-derived chromophore via a lysine residue linked to a protonated Schiff base (Palczewski et al., 2000). When the chromophore absorbs light it isomerizes and induces a conformational change in the opsin leading to G-protein mediated signal transduction through an enzyme cascade, culminating in membrane hyper-polarization. There are two types of chromophore: 11-

cis-retinal, which is an aldehyde derivative of vitamin A_1, and 11-cis-3, 4-didehydroretinal, an aldehyde derivative of vitamin A_2. Several species appear to tune their vision by switching from one chromophore to the other depending upon developmental stage or spectral environment (e.g. Temple et al., 2006). Intracellular oil droplets also influence light sensitivity by narrowing the range of wavelengths available to the opsin-chromophore complex (Bowmaker & Knowles, 1977; Partridge, 1989). Oil droplets are common in reptiles and birds, they are also found in lungfish and some amphibians (For a review see Hart et al., 2006). However, the focus of this thesis is on the modulation of color vision by the opsin genes themselves, therefore oil droplets and chromophore use will not be discussed further.

Phylogenetic analysis has determined that all vertebrate visual opsins belong to five subclasses (Yokoyama, 1994): Two short wave-sensitive opsin subclasses (SWS1 and SWS2), a rhodopsin subclass (RH1), a rhodopsin-like opsin subclass (RH2), and the long wave sensitive opsins (LWS) (Yokoyama, 2000). Orthologs of each of the five subfamilies can be found in the lamprey, *Geotria australis* (Collin & Trezise, 2004; Davies et al., 2007). This suggests that these major classes of opsins genes existed before the divergence of jawed and jawless vertebrate lineages.

Repertoire

Opsin repertoire size varies significantly as a result of gene duplication and pseudogenization among the vertebrate groups; this is something that will be extensively discussed in Chapter 3. Interestingly, polymerase chain reaction (PCR) based survey and whole genome data have indicated that many members of the ray-finned fish lineage (Actinopterygii) possess large opsin gene repertoires. The expansion of their opsin gene repertoires, in conjunction with the impact of visual capacity on life history in fish, is the reason why ray-finned fish and specifically livebearers are the focus of this research endeavour. Zebrafish (*Danio rerio*), smelt (*Plecoglossus altivelis*), cichlids and the livebearers (families Poecilidae and Anablepidae) have all been found to have especially large opsin repertoires (Rennison *et al.* unpublished). We have chosen guppies (Poeciliidae) as our model because of the possible connections between opsin genes, color vision and sexual selection.

Key sites

Gene duplication often leads to genetic and functional diversification (Ohno 1970; Taylor & Raes, 2004). In opsins, this diversification is observable as amino acid substitutions at so-called key sites. Reconstitution experiments combined with site-directed mutagenesis have provided insight into the functional consequences of opsin sequence divergence. Among the ~350 amino acid residues in an opsin there are a subset of residues that have a large impact on spectral sensitivity; these are termed 'key sites' (Yokoyama, 1995; Yokoyama & Radlwimmer, 1999). Many of the key sites interact directly with the chromophore and are located in the retinal binding pocket, within 4.5 Å of the chromophore (Palczewski et al., 2000; Ebrey & Takahashi, 2002), for a recent review see (Yokoyama, 2008). The change in spectral sensitivity as a result of amino acid substitution at key sites can range from only a few nanometers (nm) to greater than 60 nm.

Differential expression

Gene repertoire and key site substitution are important, but expression also contributes to environmental adaptation. Some vertebrate species modulate their visual capacity through differential expression (Carleton & Kocher, 2001; Takechi & Kawamura, 2005; Parry et al., 2005; Temple et al., 2008b). Differential expression of opsins was first noted in the pollack (*Pollachius pollachius*), through measuring cone frequency (using microspectrophotometry), it was found that SWS expression switches during development (Shand et al., 1988). Subsequent studies found that many other fish exhibit some type of differential opsin expression; this differential expression has been found to vary both temporally during development (Shand et al., 2008) and spatially across the retina (Takechi & Kawamura, 2005). Prior to the discovery of the fluidity of opsin expression it was thought that only the loss of a cone type or chromophore modification would be able to modify spectral sensitivity (Hárosi, 1994; Kunz et al., 1994). These findings of differential expression have demonstrated that it is essential to not only understand what opsin genes a fish possesses, but also to understand how they are used. There are several ways to measure gene expression once a repertoire has been characterized.

In situ hybridization

To obtain quantitative data on gene expression reverse transcription quantitative polymerase chain reaction (RT-qPCR) can be used. Alternatively, *in situ* hybridization allows the spatial localization of gene expression in a tissue of interest. *In situ* hybridization was first described in 1969, where it was used to localize ribosomal gene transcripts in *Xenopus* oocytes at the cellular and sub-cellular levels (Gall & Pardue, 1969). Shortly thereafter *in situ* hybridization became widely used on a diversity of sectioned/whole-mount tissue, where the goal was to determine what subpopulation of cells expressed a particular gene transcript (examples below).

In situ hybridization entails binding of a labelled single-stranded ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) probe to a single stranded messenger RNA (mRNA) target in a tissue sample of interest. Since its origin, many variations have been developed. For example, originally DNA probes were utilized, however RNA probes are now more commonly employed. Additionally, immuno-cytochemical detection may be accomplished by several methods, which fall into two categories, isotopic and non-isotopic. Non-isotopic are the most widely used methods today; these include incorporation of biotin (Langer-Safer et al., 1982; Brigati et al., 1983; Forster et al., 1985) and labelling with digoxigenin (DIG) (Herrington et al., 1989). Digoxigenin is found exclusively in the *Digitalis purpurea* plant, and therefore highly specific antibodies can be used for localization that do not interact with endogenous antigens. In this study, antibodies targeted to digoxigenin were conjugated to alkaline phosphatase, which reacts enzymatically with NBT (Nitro-Blue Tetrazolium Chloride) & BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) substrates. The product of this reaction is an insoluble purple precipitate that can be imaged using a light microscope.

In situ hybridization has been used clinically as a molecular tool for detecting the presence of viral genomes in cytological preparations and in cytogenetics to assign genes to chromosomes (Geuskens & May, 1974; Gerhard et al., 1981; Gowans et al., 1981). It has also been widely used in academic research to give an indication of where a gene of interest is actively being transcribed in a given tissue sample (e.g. (Simerly et al., 1990)). When applied to gene duplicates, this technique can indicate how expression differs

spatially or temporally and this can be used to identify examples of neo- or sub-functionalization (e.g. (Takechi & Kawamura, 2005)).

Summary

This thesis explores the molecular evolution of opsin gene duplicates at both the sequence and expression level. My focus was on guppy opsins because of the possible connection to color-based sexual selection. Previous PCR based analysis had indicated that guppies and other members of Poeciliidae, such as swordtails (*Xiphophorus* sp.), had surprisingly large opsin gene repertoires (nine or ten genes) (Hoffmann et al., 2007; Ward et al., 2008; Watson et al., 2010). I characterized the repertoire in a close relative of the guppy (Chapter 2) to determine whether or not the large repertoires of Poeciliidae were associated with color-based sexual selection on a phylogenetic tree. This research showed that more distant relatives of the guppy, in Anaplepidae, also have large, but unique repertoires and it uncovered examples of opsin gene conversion and convergent evolution. This showed that opsin gene duplication pre-dates color based sexual selection on a phylogenetic tree, suggesting that it is unlikely that the ten-opsin repertoire of the guppy evolved exclusively for color-based sexual selection. In Chapter 3, this evolutionary perspective was expanded. I discovered that opsin gene repertoire varies enormously among fishes and that large opsin repertoires are fairly common due to multiple tandem duplication events, which span the age of the taxon Teleostei. Additionally, it was found that convergent evolution at key sites has an important effect on spectral sensitivity. Chapter 4 examines the expression pattern of the nine cone opsins of the guppy (*Poecilia reticulata*) in the retina. *In situ* hybridization was used to determine how the guppy utilizes its opsin repertoire in the retina, in hopes of further understanding the basis of female mate preference and the evolution of ornate male coloration in this species. The final chapter synthesizes all of this work and discusses future directions of this investigation, in an evolutionary and ecological framework. Specifically the implications of these data on the interpretation of models of sexual selection are explored.

Chapter 2
The Opsin Repertoire Of *Jenynsia onca*: A New Perspective On Gene Duplication And Divergence In Livebearers

Abstract

The opsin gene repertoires of the four-eyed fish (*Anableps anableps*) and the guppy (*Poecilia reticulata*) have been characterized, but the relationships among some of these opsin sequences are unclear; this makes it difficult to test hypotheses that link duplication and diversification of opsins to the fascinating morphology and life history traits found in each species. Here I have sequenced opsin genes from a close relative of these two species, the one-sided livebearer (*Jenynsia onca*), in an attempt to resolve these relationships. We sequenced nine genes; LWS S180r, LWS S180, LWS P180, SWS1, SWS2A, SWS2B, RH1, RH2-1, and RH2-2. Key site analysis revealed only one unique key site haplotype in the repertoire, RH2-2. However, the amino acid substitution found only in *J. onca* is unlikely to shift maximal absorption significantly. An analysis that included opsins from guppy, the four-eyed fish and the one-sided livebearer exposed relationships that were unclear when only the guppy and four-eyed fish sequences were considered. In particular, the LWS P180 gene from *J. onca* is clearly an ortholog of the LWS P180 from guppy and the LWS gamma from the four-eyed fish. This observation moves the location of the LWS P180-S180 tandem duplication event back to the base of the Poeciliidae-Anablepidae clade, expanding the number of species possessing this unusual blue shifted LWS opsin. However, the *J. onca* LWS P180 opsin appears to have been modified by a gene conversion event with LWS S180, but it has either retained or re-evolved a proline residue at the 180 site. This gene conversion event is very similar to those that have homogenized paralogs, and disrupted evolutionary relationships among opsins in humans.

Introduction

Jenynsia onca, the one-sided livebearer, is distributed in freshwater lakes and rivers throughout southern Brazil (Lucinda et al., 2002) and has normal eyes unlike the "four-eyed" eye morphology of species in its sister group, which includes *Anableps anableps*.

Distinguishing features of a male *J. onca* include a tubular un-scaled gonopodium, which is either dextral or sinistral. Females of this species correspondingly have either a dextral or sinistral genital opening and mate only with complementary sided males. Additionally, *J. onca* has dark circular spots on the ventral portion of the flank (Lucinda et al., 2002; Nelson, 2006). Here we have used PCR to characterize the opsin genes of this species. The goal of this survey is to produce a data set that helps us characterize the relationships of opsins from guppies and the four-eyed fish *Anableps anableps*.

Vision is an interesting and dynamic sensory modality, particularly in teleost fish, a group that possess some of the greatest morphological and habitat diversity of any animal group. The first step of vision is light absorption, which occurs via opsins expressed in the rods and cones of the retina. There are five sub families of vertebrate opsins each with their maximal absorption focused on a different area of the visual spectrum (Applebury, 1994). These five subfamilies include RH1 a rod specific class (490-500 nm), LWS a long-wave sensitive class (490-570 nm), RH2 a middle-wave sensitive class (480-533 nm), SWS1 and SWS2 short-wave sensitive classes (355-440 nm and 410-490 nm respectively) (Yokoyama, 2000). Specific amino acid residues that give each opsin its unique spectral sensitivity (Yokyama et al., 1999; Yokyama, 2000; Yokoyama, 2002; Chinen et al., 2005b; Takenka & Yokoyama 2007; Yokoyama et al., 2007; Yokoyama et al., 2008). These residues are termed key sites, they are often found at positions where there is contact between the protein and the chromophore (Yokoyama, 1995; Yokoyama et al., 1999).

Opsin repertoires in fish are particularly interesting due to the extensive pattern of opsin gene duplication and divergence in the teleosts. Particularly interesting are the opsin repertoires of the livebearers. Ongoing studies of opsin gene duplication and divergence in guppies (*Poecilia reticulata*) and the four-eyed fish (*Anableps anableps*) have shown that both species have an expanded LWS repertoire. Both species possess recent species-specific duplicates and a repertoire of ten visual opsins (Hoffmann et al., 2007; Ward et al., 2008; Owens et al., 2009). Interestingly, both also have unusual morphology. In guppies this is a variable male pigmentation pattern, while *A. anableps* have unusual four-eyed morphology. Based on its phylogenetic position, *J. onca* functions as a useful out-group for both *A. anableps* and the Poeciliids (Hrbek et al.,

2007). Out-groups are used to identify synapomorphies (shared derived characters) and can indicate whether one apomorphic (unique) trait evolved before another. Here my goal is to determine whether or not particular opsin genes and gene sequences are associated with coloration in guppies and the four-eye morphology in *A. anableps*.

Opsin expression patterns are also more variable in fish than in tetrapods. For example, in zebrafish (*Danio rerio*) LWS opsin duplicates appear to have different expression domains in the retina and this pattern of expression also changes during development (Takechi & Kawamura, 2005). This has been hypothesized to be a response to the heterogeneous environment the fish live in, with spectral properties differing between the light that hits the dorsal region of the retina and that which hits the ventral region. Another example of differential expression is in cichlids, in this system a subset of the available opsin repertoire is used by each particular species to tune sensitivity in a habitat dependent way (Parry et al., 2005). The first step to characterizing these fascinating expression patterns is through the elucidation of the opsin repertoire itself.

Methods

PCR primers were designed to amplify nine opsins from the five visual opsins subfamilies (Table 2-1). These primers were complementary to regions in each opsin gene or subfamily that were conserved in guppies (*Poecilia reticulata*), and *A. anableps*. Two primer pairs were engaged for each gene.

Each primer pair was used to survey complementary DNA (cDNA) or genomic DNA in PCR reactions using Bio-Rad iProof™ high-fidelity DNA polymerase in an Eppendorf™ Mastercycler® EP Grad S thermocycler using the following conditions: Initial denaturation at 98 degrees Celsius (^{o}C) for 30 seconds (s), 35 cycles with denaturation at 98^{o}C for 5s, annealing at 55 - 65^{o}C (in 5°C intervals) for 12s, extension at 72^{o}C for 25s and a final extension at 72^{o}C for 5 minutes (min). Additional primers (1µl at 10mM) were added, at the beginning of the last PCR cycle to prevent hetero-duplex formation.

Amplicons of the predicted size were excised from agarose gels using QIAquick® Gel Extraction Kit or purified using QIAquick® PCR Purification Kit. Purified products were A-tailed using Invitrogen™ Taq polymerase and cloned using the Promega® pGEM™ - T

Easy Vector System II kit. Clones containing inserts of the correct size were sequenced using labelled M13 forward and reverse primers and a LI-COR sequencer at the University of Victoria Centre for Biomedical Research.

Live *J. onca* were obtained from a commercial supplier (The Afishionados, Winnipeg, Manitoba, Canada). Two adult *J. onca* (one male and one female) were euthanized in buffered tricaine methanesulfonate (MS222). Total RNA was isolated from the eyes using Aurum™ Total RNA Fatty and Fibrous Tissue Pack, immediately after euthanasia and enucleation cDNA was synthesized using BioRad® iScript Select cDNA Synthesis Kit from total RNA. Genomic DNA was isolated from the fish carcass using QIAquick® DNeasy Blood & Tissue Kit.

Two phylogenetic trees were reconstructed for the complete set of opsin sequences. The partial coding sequence tree included sequences from *Jenynsia onca, Anableps anableps, Poecilia reticulata, Xiphophorus pygmaeus, Lucania goodei, Oryzias latipes and Danio rerio*, *Poecilia picta, Poecilia parae, Poecilia bifurca and Tomeurus gracilis* sequence files used were 412 to 819 base pairs (bp) long. The second tree was based on 243 bp of LWS 3' coding sequence from *J. onca, A. anableps, P. reticulata, X. pygmaeus, P. picta, P. parae, P. bifurca and T. gracilis* [Accession numbers see Appendix 1]. The aligned sequences for the partial coding sequence phylogenetic tree were first used to obtain the best-fit model of evolution using Modeltest (Posada & Crandall, 1998). The phylogenetic reconstruction was done using Maximum likelihood (ML) and Neighbor-joining (NJ) (1000 bootstrap reanalyses) in PAUP* 4.8B10 and utilized the optimal model parameters (Felsenstein, 1981; Felsenstein, 1985; Saitou & Nei, 1987; Swofford, 2002). The root of the partial coding sequence tree was positioned along the branch separating the LWS opsins from all others (Okano et al., 1992). The 3' coding tree was constructed using MEGA4 using the Jukes-Cantor algorithm, NJ, and support for nodes were estimated using 500 bootstrap reanalyses (Jukes & Cantor, 1969; Felsenstein, 1985; Saitou & Nei, 1987; Tamura et al., 2007). Pair-wise deletion was used in the case of missing nucleotides for the analysis.

Gene conversion detection was undertaken using GENECONV version 1.81A (Sawyer, 1999). We used the program's default values. The input for analysis was a coding sequence nucleotide alignment containing *A. anableps* LWS S180α and LWS S180γ as

well as *J. onca*, *X. pygmaeus*, *P. picta, P. parae, P. reticulata, and P. bifurca* LWS S180 and LWS P180.

Table 2-1 Primers used for *Jenynsia onca* cDNA and genomic PCR.

Opsin category	Primer Name	Sequence
SWS1	SWS1Fw1	5'- AACTACATCYTGGTMAACATCTCC-3'
	SWS1Rev2	5'-GAACTGTTTGTTCATGAAGGCG-3'
SWS2	SWS2Fw1	5'-GYACWATTCAATACAAGAARC-3'
	SWS2Rev2	5'-TCTCWGCCTTCTGGGTKGAGGC-3'
	SWS2AFw1	5'-GTCCACCCGAGTCATAGAGC-3'
	SWS2ARev2	5'-GCCCACGGTTGTTGACAAC-3'
RH2	RH2Fw1	5'- AACTTCTAYATCCCGWTGTCC-3'
	RH2Rev1	5'- AGCATGCAGTTACGGACTG -3'
	RH2-2Fw1	5'-CAACAGGACGGGCTGGTGAGG-3'
	RH2-2Rev3	5'-ACCCATTCCAATTGTTGCC-3'
RH1	RH1Fw2	5'-GGAGTCCTTATGAATATCCTCAG-3'
	RH1Rev2	5'-CCTGTTGCTCCATTTATGCAGG-3'
LWS	Fw100	5'-GATCCCTTTGAAGGACCAAACT-3'
	Fw1a	5'-TCTTATCAGTCTTCACCAACGG-3'
	RevEnd	5'-TTATGCAGGAGCCACAGAGG-3'
	Rev8	5'-GCCCACCTGTCGGTTCATGAAG-3'

Results and Discussion

PCR screening using gene specific primers (Table 2-1) amplified nine visual opsins: LWS S180r, LWS S180, LWS P180, SWS1, SWS2A, SWS2B, RH1, RH2-1, & RH2-2. All opsins except LWS S180r and S180 are expressed, having been amplified from eye cDNA derived from one adult male and one adult female *J. onca*. LWS S180r and S180 opsins were amplified only from genomic DNA. However, the reason that LWS S180 and S180r could not be retrieved from cDNA could be attributed to life stage, as only adults were used in this study. A particularly interesting finding in this repertoire was the LWS P180 opsin. An alignment that included LWS opsins from *J. onca*, *P. reticulata*, and *A. anableps* reveals a region with many shared amino acid substitutions. My conclusion that these genes are orthologs moves the point of LWS duplication farther back within Cyprinodontiformes. I did not find any species-specific gene duplication events in *J. onca*, but my PCR based survey might have missed some genes.

The *J. onca* opsins were aligned to orthologous sequences from other fish species. Sequences in the alignment were 573 to 930 bp long. We used PAUP* 4.0B10 to calculate genetic distances based on the modeltest best-fit model of sequence evolution and to reconstruct a Neighbor joining (NJ) tree (Figure 2-1) (Felsenstein, 1981; Saitou & Nei, 1987; Swofford, 2002). Sequences from each opsin subfamily formed well-supported monophyletic groups, with bootstrap support ≥97% (1000 replicates) (Felsenstein, 1985).

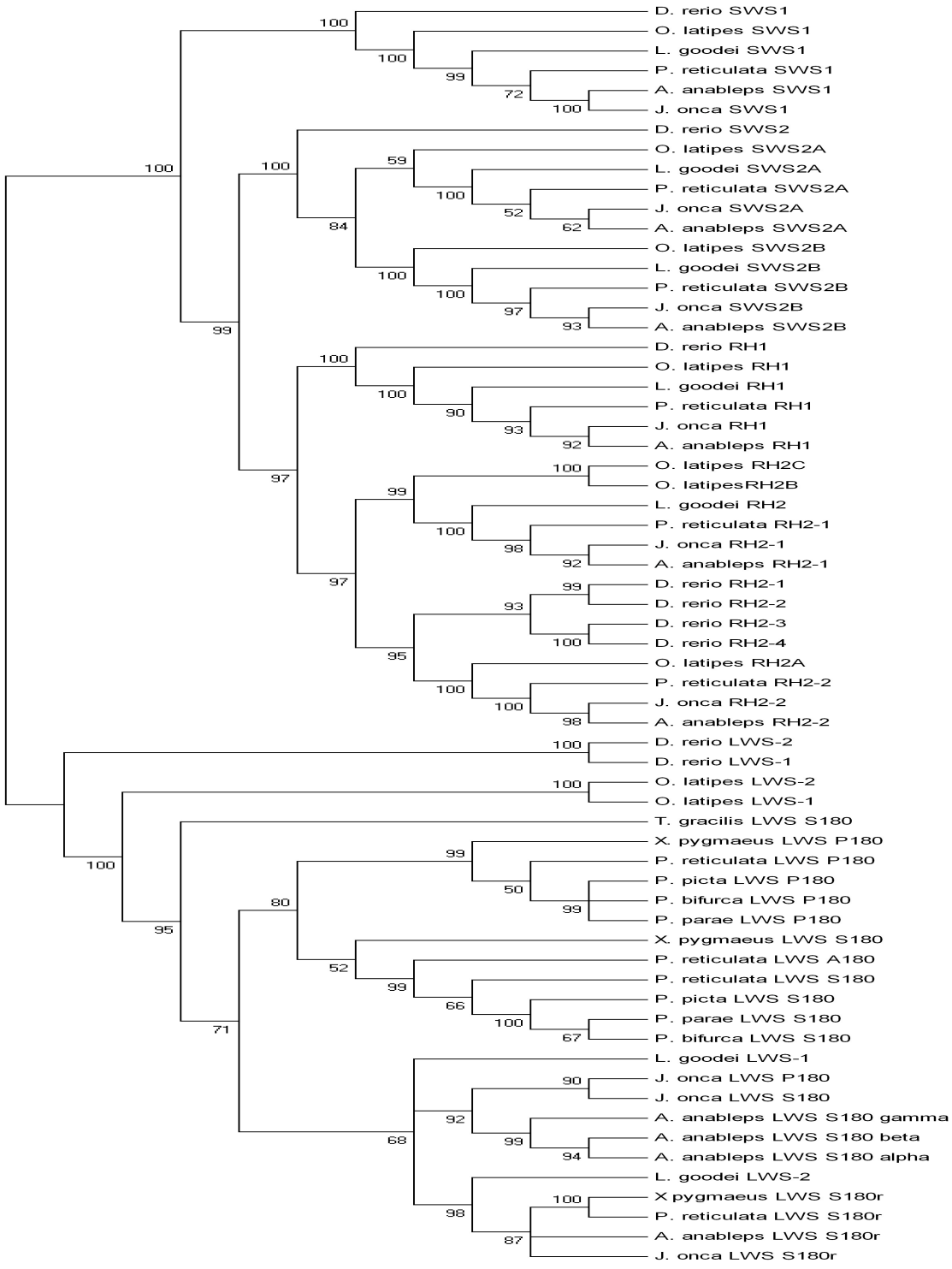

D. rerio SWS1
O. latipes SWS1
L. goodei SWS1
P. reticulata SWS1
A. anableps SWS1
J. onca SWS1
D. rerio SWS2
O. latipes SWS2A
L. goodei SWS2A
P. reticulata SWS2A
J. onca SWS2A
A. anableps SWS2A
O. latipes SWS2B
L. goodei SWS2B
P. reticulata SWS2B
J. onca SWS2B
A. anableps SWS2B
D. rerio RH1
O. latipes RH1
L. goodei RH1
P. reticulata RH1
J. onca RH1
A. anableps RH1
O. latipes RH2C
O. latipesRH2B
L. goodei RH2
P. reticulata RH2-1
J. onca RH2-1
A. anableps RH2-1
D. rerio RH2-1
D. rerio RH2-2
D. rerio RH2-3
D. rerio RH2-4
O. latipes RH2A
P. reticulata RH2-2
J. onca RH2-2
A. anableps RH2-2
D. rerio LWS-2
D. rerio LWS-1
O. latipes LWS-2
O. latipes LWS-1
T. gracilis LWS S180
X. pygmaeus LWS P180
P. reticulata LWS P180
P. picta LWS P180
P. bifurca LWS P180
P. parae LWS P180
X. pygmaeus LWS S180
P. reticulata LWS A180
P. reticulata LWS S180
P. picta LWS S180
P. parae LWS S180
P. bifurca LWS S180
L. goodei LWS-1
J. onca LWS P180
J. onca LWS S180
A. anableps LWS S180 gamma
A. anableps LWS S180 beta
A. anableps LWS S180 alpha
L. goodei LWS-2
X pygmaeus LWS S180r
P. reticulata LWS S180r
A. anableps LWS S180r
J. onca LWS S180r

Figure 2-1 Phylogenetic analysis of *Jenynsia onca* opsins.

A neighbor-joining bootstrap tree that uses opsin coding sequence from *J. onca* and relatives. The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is reported at the nodes. PAUP* 4.0B10 was used, the tree is based on modeltest's best-fit model of evolution, and complete phylogenetic analysis (Felsenstein, 1985; Saitou & Nei, 1987; Posada & Crandall, 1998; Swofford, 2002) [For accession numbers see Appendix 1]. All codon positions were included. Pair-wise deletion was used in the case of missing nucleotides for the analysis.

The phylogenetic analysis based upon opsin gene sequences was consistent with regard to both the subfamily that the genes fell into and with species taxonomy. The LWS subfamily can be further grouped into haplotype clades, however, *J. onca* LWS S180 and LWS P180 did not fall out on the tree where they would be predicted to based on haplotype identity (Figure 2-1). LWS P180 was very similar to *A. anableps* LWS S180γ and Poeciliidae LWS P180 over its 3' end (Figure 2-2). When only the 3' region of these two genes and the Poeciliidae LWS are used in phylogenetic analysis two distinct clades with 60% bootstrap support are observed separating LWS S180 from LWS P180 (including *A. anableps* LWS S180γ) (Figure 2-3).

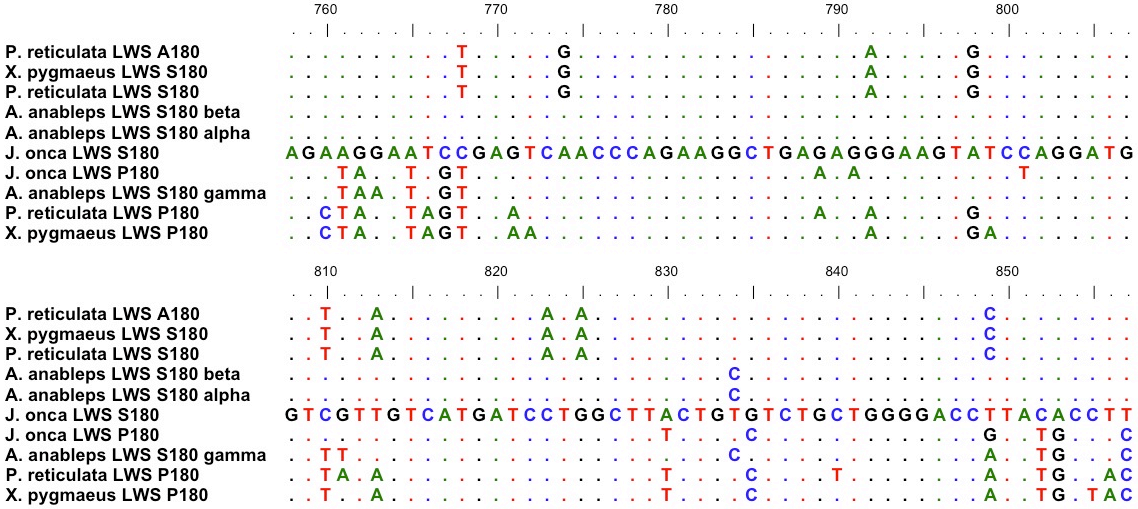


Figure 2-2 Sequence alignment of conserved LWS P180 sequence.

A sequence alignment of a 100 base pair portion (nucleotide 758 - 857) of the *J. onca* LWS P180 and *A. anableps* LWS S180γ and Poeciliid LWS genes. Highlighted is the *J.onca* S180. This exemplifies the similarity among the *J. onca* LWS P180, Poeciliid LWS P180 and *A. anableps* LWS S180γ [Accession numbers for these sequences are listed in Appendix 1].

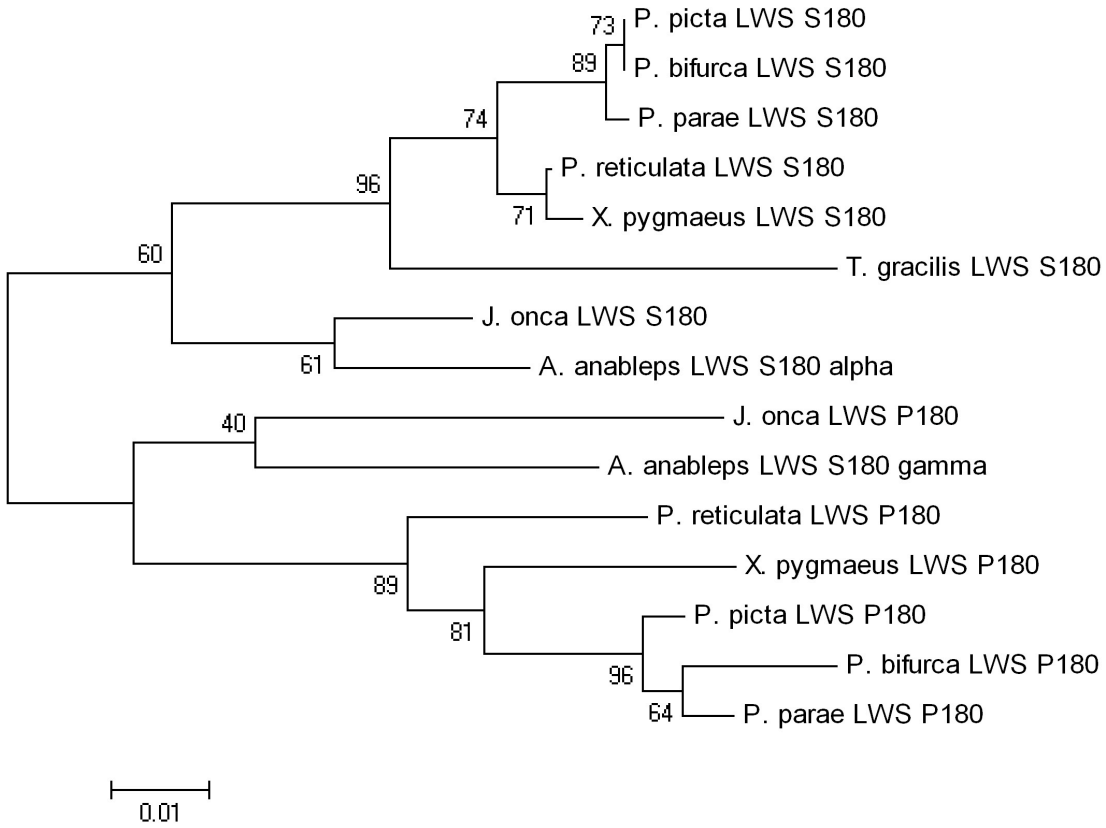


Figure 2-3 Phylogenetic analysis of shared 3' LWS P180 sequence.

A neighbor-joining bootstrap consensus tree of a 243 base pair portion of 3' LWS S180 and LWS P180 opsins from *J. onca* and relatives. The percentage of trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is reported at the nodes (Felsenstein, 1985). The Jukes-Cantor algorithm was used and all codon positions were included (Jukes & Cantor, 1969). Pair-wise deletion was used in the case of missing nucleotides for the analysis. Phylogenetic analyses used MEGA4 (Tamura et al., 2007) [For accession numbers see Appendix 1].

I hypothesize that two recent gene conversion events explain the pattern observed above (Figure 2-3). One event would have occurred between the two *J. onca* genes, from LWS S180 to LWS P180. This was an incomplete conversion and resulted in a LWS P180 gene with a short untouched sequence string at the 3' end. This conversion event in *J. onca* also explains why the sequence surrounding the proline at amino acid position 180, which occurs before the 3' end, is very similar to the *J. onca* LWS S180. Indeed, based on the pattern of similarity it seems likely that the P180 substitution occurred independently in *J. onca*. The second conversion event appears to have occurred recently in the *A. anableps* the majority of LWS S180γ, was over-written by LWSS180α. This event explains why LWS S180α and LWS S180γ are nearly identical and it also explains why LWS S180γ groups with the *J.onca* and poeciliid LWS P180 genes only when exclusively 3' sequences are used in phylogenetic reconstruction (Figure 2-4). This conversion event also clarifies why the *A. anableps* LWS S180γ does not have a proline at the 180 amino acid site. My hypothesis that gene conversion has influenced sequence variation among opsin paralogs is supported by an analysis using GENECONV 1.81A, which detects sequence pairs that have abnormally long continuous regions of high sequence similarity found within regions of lower similarity overall (Li, 1993; Sawyer 1999). GENECONV detected a gene conversion event between LWS S180 and LWS P180/ S180γ in *A. anableps* from the beginning of the sequence to 629 bp ($p = 0.012$) and in *J. onca* from the start of the sequence to 567 bp ($p = 0.036$), which correspond to my predicted conversion events.

An alternative explanation for this pattern of evolution would be convergent evolution at the level of amino acid sequence and codon over this 3' region (Figure 2-3). However convergence in non-coding nucleotide positions seems unlikely and given the previous observation that gene conversion between linked opsins is common, independent evolution of the region shown in Figure 2-3 is not my preferred hypothesis. Homogenized key site haplotypes have been observed not only in fish, such as the guppy where the LWS A180 is a product of duplication followed by partial gene conversion, but also in humans where conversion events are often detrimental (Winderickx et al., 1993; Reyniers et al., 1995; Ward et al., 2008). The conversion events I propose in livebearers may have been facilitated between LWS P180 and LWS S180 due to their position as tandem

inverted duplicates, something that has been confirmed in both *Poecilia* and *Xiphophorous* (Ward et al., 2008; Watson et al., 2010).

As mentioned above, there are key sites within each opsin subclass, which may be used to estimate the opsin maximal absorption. I investigated the key site haplotypes of these nine opsins and based on the LWS haplotypes I have estimated the LWS maximal absorption values (Yokoyama, 2008) [see Appendix 2]. The key site substitutions seen in the LWS P180 although found in other species, confer a significant change in maximal absorption; the serine to proline substitution alone results in a -19 nm shift (Davies et al., 2009). Only one key site substitution is not seen in *J. onca*'s relatives; RH2-2 deviates from the isoleucine consensus residue to a valine residue in amino acid site 65 (based on human LWS amino acid numbering (Nathans et al., 1986b), although this would likely not significantly shift the maximal absorption (Takenaka & Yokoyama, 2007). The most interesting point to glean from the haplotype comparison is that despite significant nucleotide divergence between *J. onca* and its relatives there is a large degree of amino acid conservation at key sites between orthologs.

Conclusion

Jenynsia onca has nine visual opsins: three LWS genes, one RH1 gene, two RH2 genes, an SWS1 gene and two SWS2 genes. There is significant phenotypic (maximal absorption) conservation among the three species compared here, with only one instance of amino acid key site residue substitution. These *J. onca* data will help us identify differences in opsin expression that are associated with the unusual eyes of *A. anableps* and with the remarkable coloration of guppies by acting as an out-group.

Contributions for this chapter

This chapter is published in BMC Research Notes 2009, 2:159 Windsor D.J & Owens G.L, under the title of "The opsin repertoire of *Jenynsia onca*: a new perspective on gene duplication and divergence in livebearers". There have been minor changes in the version presented here. The author contributions of this paper are as such: Diana J. Rennison (Windsor) & Gregory L. Owens carried out the PCR survey of cDNA and genomic DNA, completed the sequence alignment, and created the phylogenies. Diana J. Rennison drafted the manuscript.

Chapter 3
Opsin Gene Duplication and Divergence in Ray-finned Fish

Abstract

Ray-finned fish (Actinopterygians) possess surprisingly large opsin repertoires, significantly larger than those found in their sarcopterygian (lobed finned fish and tetrapods) relatives. In chapter three I provide the first large-scale analysis and review of ray-finned fish opsin sequence data. I also characterized the duplication events that have lead to the ten gene repertoire in my focal species, the Cumana guppy (*Poecilia reticulata*). I reconstructed the fish opsin gene phylogeny and detected a total of forty-two duplication nodes. These duplication events span the age of the taxon Actinopterygii, but are not evenly distributed among the five opsin subfamily clades. Tandem duplication appears to be the most prevalent mechanism of gene duplication. Surprisingly, the 3R whole genome duplication that occurred in the ancestor of ray-finned fish does not appear to have contributed to opsin amplification in this lineage. In some cases opsins gene loss has taken place after long periods of retention. By mapping amino acid substitutions at key sites onto subfamily phylogenies, I reveal many examples of convergent evolution, i.e., the same residues and sometimes even the same key site haplotypes have evolved more than once. Gene conversion also appears to have played a role in opsin diversification, specifically in the LWS opsin subfamily. By correlating key site substitution and maximal absorption, I find that key site substitution does not always explain variation in spectral sensitivity.

Introduction

Opsin genes encode the protein component of visual pigments found in retinal photoreceptors. They function as light receptors when bound to one of the two chromophores (vitamin A1 or A2 derivatives). The size of the opsin gene family varies among vertebrate species. For example, most mammals have three genes, whereas some ray-finned fish have ten. Furthermore, direct connections can be made between sequence variation and function (i.e., wavelength sensitivity). These factors, copy number variation, sequence variation, and knowledge of the functional implications of sequence

variation, make the opsin gene family an unusually good model for investigations into gene duplication and divergence (Jacobs et al., 1998; Yokoyama & Radlwimmer, 1999).

Opsin gene duplication is not only important for sensitivity to a diversity of colors; it is also the key to discriminating among these colors. This is because color vision requires the comparison of photon absorption from multiple distinct photo-pigments. Most mammals possess a short-wavelength sensitive (SWS1) opsin, a middle wave-sensitive opsin used in dim-light (RH1), and a long-wavelength sensitive (LWS) opsin (Jacobs, 1993). Only the SWS1 and LWS opsin are expressed in cone cells and used for daytime (bright light) vision. However, as a result of LWS gene duplication and divergence in the ancestor of great apes, humans and other great apes acquired trichromacy, a term describing the three-receptor basis of color vision (Ibbotson et al., 1992). Trichromacy improves wavelength discrimination in these species compared to mammals with two cone cell receptors (dichromats) and compared to so-called color deficient humans that have lost the use of one of their two LWS opsin genes (Nathans et al., 1986a; Jacobs et al., 1996).

Mammals, even great apes with their LWS duplicates, have fewer opsin genes than most vertebrates (Yokoyama, 1994). A second shortwave sensitive subclass (SWS2) and a rhodopsin-like (RH2) subclass were discovered in surveys of goldfish (*Carassius auratus*) (Johnson et al., 1993), Carolina anole (*Anolis carolinesis*) (Kawamura & Yokoyama 1995; Kawamura & Yokoyama 1996) and chicken (*Gallus gallus*) (Okano et al., 1992). In some taxa these opsin subclasses have experienced subsequent duplications; zebrafish for example have four RH2 opsin genes (Vihtelic et al., 1999; Chinen et al., 2003). PCR-based and whole genome sequence surveys have now examined enough ray-finned fish (actinopterygians) to provide confidence in the conclusion that many species in this group possesses more opsins than their lobe-finned fish (sarcopterygian) relatives. However, so far these data have not been included in a single large-scale analysis.

In this study opsin gene duplication events were mapped onto a fish phylogeny. For some species, genomic data were available that allowed the determination of the mechanism of gene duplication. After identifying duplication nodes, questions about post duplication divergence were addressed. For example, as function in opsins (spectral sensitivity) can be linked to amino acid residues at key sites, these sites can tell us

whether or not opsins gain new function (sensitivities) post duplication. By mapping key site substitutions onto the phylogeny I identified many instances of convergent neo-functionalization and linked key site haplotype to phenotype. Another post-duplication gene fate I investigated was gene conversion. Gene conversion is a form of homologous recombination; it can occur between homologous chromosomes, sister chromatids or highly similar sequences and generally occurs in newly generated duplicates (Chen et al., 2007). This process entails the one-way transfer of gene sequence from a donor sequence to an acceptor sequence. During this process, the acceptor sequence is overwritten, while the donor sequence remains unchanged. These events can either be complete or partial overwrites of the acceptor sequence. Gene conversion has been shown to play an important part in the concerted evolution of multi-gene families (Liao, 1999). However, gene conversion also complicates phylogenetic reconstruction, as gene-converted paralogs often appear to be more closely related to each other than their orthologs (something that can also be attributed to recent duplication).

In this survey I found that there are forty-two opsin gene duplication nodes and the most prevalent mechanism of gene duplication is tandem duplication. However, whole genome duplication and retro-duplication have also played a role in opsin repertoire expansion. There were many examples of neo-functionalization as indicated by post-duplication modification of spectral sensitivity seen in many duplicates. Mapping key-site amino acid substitutions also revealed multiple examples of convergent evolution. Close examination of sequence structure revealed the prevalence of gene conversion in ray-finned fish opsin, particularly in tandem LWS genes. Pseudogenization and gene loss events were also found to have taken place in some of the surveyed species. Given the diversity of ray-finned fish species included in this survey, it also provided me with evolutionary context to the opsin repertoires of livebearers. The ten opsin repertoire of the guppy (*Poecilia reticulata*) seems exorbitant without the knowledge that close relatives, and more distantly related species have similar sized repertoires.

Methods

Species tree construction

A species tree was used to summarize the duplication data for the species found in each of the opsin subfamilies trees. RH1 sequences were collected using a *Danio rerio* RH1 querey sequence in a BLASTn (default parameters) search of the NCBI nucleotide database. These sequences were then hand aligned and analyzed by the maximum likelihood method in PAUP* 4.0B10, using a best-fit model of evolution generated in modeltest (Felsenstein, 1981; Posada & Crandall, 1998; Swofford, 2002). Duplication nodes were removed and terminal branches were added for *Jordanella floridae*, *Scophthalmus maximus*, *Zacco pachycephalus*, *Candidia barbatus*, *Clupea harengus* and *Nannostomus beckfordi.* These species lacked RH1 gene sequences; therefore terminal branches were added in a manner that was consistent with fish taxonomy (Nelson, 2006). To summarize duplication events five ML trees were generated (one for each subfamily), the nodes representing gene duplication events were counted and the positions of these nodes relative to speciation events were noted. Identified pseudogenization events were also mapped onto the species tree.

Opsin subfamily tree construction and key-site mapping

Default BLASTn parameters were used in the query of the NCBI nucleotide database and Ensembl genome sequence databases using *Danio rerio* sequences from each subfamily (Altschul et al., 1990). Coding sequences (DNA) were manually aligned in BioEdit (Hall, 2001), [see Appendix 3 for accession numbers of included species]. As the 5' and 3' ends sequences were variable in length, sequences were trimmed of regions that were not found in all species. See table 3.1 for sequence lengths and species numbers. Neighbor-joining phylogenies were generated from each multiple sequence alignment using a Jukes and Cantor model of evolution (Jukes & Cantor, 1969; Saitou & Nei, 1987). The un-rooted tree generated is presented with lamprey sequences as a sister group to the actinopterygian (ray-finned fishes) sequences. Subsequently, substitutions at key sites were parsimoniously mapped onto each subfamily NJ phylogeny. These key sites had been previously demonstrated to be important for wavelength sensitivity in each respective subfamily Sun et al., 1997; Yokoyama & Radlwimmer, 1998; Yokoyama et

al., 1999; Yokoyama, 2000; Babu et al., 2001; Yokoyama, 2002; Takahashi & Ebrey, 2003; Yokoyama & Tada, 2003; Parry et al., 2004; Chinen et al., 2005a; Chinen et al., 2005b; Takahashi & Yokoyama, 2005; Takenaka & Yokoyama, 2007; Yokoyama et al., 2007; Yokoyama, 2008.

Maximal absorption spectral distribution

Maximal absorption data previously generated by microspectrophotometry and *in vitro* reconstitution for species in which amino acid sequences were available was collected (see Appendix 4 for values and references). Maximal absorption data were plotted by subfamily. Assignments of wavelength of maximal absorption to subfamily used were those made by the original collectors.

Table 3-1 Sequence information for opsin subfamily trees.

Opsin subfamily	Sequence length for multiple sequence alignment	Number of ray-finned fish species included	Number of sequences included
SWS1	793 - 1053 bp	47	48
SWS2	680 – 1095 bp	50	59
RH1	726 - 1068 bp	70	82
RH2	712 - 1068 bp	54	88
LWS	411 - 1111 bp	58	81

Results

Gene duplication in surveyed ray-finned fish

Figure 3-1 shows the relationship between the major orders of ray-finned fish. Forty-two opsin duplication events were mapped onto the ray-finned fish phylogeny Figure 3-2. Two of these duplication events occurred by retro-transposition, one in LWS subclass and one in RH1 subclass. Eleven of the forty-two events are tandem duplication events.

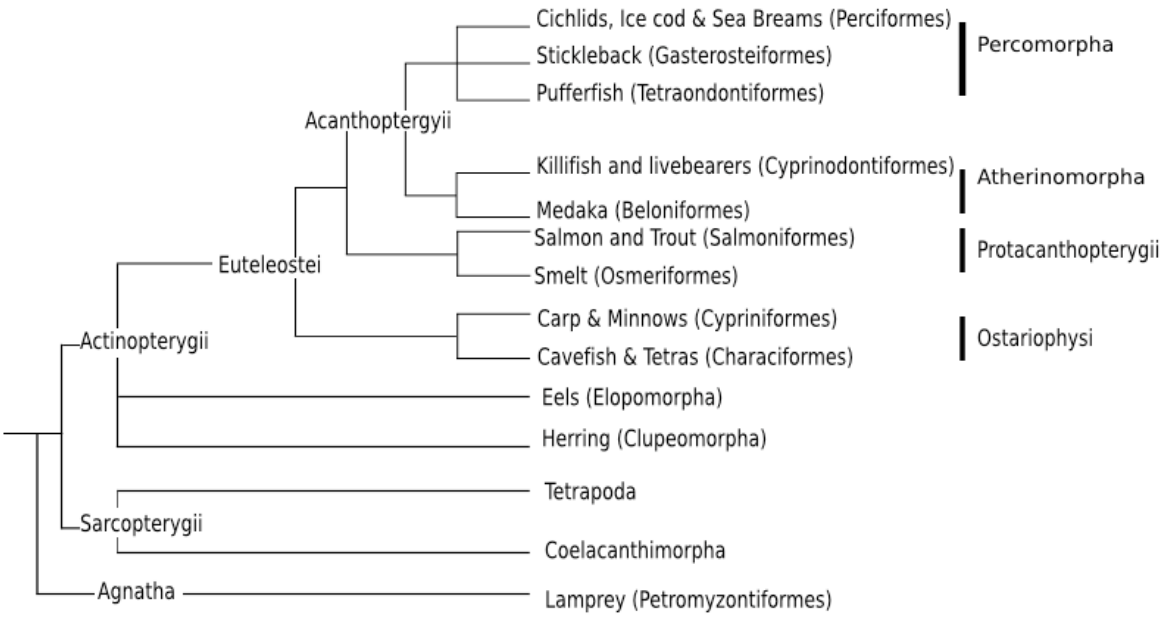


Figure 3-1 Phylogenetic relationships of representative ray-finned fish orders, as classified by Nelson 2006.

In the fifty-eight fish species with LWS opsin sequences, there have been twelve duplication events; four of these were tandem duplications. A large number of LWS opsin duplication events occur in the order Cyprinidontiformes, which includes the livebearers (e.g., guppies, swordtails, four-eyed fish and one-sided livebearer), splitfins (Goodeidae), flagfish (Cyprinodontidae) and killifish (Fundulidae). Within the LWS clade there is the LWS S180r duplicate (Ward et al., 2008). LWS S180r occurs in intron XI of the gephryin gene and has only the first of the five introns typically found in LWS opsins (Watson et al., 2010). This indicates that LWS S180r was likely generated by retroduplication. The LWS S180r gene has been found in fish in the families Poeciliidae, Anablepidae, and Fundulidae. Representative species from the families Poeciliidae and Anablepidae also share the inverted tandem duplicates LWS S180 and LWS P180. However, the Anablepid LWS P180 orthologs in *Jenynsia onca* (LWS P180) and *Anableps anableps* (S180γ) occur in a separate clade. These sequences are found as sister sequences to their LWS S180 paralogs due to gene conversion, as explained in Chapter 2 (Windsor & Owens, 2009). The guppy (*Poecilia reticulata*) also has tandem duplicates LWS S180 and LWS A180. Aside from these livebearer LWS duplicates there have been

independent duplication events in the American flag fish (*Jordanella floridae*), medaka (*Oryzias latipes*), Ayu smelt (*Plecoglossus altivelis*) and zebrafish (*Danio rerio*). There are two shared duplication events in Mexican cavefish (*Astyanax fasciatus*) and neon tetra (*Paracheirodon innesi*); the older of the two events is also shared with *Nannostomus beckfordi*. Another shared LWS opsin duplication is in *Carassius auratus* and *Cyprinus carpio,* which is thought to be a result of either whole genome duplication or chromosome duplication (Li et al., 2009).

The RH2 subclass contains twenty-one duplication events, six of these have been found to be tandem. One of these duplications generated the paralogous RH2A and RH2B genes. This tandem duplication occurred in the ancestor of fish in the taxon Neoteleosti (i.e., in Euteleostei after procanthopterygians such as smelt and salmonids diverged). Six RH2 duplication events are found in Ostariophysi alone. Some RH2 duplication events appear to have occurred early in ray-finned fish evolution. Two of these occurred in the common ancestor of the cyprinids, zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), the thickhead chub (*Zacco pachycephalus*), and the dace (*Candidia barbatus*). The degree of sequence divergence suggests that the first of these events might have occurred in the ancestor of all species in taxon Ostariophysi (Spady 2006). There have also been recent RH2 duplication events in the scabbard fish (*Lepidopus fitchi*), sockeye salmon (*Oncorhynchus kisutch*) (which appears to be derived from whole genome duplication) (Temple et al., 2008b), *Danio rerio*, *Stenobrachius leucopsarus*, *Gasterosteus aculeatus*, *Scophthalmus maximus*, *Acanthopagrus,* and *Oryzias latipes.* A duplication event is shared by multiple cichlid species and there us also a shared RH2 duplication event in *Carassius auratu*s and *Cyprinus carpio*.

For the thirty-nine species included in this study two gene duplication events were found in the SWS2 subclass. One of which is an ancient tandem duplication event shared by many fish in the superorder Acanthopterygii. Another duplication event is shared by *Carassius auratus* and *Cyprinus carpio.* This duplication is thought to be due to either whole genome or chromosomal duplication and likely is from the same event as the LWS duplication event found in these species (Li et al., 2009).

There is only one SWS1 duplication event found in ray-finned fish. These duplicates are found in Ayu smelt (*Plecoglossus altivelis*), a species in the family Osmeridae. These

duplicates are 85% identical (over a 1025 bp alignment), an observation indicating that this duplication occurred early in the evolution of osmerids and that other species in the family will probably be found to possess these duplicates.

Within the seventy species of ray-finned fish that have been surveyed, the RH1 subclass has been duplicated six times. One of these duplication events was a retro-transposition event, which formed the errlo (intron-containing) and RH1 (intron-less) gene lineages. This RH1 retro-duplication appears to have occurred in the common ancestor of all actinopterygians, as sturgeon, paddlefish, and bowfin all possess the intron-less RH1. Subsequent single intron-less RH1 duplications are distributed across the phylogeny and duplication has not occurred multiple times in any given lineage.

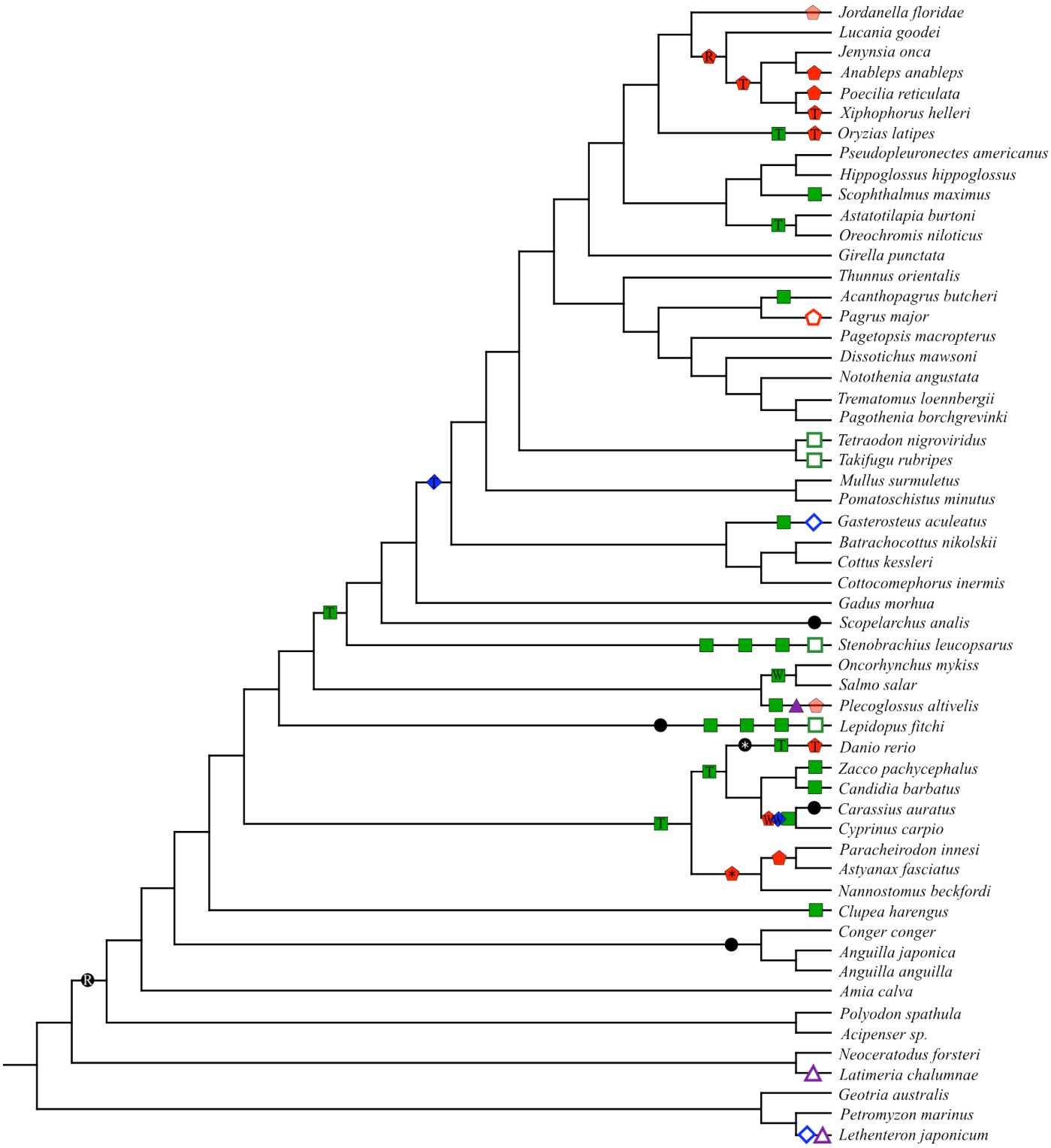

Jordanella floridae
Lucania goodei
Jenynsia onca
Anableps anableps
Poecilia reticulata
Xiphophorus helleri
Oryzias latipes
Pseudopleuronectes americanus
Hippoglossus hippoglossus
Scophthalmus maximus
Astatotilapia burtoni
Oreochromis niloticus
Girella punctata
Thunnus orientalis
Acanthopagrus butcheri
Pagrus major
Pagetopsis macropterus
Dissotichus mawsoni
Notothenia angustata
Trematomus loennbergii
Pagothenia borchgrevinki
Tetraodon nigroviridus
Takifugu rubripes
Mullus surmuletus
Pomatoschistus minutus
Gasterosteus aculeatus
Batrachocottus nikolskii
Cottus kessleri
Cottocomephorus inermis
Gadus morhua
Scopelarchus analis
Stenobrachius leucopsarus
Oncorhynchus mykiss
Salmo salar
Plecoglossus altivelis
Lepidopus fitchi
Danio rerio
Zacco pachycephalus
Candidia barbatus
Carassius auratus
Cyprinus carpio
Paracheirodon innesi
Astyanax fasciatus
Nannostomus beckfordi
Clupea harengus
Conger conger
Anguilla japonica
Anguilla anguilla
Amia calva
Polyodon spathula
Acipenser sp.
Neoceratodus forsteri
Latimeria chalumnae
Geotria australis
Petromyzon marinus
Lethenteron japonicum

Figure 3-2 Summary of opsin duplication nodes mapped onto a species tree.
Tree was constructed as a composite of a Maximum Likelihood RH1 gene tree and established species taxonomy. Gene duplication events are mapped onto the tree. Filled purple triangles, blue diamonds, black circles, green squares and red pentagons represent duplications in the SWS1, SWS2, RH1, RH2 and LWS subfamilies respectively. A 'T' within a shape represents that the duplication is known to be a tandem duplication while 'R' means that it is a retro-transposition event and 'W' indicates origin by whole genome duplication. Hollow shapes represent identified pseudogenization events, while transparent shapes represent gene pairs that might be alleles. A star indicates that the duplication event is likely older than what the phylogeny indicates.

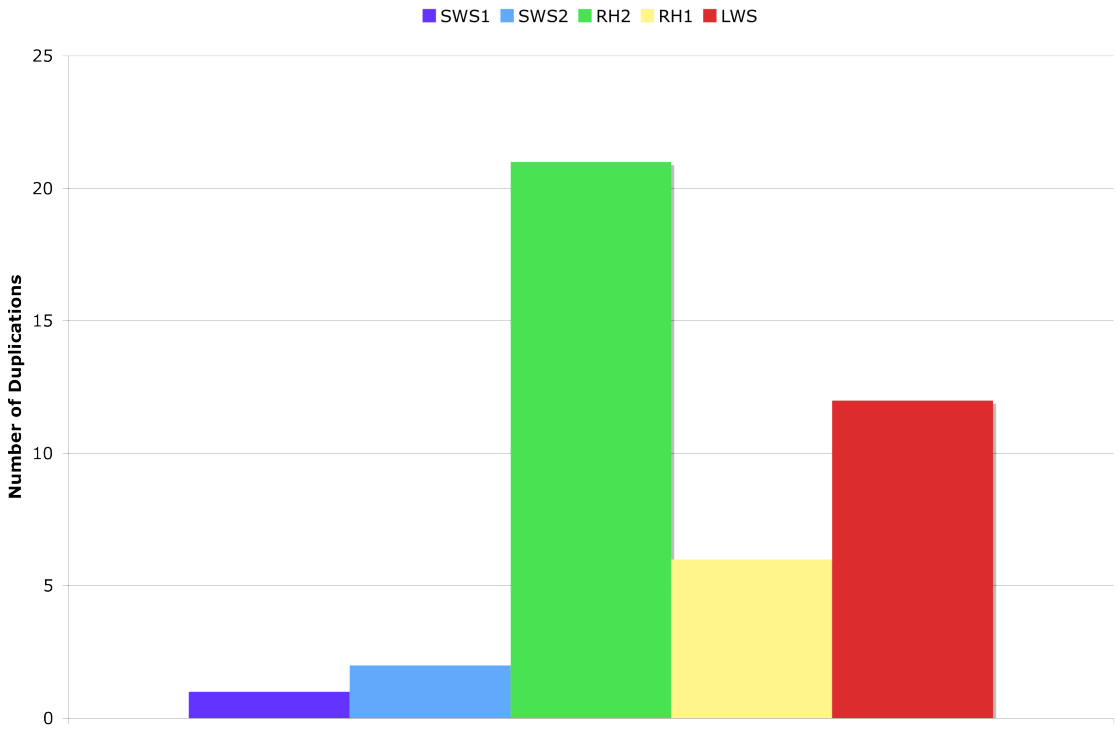


Figure 3-3 Number of gene duplications in ray-finned fish, by opsin subfamily.

Pseudogenes and gene loss

A large number of gene duplication nodes have been identified in ray-finned fish, as seen in Figure 3-2. While some species have retained both products of these duplication events, many have only retained one of the two paralogs. In most cases, the lack of one gene cannot be considered evidence for gene loss; rather it is more likely that it has yet to be sequenced. However, some opsin pseudogenization events have been described. In ray-finned fish, pseudogenization has been demonstrated in the SWS2, LWS and RH2 subfamilies. See Figure 3-2 for the location of the nodes where these events have been indicated.

The SWS2 subfamily has one pseudogene, which was identified in this study. I found a SWS2B pseudogene in the three-spine stickleback genome (*Gasterosteus aculeatus*). This sequence is linked to SWS2A, a position observed for the SWS2A and SWS2B paralogs in other species. Exon two of the SWS2B pseudogene is the only alignable portion of this gene the other exons are unrecognizable. In the RH2 subfamily four pseudogenes have been characterized. In each case, loss of function occurred after gene duplication. That is, so far no ray-finned fish species with an RH2 pseudogene lacks a functional RH2 opsin. Both puffer fish species surveyed (*Tetraodon nigroviridis* and *Takifugu rubripes*) have an RH2B pseudogene, but these gene loss events appear to be independent. RH2B in *T. rubripes* is disrupted by a non-long terminal repeat retro-element insertion and a deletion that has truncated the N-terminal of the gene (Neafsey & Hartl, 2005). The *T. nigroviridis* RH2B gene is highly degenerate (mechanism of pseudogenization is unknown) and is thought to have been pseudogenized shortly after the *Tetraodon* and *Takifugu* lineages diverged, given the more advanced stage of degeneracy. Interestingly, five other species in the genus *Takifugu* have maintained functional RH2A and RH2B genes (though only short portions of each gene have been characterized to date) (Neafsey & Hartl, 2005). As RH2A and RH2B genes are derived from an event in the common ancestor of fish in the taxon Neoteoleosti (see above), the RH2B gene in *Takifugu rubripes* appears to have been functional for many millions of years before it was pseudogenized. In a cDNA library screen neither pseudogene was represented, suggesting a lack of expression and further supporting the hypothesis of pseudogenization (Neafsey & Hartl, 2005). There are also RH2 pseudogenes in the

northern lanternfish, *Stenobrachius leucopsarus*, and the scabbardfish, *Lepidopus fitchi*. One of the four RH2 genes in the scabbardfish, RH2-D has a frame shift due to a premature stop codon at site 177. RH2-C in the lanternfish is one of four RH2 genes in this species (Yokoyama & Tada, 2010). RH2-C also has a premature stop codon at codon 175 and has a change in the splicing signal of exon two that has lead to a loss of function. In comparison to these other opsin subclasses, there is only one ray-finned fish LWS opsin pseudogene and no SWS1 pseudogenes that have been characterized. The red seabream (*Pagrus major*) possesses a LWS gene that has been non-functionalized by a frame shift mutation (Wang et al., 2009).

Complete gene loss has also been demonstrated in ray-finned fish. The SWS1, SWS2, LWS and RH2 subfamilies but not RH1 have characterized gene losses. These gene losses have been identified through whole genome surveys, screening genomic DNA libraries and southern blot studies, in which there has been an inability to detect the presence of these genes (Minamoto & Skimizu, 2005; Neafsey & Hartl, 2005; Pointer et al., 2005). One such gene loss was identified in the smelt (*Plecoglossus altivelis*), where SWS2 could not be detected in either PCR or southern blot screens (Minamoto & Shimizu, 2005). In two puffer fish (*T. rubripes* and *T. nigroviridis*) there has been a demonstrated loss of the SWS1 and SWS2A genes. Neither gene can be located in the whole genome sequencing projects of either pufferfish species (Neafsey & Hartl, 2005). Another example is in the Antarctic notothenioid, *Dissostichus mawsoni,* which is thought to lack LWS, as this gene was not detected by PCR screen or southern blotting (Pointer et al., 2005). Additionally, in this study I have found that the three-spine stickleback (*Gasterosteus aculeatus*) lacks RH2A, as it could not be retrieved in a search of the whole genome sequence assembly. Synteny data also support gene loss for the SWS2A & SWS1 opsin sequences of pufferfish and RH2A of stickleback.

Repertoire comparison

This opsin repertoire survey demonstrates that on average ray-finned fish (actinopterygians) have more visual opsins than their sarcopterygian or agnathan relatives. This is due to the retention of the five ancestral opsin subclasses in many ray-finned fish species and the striking number of gene duplication events. There are three

surveyed agnathans; all are lampreys, members of Petromyzontidae. *Geotria australis* has been found to possess five genes and *Petromyzon marinus* and *Lethenteron japonicum* both have four genes, however two of *P. marinus*' and *L. japonicum*'s genes are pseudogenes (Davies et al., 2009). Many sarcopterygian relatives have also been surveyed and it appears that there have been numerous independent opsin gene losses in the members of this clade. For example two surveyed coelacanth species (*Latimeria menadoensis* and *Latimeria chalumnae*) have been found to possess only two functional visual opsin genes (RH1 and RH2) (Yokoyama & Tada, 2000; Bailes et al., 2007). The Australian lungfish (*Neoceratodus forsteri*) in contrast has retained the ancestral five visual opsins (one from each of the five subclasses). Reptiles and birds have also been shown to possess a single opsin from each of the ancestral five opsin subclasses (Okano et al., 1992; Kawamura & Yokoyama, 1993; Kawamura & Yokoyama, 1995; Kawamura & Yokoyama, 1996). While, the two amphibian sequencing projects of *Xenopus tropicalis* and *Ambystoma tigrinum*, indicate that amphibians lack the RH2 subclass all together, they have retained genes from the other four subclasses. Placental mammals generally possess three or fewer visual opsins (RH1, LWS, SWS1) (Jacobs & Rowe, 2004; Bowmaker & Hunt, 2006). However, there are exceptions due to recent gene duplication. Old world primates, the howler monkey (*Alouatta seniculus*) and the bat species *Haplonycteris fischeri* possess four visual opsins due to independent LWS gene duplication events (Nathans et al., 1986b; Ibbotson et al., 1992; Jacobs et al., 1996; Wang et al., 2004). Marsupials, like placental mammals, possess RH1, LWS and SWS1 genes (Strachan et al., 2004; Arrese et al., 2006). Monotremes have also been found to only possess three visual opsins, however they have retained the SWS2 subclass instead of the SWS1 (Davies et al., 2007; Wakefield et al., 2008).

Gene Conversion

There are at least three examples of gene conversion among the species included in this study, all of which occur in the LWS subfamily. As reported in Chapter 2, two LWS paralogs in the one-sided livebearer (*Jenynsia onca*) and the four-eyed fish (*Anableps anableps*) appear to have undergone gene conversion. This gene conversion has resulted in a high degree of sequence similarity (95% identical) between large portions of their

LWS duplicates (Windsor & Owens, 2009). Other regions of the gene are 70% identical, as these portions were not overwritten during the gene conversion event and represent prior sequence divergence. There is also an example of gene conversion in the guppy (*Poecilia reticulata*). A population from Cumana Venezuela possesses a hybrid LWS locus, which may or may not be present in other populations. This locus appears to be the product of a duplication and gene conversion event between the LWS A180 and LWS P180 loci (Ward et al., 2008). It has also noted that the *Danio rerio* LWS gene paralogs exhibit high sequence similarity (95%) except in exon one where LWS-2 is only 57% identical to LWS-1. Thus it is possible that the pair is a product of a more ancient tandem duplication followed by a recent and near complete gene conversion. Indeed, a phylogenetic tree produced using only LWS first exons places the *D. rerio* LWS-2 outside the cyprinid clade (Data not shown).

Key sites

Key sites are amino acid positions that have been demonstrated through site-directed mutagenesis to have a significant effect on spectral sensitivity. Many of these sites are polymorphic. This variation often involves two or three possible amino acids (i.e., many sites are dimorphic or trimorphic). Interestingly, it appears that at many of these polymorphic sites there are some amino acids, which 'toggle' back and forth across the phylogeny. Amino acid substitutions at key sites were mapped onto each subfamily opsin phylogeny. This was done in order to expose instances of convergent evolution at these sites. The recurrence of these amino acid substitutions in distantly related organisms is an interesting example of convergent evolution.

Within the LWS subclass, three of the five key sites are polymorphic in ray-finned fish. There have been at least twenty-three key site substitutions at these three sites in ray-finned fish. Amino acid identity at key site 164 (180 in human amino acid residue numbering) varies across the phylogeny; this site is trimorphic (Figure 3-4). Serine is the ancestral residue at the 164 site and this amino acid has been conserved in many species. There have also been independent substitutions to alanine or proline in a number of species. Six independent serine to proline substitution events have occurred at the 164 site in ray-finned fish (a S164P substitution is also observed in lamprey), including

multiple livebearers (Guppy, *Xiphophorus malinche* and *X. birchmani* and *Jenynsia onca*). The S164P is a significant functional substitution, shifting maximal absorption -19 nm (Davies et al., 2009). Alanine substitution at the 164 site has occurred nine times in ray-finned fish. The S164A substitution has been shown to shift maximal absorption by -7 nm (Yokoyama & Radlwimmer, 1998). This substitution is also seen in the human MWS (LWS2) (Nathans et al., 1986b). There has been one reversion to the ancestral S164; this is seen in the cichlid *Pseudotropheus acei*. The other two key sites in this subfamily that vary in fish are amino acids 261 and 269. The Y261F (-10nm) and T269A (-16nm) substitutions are found together. These substitutions have occurred once in the Characidae and once in the Poeciliidae families. Interestingly, all characterized key site substitutions in the LWS subfamily are blue shifting relative to the ancestral state (Yokoyama & Radlwimmer, 1998).

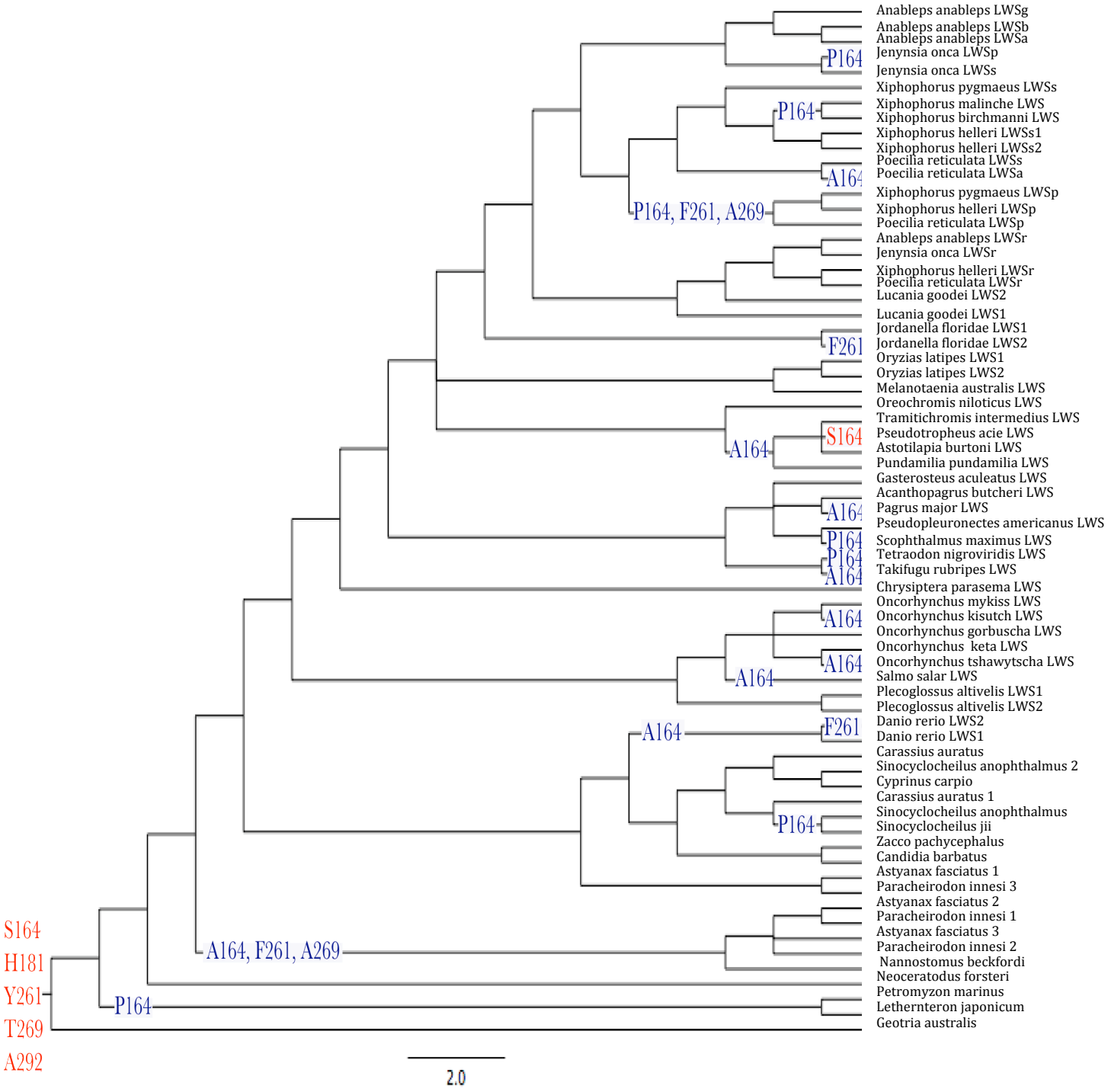

Anableps anableps LWSg
Anableps anableps LWSb
Anableps anableps LWSa
P164
Jenynsia onca LWSp
Jenynsia onca LWSs
Xiphophorus pygmaeus LWSs
P164
Xiphophorus malinche LWS
Xiphophorus birchmanni LWS
Xiphophorus helleri LWSs1
Xiphophorus helleri LWSs2
Poecilia reticulata LWSs
A164
Poecilia reticulata LWSa
Xiphophorus pygmaeus LWSp
P164, F261, A269
Xiphophorus helleri LWSp
Poecilia reticulata LWSp
Anableps anableps LWSr
Jenynsia onca LWSr
Xiphophorus helleri LWSr
Poecilia reticulata LWSr
Lucania goodei LWS2
Lucania goodei LWS1
Jordanella floridae LWS1
F261
Jordanella floridae LWS2
Oryzias latipes LWS1
Oryzias latipes LWS2
Melanotaenia australis LWS
Oreochromis niloticus LWS
Tramitichromis intermedius LWS
S164
Pseudotropheus acie LWS
A164
Astotilapia burtoni LWS
Pundamilia pundamilia LWS
Gasterosteus aculeatus LWS
Acanthopagrus butcheri LWS
A164
Pagrus major LWS
Pseudopleuronectes americanus LWS
P164
Scophthalmus maximus LWS
P164
Tetraodon nigroviridis LWS
A164
Takifugu rubripes LWS
Chrysiptera parasema LWS
Oncorhynchus mykiss LWS
A164
Oncorhynchus kisutch LWS
Oncorhynchus gorbuscha LWS
Oncorhynchus keta LWS
A164
Oncorhynchus tshawytscha LWS
A164
Salmo salar LWS
Plecoglossus altivelis LWS1
Plecoglossus altivelis LWS2
A164
F261
Danio rerio LWS2
Danio rerio LWS1
Carassius auratus
Sinocyclocheilus anophthalmus 2
Cyprinus carpio
Carassius auratus 1
P164
Sinocyclocheilus anophthalmus
Sinocyclocheilus jii
Zacco pachycephalus
Candidia barbatus
Astyanax fasciatus 1
Paracheirodon innesi 3
Astyanax fasciatus 2
Paracheirodon innesi 1
Astyanax fasciatus 3
Paracheirodon innesi 2
Nannostomus beckfordi
Neoceratodus forsteri
Petromyzon marinus
Lethernteron japonicum
Geotria australis
S164
H181
Y261
T269
A292
A164, F261, A269
P164
2.0

Figure 3-4 Neighbor-joining bootstrap consensus tree of the LWS cone opsins of ray-finned fish species with mapped key-site substitutions.

Nucleotide sequences were hand aligned and the tree was generated by the neighbor-joining method using a Jukes and Cantor model of evolution with 1000 bootstrap replications in PAUP*4b10 (Felsenstein, 1985; Jukes & Cantor, 1969; Saitou & Nei, 1987; Swofford, 2002). Substitutions at previously identified key sites were parsimoniously mapped onto the tree. Color indicates the direction of the spectral shift as a result of the given substitution, relative to the ancestral state; Red, is longer wavelength, and blue, shorter wavelength. Accession numbers are listed in Appendix 3

In the SWS1 subclass eleven of the thirteen key sites are found to vary in ray-finned fish Figure 3-5. Cumulatively, forty-five key site substitution events in the SWS1 subclass have occurred in ray-finned fish. A very important key site modification is found in *Lepidopus fitchi*, where amino acid 86 has been deleted. This deletion, results in a shift from ultraviolet (UV) sensitivity to violet sensitivity (Tada et al., 2009). Many key site substitutions are found in *Cyprinus carpio* SWS1, *Plecoglossus altivelis* SWS1-A, *Lepidopus fitchi* and SWS1 sequences in the Nototheniidae family. There have been multiple substitutions at key site 97 and this site is trimorphic (C, S, A). This site exemplifies convergent evolution, as there have been six independent substitutions to Serine at amino acid 97 and two independent substitutions to cysteine. Amino acid 114 is also extremely variable, with nine independent substitutions, eight of these are convergent substitutions to serine from the ancestral A114, and there is also one substitution to threonine.

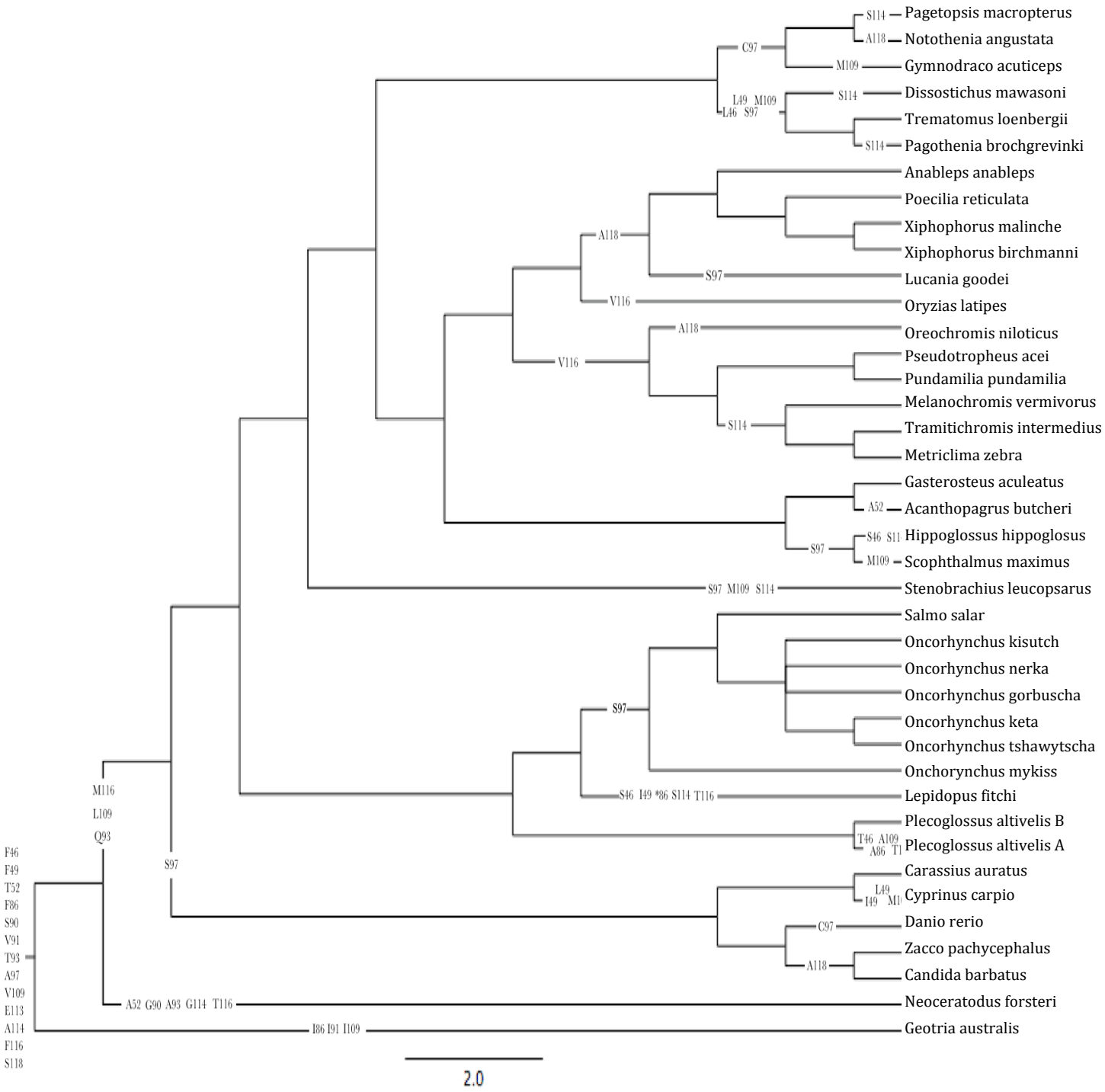

S114
A118
C97
M109
L49 M109
L46 S97
S114
S114
A118
S97
V116
A118
V116
S114
A52
S46 S11
S97
M109
S97 M109 S114
S97
S46 I49 *86 S114 T116
T46 A109
A86 T1
M116
L109
Q93
S97
L49
I49 M1
C97
A118
A52 G90 A93 G114 T116
I86 I91 I109
F46
F49
T52
F86
S90
V91
T93
A97
V109
E113
A114
F116
S118
2.0
Pagetopsis macropterus
Notothenia angustata
Gymnodraco acuticeps
Dissostichus mawasoni
Trematomus loenbergii
Pagothenia brochgrevinki
Anableps anableps
Poecilia reticulata
Xiphophorus malinche
Xiphophorus birchmanni
Lucania goodei
Oryzias latipes
Oreochromis niloticus
Pseudotropheus acei
Pundamilia pundamilia
Melanochromis vermivorus
Tramitichromis intermedius
Metriclima zebra
Gasterosteus aculeatus
Acanthopagrus butcheri
Hippoglossus hippoglosus
Scophthalmus maximus
Stenobrachius leucopsarus
Salmo salar
Oncorhynchus kisutch
Oncorhynchus nerka
Oncorhynchus gorbuscha
Oncorhynchus keta
Oncorhynchus tshawytscha
Onchorynchus mykiss
Lepidopus fitchi
Plecoglossus altivelis B
Plecoglossus altivelis A
Carassius auratus
Cyprinus carpio
Danio rerio
Zacco pachycephalus
Candida barbatus
Neoceratodus forsteri
Geotria australis

Figure 3-5 Neighbor-joining bootstrap consensus tree of the SWS1 cone opsins of ray-finned fish species with mapped key-site substitutions.

Nucleotide sequences were hand aligned and the tree was generated by the neighbor-joining methods using a Jukes and Cantor model of evolution with 1000 bootstrap replications in PAUP*4b10 (Felsenstein, 1985; Jukes & Cantor, 1969; Saitou & Nei, 1987; Swofford, 2002). Substitutions at previously identified key sites were parsimoniously mapped onto the tree. Accession numbers are listed in Appendix 3

In the SWS2 subclass, twelve of the thirteen key sites vary in the surveyed ray-finned fish (Figure 3-6.) There have been cumulatively forty-two substitutions among these twelve sites in ray-finned fish. A substitution is present that defines the SWS2B sequences; an A94C substitution is shared by all SWS2B sequences, and is not present in the SWS2A subtype. A highly variable key site in the SWS2 subclass is amino acid 116. There are thirteen independent substitutions at this key site and it is pentamorphic; at this site there have been substitutions from the ancestral threonine to valine (four times), isoleucine (two times), alanine (two times), methionine (three times) and leucine (three times). The SWS2 sequence in *Anguilla anguilla* is very divergent with four key site substitutions. The *Lucania goodei* SWS2A sequence is also highly divergent with three key site substitutions.

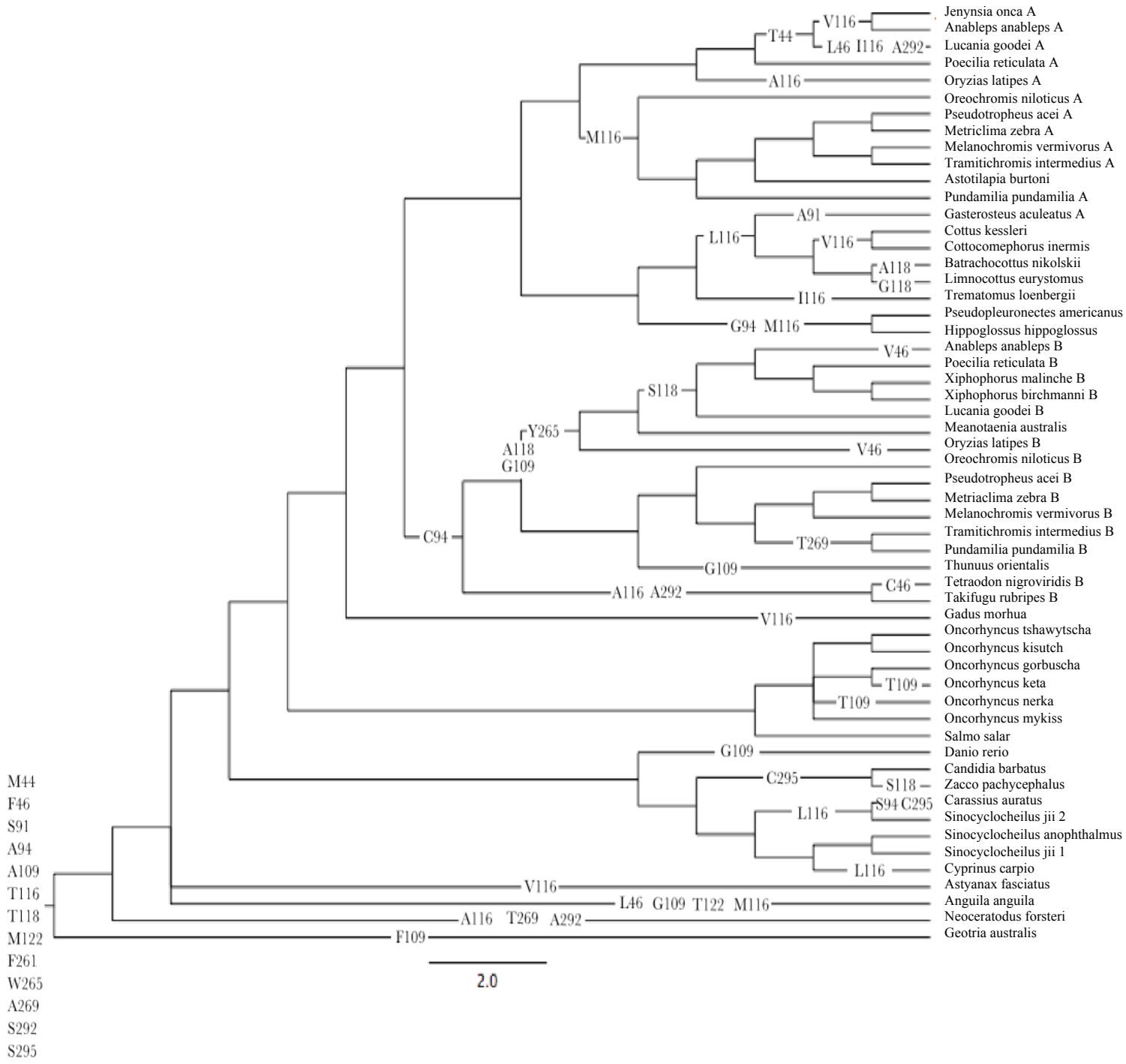

Jenynsia onca A
Anableps anableps A
Lucania goodei A
Poecilia reticulata A
Oryzias latipes A
Oreochromis niloticus A
Pseudotropheus acei A
Metriclima zebra A
Melanochromis vermivorus A
Tramitichromis intermedius A
Astotilapia burtoni
Pundamilia pundamilia A
Gasterosteus aculeatus A
Cottus kessleri
Cottocomephorus inermis
Batrachocottus nikolskii
Limnocottus eurystomus
Trematomus loenbergii
Pseudopleuronectes americanus
Hippoglossus hippoglossus
Anableps anableps B
Poecilia reticulata B
Xiphophorus malinche B
Xiphophorus birchmanni B
Lucania goodei B
Meanotaenia australis
Oryzias latipes B
Oreochromis niloticus B
Pseudotropheus acei B
Metriaclima zebra B
Melanochromis vermivorus B
Tramitichromis intermedius B
Pundamilia pundamilia B
Thunuus orientalis
Tetraodon nigroviridis B
Takifugu rubripes B
Gadus morhua
Oncorhyncus tshawytscha
Oncorhyncus kisutch
Oncorhyncus gorbuscha
Oncorhyncus keta
Oncorhyncus nerka
Oncorhyncus mykiss
Salmo salar
Danio rerio
Candidia barbatus
Zacco pachycephalus
Carassius auratus
Sinocyclocheilus jii 2
Sinocyclocheilus anophthalmus
Sinocyclocheilus jii 1
Cyprinus carpio
Astyanax fasciatus
Anguila anguila
Neoceratodus forsteri
Geotria australis
T44
V116
L46 I116 A292
A116
M116
A91
L116
V116
A118
G118
I116
G94 M116
V46
S118
Y265
A118
G109
V46
C94
T269
G109
A116 A292
C46
V116
T109
T109
G109
C295
S118
L116
S94 C295
L116
V116
L46 G109 T122 M116
A116 T269 A292
F109
2.0
M44
F46
S91
A94
A109
T116
T118
M122
F261
W265
A269
S292
S295

Figure 3-6 Neighbor-joining bootstrap consensus tree of the SWS2 cone opsins of ray-finned fish species with mapped key-site substitutions.

Nucleotide sequences were hand aligned and the tree was generated by the neighbor-joining methods using a Jukes and Cantor model of evolution with 1000 bootstrap replications in PAUP*4b10 (Jukes & Cantor, 1969; Felsenstein, 1985; Saitou & Nei, 1987; Swofford, 2002). Substitutions at previously identified key sites were parsimoniously mapped onto the tree. Accession numbers are listed in Appendix 3.

In the RH2 subclass seven of the nine key sites vary in ray-finned fish (Figure 3-7. Among these sites there are forty-six independent key site substitutions. Three key-site substitutions occurred at the base of ray-finned fish, L49C, D83H and A164M. The substitution F52T is shared by all RH2A sequences and is not found in the RH2B sequences. The E122Q is an example of convergent evolution, as this substitution has occurred independently fifteen times in ray-finned fish. This substitution results in a 20 nm blue shift.

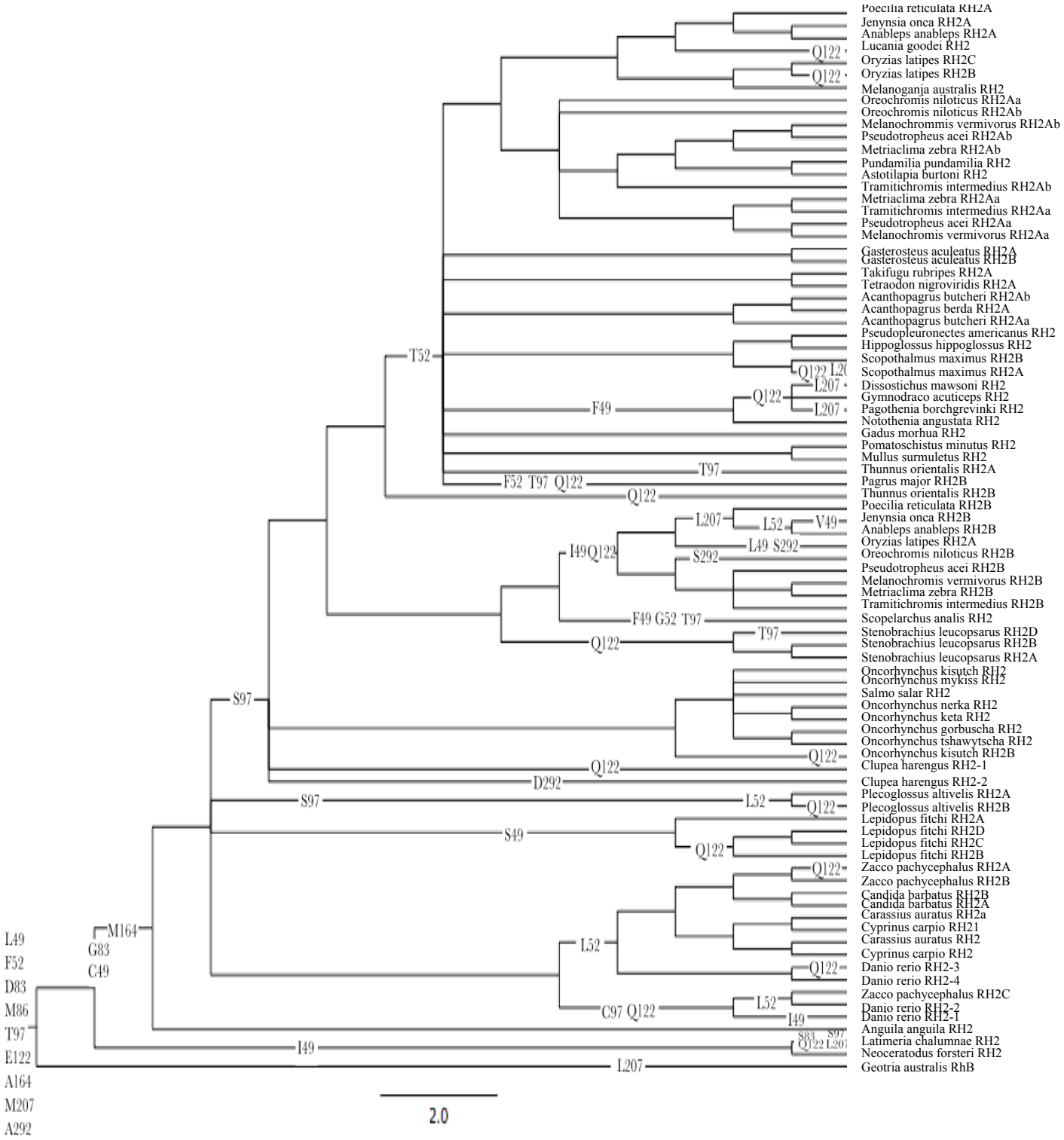

Poecilia reticulata RH2A
Jenynsia onca RH2A
Anableps anableps RH2A
Lucania goodei RH2
Oryzias latipes RH2C
Oryzias latipes RH2B
Melanoganja australis RH2
Oreochromis niloticus RH2Aa
Oreochromis niloticus RH2Ab
Melanochrommis vermivorus RH2Ab
Pseudotropheus acei RH2Ab
Metriaclima zebra RH2Ab
Pundamilia pundamilia RH2
Astotilapia burtoni RH2
Tramitichromis intermedius RH2Ab
Metriaclima zebra RH2Aa
Tramitichromis intermedius RH2Aa
Pseudotropheus acei RH2Aa
Melanochromis vermivorus RH2Aa
Gasterosteus aculeatus RH2A
Gasterosteus aculeatus RH2B
Takifugu rubripes RH2A
Tetraodon nigroviridis RH2A
Acanthopagrus butcheri RH2Ab
Acanthopagrus berda RH2A
Acanthopagrus butcheri RH2Aa
Pseudopleuronectes americanus RH2
Hippoglossus hippoglossus RH2
Scopothalmus maximus RH2B
Scopothalmus maximus RH2A
Dissostichus mawsoni RH2
Gymnodraco acuticeps RH2
Pagothenia borchgrevinki RH2
Notothenia angustata RH2
Gadus morhua RH2
Pomatoschistus minutus RH2
Mullus surmuletus RH2
Thunnus orientalis RH2A
Pagrus major RH2B
Thunnus orientalis RH2B
Poecilia reticulata RH2B
Jenynsia onca RH2B
Anableps anableps RH2B
Oryzias latipes RH2A
Oreochromis niloticus RH2B
Pseudotropheus acei RH2B
Melanochromis vermivorus RH2B
Metriaclima zebra RH2B
Tramitichromis intermedius RH2B
Scopelarchus analis RH2
Stenobrachius leucopsarus RH2D
Stenobrachius leucopsarus RH2B
Stenobrachius leucopsarus RH2A
Oncorhynchus kisutch RH2
Oncorhynchus mykiss RH2
Salmo salar RH2
Oncorhynchus nerka RH2
Oncorhynchus keta RH2
Oncorhynchus gorbuscha RH2
Oncorhynchus tshawytscha RH2
Oncorhynchus kisutch RH2B
Clupea harengus RH2-1
Clupea harengus RH2-2
Plecoglossus altivelis RH2A
Plecoglossus altivelis RH2B
Lepidopus fitchi RH2A
Lepidopus fitchi RH2D
Lepidopus fitchi RH2C
Lepidopus fitchi RH2B
Zacco pachycephalus RH2A
Zacco pachycephalus RH2B
Candida barbatus RH2B
Candida barbatus RH2A
Carassius auratus RH2a
Cyprinus carpio RH21
Carassius auratus RH2
Cyprinus carpio RH2
Danio rerio RH2-3
Danio rerio RH2-4
Zacco pachycephalus RH2C
Danio rerio RH2-2
Danio rerio RH2-1
Anguila anguila RH2
Latimeria chalumnae RH2
Neoceratodus forsteri RH2
Geotria australis RhB
T52
F49
T97
F52 T97 Q122
Q122
L207
L52
V49
L49 S292
S292
I49Q122
F49 G52 T97
T97
Q122
S97
Q122
D292
S97
L52
Q122
S49
Q122
Q122
Q122
L52
C97 Q122
L52
I49
I49
L207
M164
G83
C49
L49
F52
D83
M86
T97
E122
A164
M207
A292
2.0

Figure 3-7 Neighbor-joining bootstrap consensus tree of the RH2 cone opsins of ray-finned fish species with mapped key-site substitutions.

Nucleotide sequences were hand aligned and the tree was generated by the neighbor-joining methods using a Jukes and Cantor model of evolution with 1000 bootstrap replications in PAUP*4b10 (Jukes & Cantor, 1969; Felsenstein, 1985; Saitou & Nei, 1987; Swofford, 2002). Substitutions at previously identified key sites were parsimoniously mapped onto the tree. Accession numbers are listed in Appendix 3.

In the RH1 subclass ten of the twelve key sites vary in ray-finned fish (Figure 3-8). Among these sites there have been forty-nine key site substitutions. The errlo orthologs all share a L194P substitution. Interestingly, this substitution is also found in the *Danio rerio* RH1 duplicate and the freshwater RH1 sequences from eels (*Anguilla japonica* and *A. anguilla*). A K195N substitution also occurs with the P194 substitution in both the *Danio rerio* RH1 duplicate and the eel freshwater RH1 sequences. A D83N has occurred independently twelve times among ray-finned fish; the D83N substitution shifts maximal absorption 6 nm toward blue (Nathans, 1990). This substitution is also a reversion to what appears to be the ancestral residue in this subclass, based on its occurrence in *Geotria australis* RhA, *Petromyzon marinus* RhA, *Lethenteron japonicum* RhA, *Xenopus laevis* and *Anolis carolinesis*.

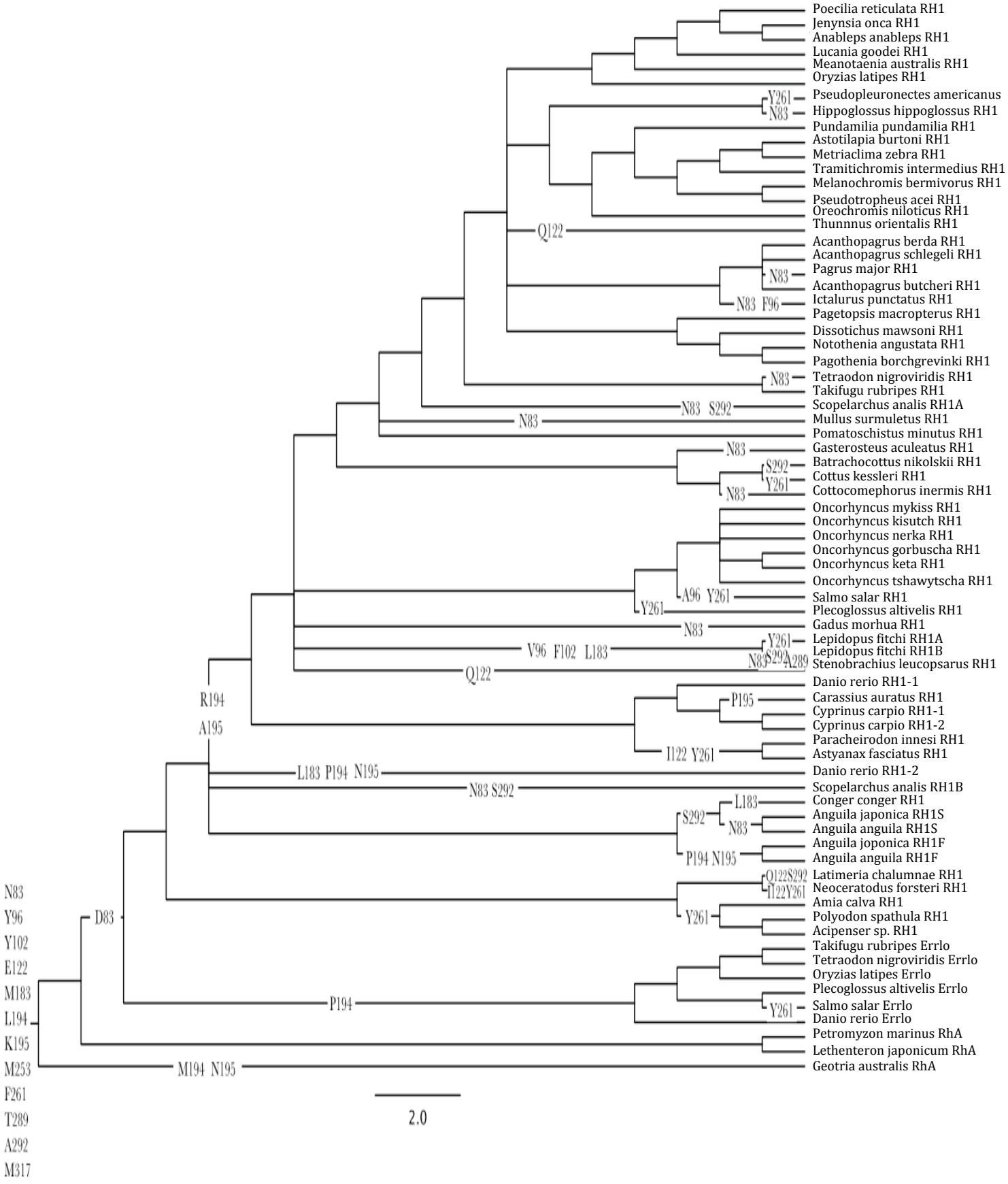

Poecilia reticulata RH1
Jenynsia onca RH1
Anableps anableps RH1
Lucania goodei RH1
Meanotaenia australis RH1
Oryzias latipes RH1
Y261
Pseudopleuronectes americanus
N83
Hippoglossus hippoglossus RH1
Pundamilia pundamilia RH1
Astotilapia burtoni RH1
Metriaclima zebra RH1
Tramitichromis intermedius RH1
Melanochromis bermivorus RH1
Pseudotropheus acei RH1
Oreochromis niloticus RH1
Q122
Thunnnus orientalis RH1
Acanthopagrus berda RH1
Acanthopagrus schlegeli RH1
N83
Pagrus major RH1
Acanthopagrus butcheri RH1
N83 F96
Ictalurus punctatus RH1
Pagetopsis macropterus RH1
Dissotichus mawsoni RH1
Notothenia angustata RH1
Pagothenia borchgrevinki RH1
N83
Tetraodon nigroviridis RH1
Takifugu rubripes RH1
N83 S292
Scopelarchus analis RH1A
N83
Mullus surmuletus RH1
Pomatoschistus minutus RH1
N83
Gasterosteus aculeatus RH1
S292
Batrachocottus nikolskii RH1
Y261
Cottus kessleri RH1
N83
Cottocomephorus inermis RH1
Oncorhyncus mykiss RH1
Oncorhyncus kisutch RH1
Oncorhyncus nerka RH1
Oncorhyncus gorbuscha RH1
Oncorhyncus keta RH1
Oncorhyncus tshawytscha RH1
A96 Y261
Salmo salar RH1
Y261
Plecoglossus altivelis RH1
N83
Gadus morhua RH1
Y261
Lepidopus fitchi RH1A
V96 F102 L183
Lepidopus fitchi RH1B
N83 S292 A289
Stenobrachius leucopsarus RH1
Q122
Danio rerio RH1-1
R194
P195
Carassius auratus RH1
Cyprinus carpio RH1-1
A195
Cyprinus carpio RH1-2
Paracheirodon innesi RH1
I122 Y261
Astyanax fasciatus RH1
L183 P194 N195
Danio rerio RH1-2
N83 S292
Scopelarchus analis RH1B
L183
Conger conger RH1
S292
Anguila japonica RH1S
N83
Anguila anguila RH1S
Anguila joponica RH1F
P194 N195
Anguila anguila RH1F
Q122S292
Latimeria chalumnae RH1
I122Y261
Neoceratodus forsteri RH1
N83
Amia calva RH1
Y96
D83
Y261
Polyodon spathula RH1
Y102
Acipenser sp. RH1
E122
Takifugu rubripes Errlo
M183
Tetraodon nigroviridis Errlo
Oryzias latipes Errlo
L194
P194
Plecoglossus altivelis Errlo
Y261
Salmo salar Errlo
K195
Danio rerio Errlo
Petromyzon marinus RhA
M253
M194 N195
Lethenteron japonicum RhA
Geotria australis RhA
F261
T289
2.0
A292
M317

Figure 3-8 Neighbor-joining bootstrap consensus tree of the RH1 rod opsins of ray-finned fish species with mapped key-site substitutions.

Nucleotide sequences were hand aligned and the tree was generated by the neighbor-joining methods using a Jukes and Cantor model of evolution with 1000 bootstrap replications in PAUP*4b10 (Jukes & Cantor, 1969; Felsenstein, 1985; Saitou & Nei, 1987; Swofford, 2002). Substitutions at previously identified key sites were parsimoniously mapped onto the tree. Accession numbers are listed in Appendix 3

Wavelength of maximal absorption

The distribution of maximum absorption (based upon microspectrophotometry and *in vitro* reconstruction studies) for ray-finned fish opsins is plotted in Figure 3-9. The majority of LWS sensitivity occurs between 547 nm-578 nm. SWS1 sensitivity appears to be restricted to the narrow UV range of 356-378 nm, with one exception in *Lepidopus fitchi,* which has violet sensitivity at 423nm. There is less variation in spectral sensitivity among RH1 sequences than is seen in other opsin subclasses. Most RH1 maximal absorptions (69.2%) fall within 6 nm of 503 nm Figure 3-9. RH2 sensitivities have the greatest overlap with other subclasses; RH2 sensitivities overlap with RH1, LWS and SWS2 sensitivity. RH2 maximal absorptions fall within 467 nm to 555 nm with a blue shifted exception in *Oryzias latipes* at 452 nm. SWS2 maximal absorption falls quite continuously within the range of 405 nm to 475 nm. Interestingly, a region of the spectrum (379-405 nm) is not represented in the distribution of maximal absorption values for all five subfamilies, otherwise the distribution is continuous.

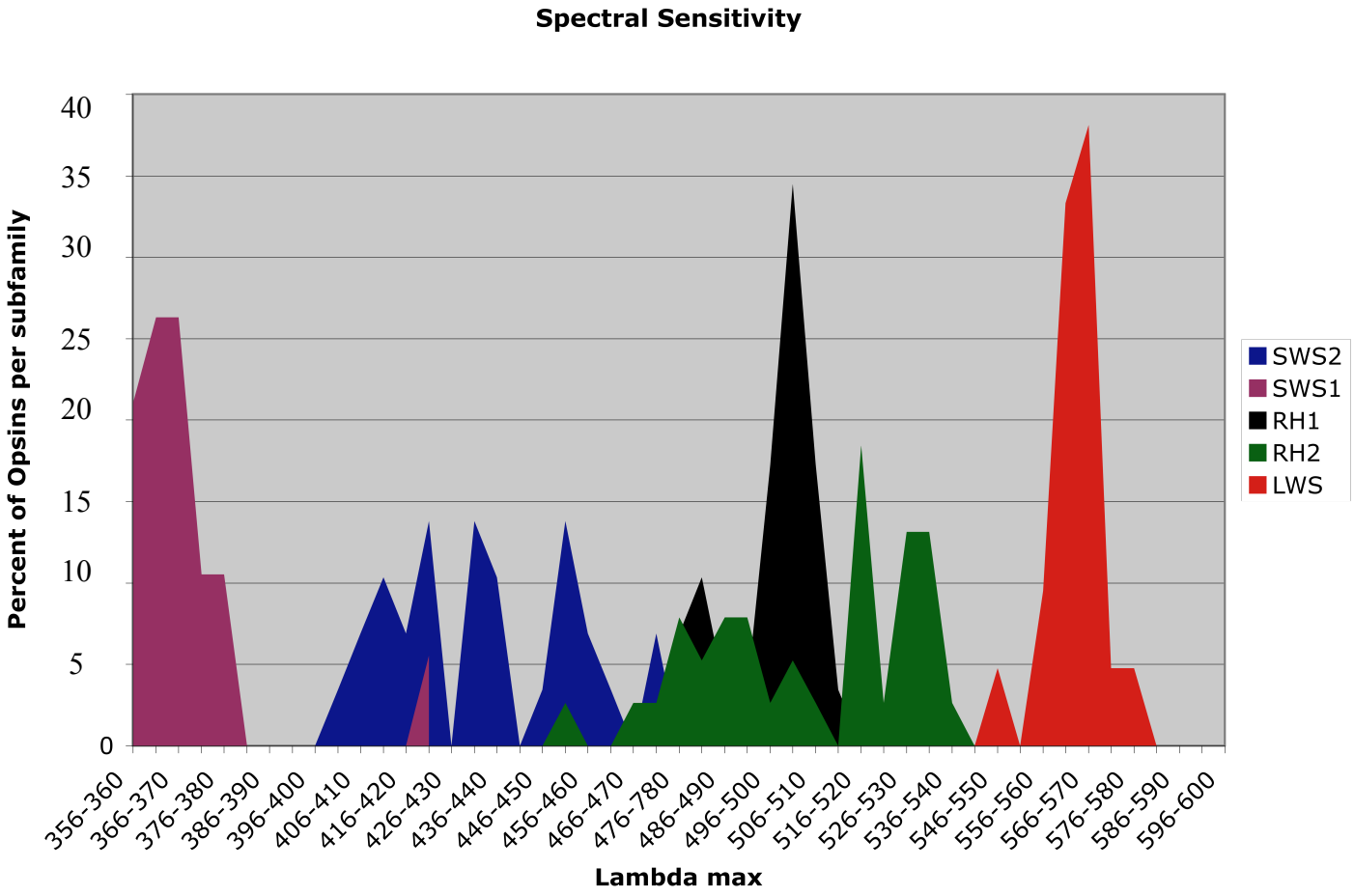


Figure 3-9 Maximal absorption distribution in ray-finned fish for the five subfamilies of visual opsins.

Maximal absorption values were determined by MSP or *in vitro* reconstitution [see Appendix 4 for values and references]. Purple, Blue, Black, Green and Red represent SWS1, SWS2, RH1, RH2 and LWS genes respectively.

Discussion

Duplication ages and repertoire size.

The forty-two opsin duplications in actinopterygians far exceed what has been seen in sarcopterygians. In sarcopterygians there have been three confirmed opsin duplication events. All occurred in the LWS subfamily, they are seen great apes (humans and howler monkeys) and the bat species *Haplonycteris fischer* (Jacobs et al., 2004; Wand et al., 2004). The large opsin repertoires found in many ray-finned fish are the result of a combination of a few recent (less than 75 MYA) duplication events and the

retention of multiple ancient duplicates (200-450 MYA). For example the guppy opsin repertoire is the result of six duplication events, three ancient duplication events and three more recent events.

The first ancient event is a retro-transposition event, which produced the errlo and RH1 duplicates. Given that the mechanism of this last duplication is retro-transposition the presence or absence of introns in the RH1 gene for a given species can be used to approximate the date of this duplication. The duplication that produced errlo and RH1 would have occurred ~407 MYA (Near & Miyab, 2009); this is the predicted divergence time between Polypteriformes that possesses a RH1 with introns and Actinopteri that possess an intron-less copy of RH1. The second ancient duplication is the RH2 duplication, which produced RH2A and RH2B, occurred ~229 MYA (+/- 29 MY) (Spady 2006). Spady estimated the timing of opsin gene duplications by using three rate calibrations in conjunction with established mitochondrial-based divergence times for particular lineages. These dates were then applied to duplication nodes on a phylogeny. The third ancient duplication is the SWS2 duplication that produced SWS2A and SWS2B having occurred ~198 MYA (+/- 8 MY (Million years)) (Spady 2006). Lastly, the four LWS duplicates found in guppy are the product of three independent duplication events having occurred within the last 72 MY (+/- 15 MY) (Spady 2006). This combination of recent duplication and retained ancient duplicates is a common observation. For example cichlids share the ancient SWS2, RH2 and RH1 duplication events with guppies but, they also have a recent RH2 duplication event predicted to have occurred 29 MYA (+/- 13 MY) (Spady 2006. Zebrafish (*Danio rerio*) also exemplify this trend of ancient and recent duplication events producing a large opsin repertoire. In the case of zebrafish there have been six duplication events. The two LWS genes in zebrafish were produced in a duplication event ~14 MY (+/-3) ago (Spady 2006). The four RH2 genes found in the zebrafish repertoire were produced by three independent duplication events. The original duplication is thought to have taken place 206 MYA (+/- 28 MY), two subsequent duplication events produced RH2-1 and RH2-2 29 MYA (+/- 4 MY) and RH2-3 and RH2-4 occurred 94 MYA (+/- 12 MY) respectively (Spady 2006). Zebrafish also have three RH1 (including errlo, dated above) genes. The duplication event, which produced

the intronless RH1-1 and RH1-2 duplicates, has not been dated (it currently has no known orthologs).

Interestingly, none of the large opsin repertoires yet characterized have been attributed to multiple species-specific (recent) gene duplication events. This observation is apparent upon inspection of the opsin phylogeny as there are generally more orthologs of a given gene than paralogs. This is a trend not observed in all G-protein coupled receptor families. For example in chemo-receptors, a given species can have up to 800 genes and pseudogenes, that are the result of many recent duplication events rather than the accumulation of old duplicates (Robertson, 1998).

Gene duplication is more prevalent in some opsin subclasses than others (Figure 3-3). The LWS and RH2 subclass contain the largest number of paralogs, while paralogs are fairly rare in the SWS1 and SWS2 sub-groups. There have been some hypotheses put forth to explain the subclass level differences in duplication frequency. For example Gojobori and Innan (2009) suggested that there are limitations imposed on functional diversification of genes sensitive to light at either end of the spectrum (short-wavelengths and long-wavelengths) (Gojobori & Innan, 2009). They thus propose that there are fewer retained gene duplications in the SWS1 and LWS subclasses due to this functional constraint. Correspondingly, it is suggested that the SWS2 and RH2 subclasses have a larger number of duplications due to a lack of functional constraint, as they are free to diversify within the middle-wavelengths. However, my data does not support their hypothesis: The LWS subfamily has the second largest number of retained duplicates and that the SWS2 subclass has only retained duplicates from two duplication events. Although their hypothesis on the constraints of SWS1 sequences due properties of UV light could explain the lack of duplicates in this subclass, there is no support for their hypothesis of constraint in the LWS. The increased duplication and retention of LWS and RH2 genes may also hint that there is a possible functional or adaptive explanation.

Surprisingly, there does not appear to be a clear connection between opsin repertoire size (or correspondingly opsin duplication) and habitat. When habitat depth or salinity is overlaid onto an opsin phylogeny a clear trend does not emerge. Even when comparing species inhabiting the bathypelagic region to those that are surface dwelling a correlation is not apparent; species occupying both habitat types possess duplicated opsins genes

(particularly RH2 genes), despite the distinct spectral regimes associated with great depths. This may be attributed to complicated life history traits such as vertical migrations undertaken by many of the bathypelagic species that have been surveyed thus far (e.g. *Lepidopus fitchi* and *Stenobrachius leucopsarus*). Even when comparing species occupying similar environments, for example shallow freshwater habitats (livebearers vs. cyprinids) I find that differences. Specifically, despite both clades possessing large opsin repertoires, these repertoires differ in composition (i.e. different subfamilies have undergone duplication); in cyprinids RH2 duplication is fairly common, whereas in the livebearers there have been multiple LWS duplication events. Future work including a more phylogenetically representative survey of opsin repertoires, as well as, precise characterization of spectral habitat will be required before a clear link between environment and repertoire will emerge.

Mechanism of duplication

Gene duplication can occur by whole genome duplication, chromosomal duplication, tandem duplication and retro-transposition. Whole genome duplication appears to have played an important role early in vertebrate opsin gene evolution (Larhammar et al., 2009), but does not appear to be a primary contributor to opsin gene amplification in ray-finned fish. Data from whole genome sequencing projects indicate that tandem duplication has played an important role in opsin repertoire expansion. Retroduplication plays a role, but it is important to note that many of the opsin repertoire surveys have been undertaken at the cDNA level, thus the prevalence of retro-transposition cannot be accurately estimated at this time.

Tandem duplication

Tandem duplication is thought to occur by two mechanisms, either through unequal cross-over during meiosis or unequal sister chromatid exchange during mitosis (Sturtevant, 1925; Hu & Worton, 1992). This mechanism often allows for the duplication of nearby regulatory regions, maintaining expression. Given that tandem duplication results in duplicates that are in relatively close proximity to one another, it's a fairly easily identified mechanism of duplication. This study indicates that tandem duplication

appears to be the most common method of opsin gene duplication in ray-finned fish. Tandem duplication has also been demonstrated to play an important role in the expansion of other gene families such as the olfactory receptors (Niimura & Nei, 2003). It has been suggested that there is a higher duplicability for tandem duplicates than retroduplicates and that tandem duplicates are more likely to be retained, at least in human and mouse genomes (Wang et al., 2010). My finding of more tandem duplication than retro-duplication in opsins is consistent with this suggestion.

Retroduplication

Retroduplication occurs when the mRNA of a parental gene is reverse transcribed into cDNA and is then inserted into chromosomal DNA at a distinct location (Brosius, 1999). Thus this mechanism creates an intronless paralog of the 'parental' gene. However, successful duplication requires the generation or maintence of regulatory regions. This is not always achieved during tandem duplication and perhaps is even more rare when retro-transposition is the mechanism of duplication. Retro-duplicated genes are often transcriptionally inactive; this is because the promoter is not generally transcribed, as transcription is initiated downstream from a core promoter region. However, it has been found that occasionally core promoters can be maintained through joint transcription or generated by one of at least three mechanisms (Okamura & Nakai, 2008). The promoter can be maintained from the original source gene through transcription and reverse transcription with the exonic downstream sequence. Alternatively, a portion of the coding sequence of the gene may become the promoter. A chimera can also be produced combining the exonic sequence of the source gene with a copy of a promoter from an unrelated gene. Given that promoters can be acquired, it is not surprising that example have been found where retro-duplicated genes have acquired novel functions or novel expression patterns (Soares et al., 1985; Marques et al., 2005). Both identified opsin retro-genes RH1 and LWS-S180r are expressed in the eye, thus they have acquired or retained promoters presumably through one of the above mechanisms. Interestingly, the LWS-S180r gene has maintained the first intron, which may have facilitated the post retro-duplication expression of this gene. Another requirement of successful retro-duplication is that the gene must be expressed in the germ-line in order for the retro-

duplicate to be inherited. Given this requirement it is somewhat interesting that RH1 and LWS-S180r have been attributed to retro-duplication. This may indicate a broad low-level expression profile for opsin genes (or gamete expression), particularly for the source genes of the RH1 and LWS-S180r genes.

Whole genome duplication

Whole genome duplication appears to have had only a minor role in the amplification of ray-finned fish opsin repertoires. Indeed, it is surprising that it doesn't appear that any opsin duplications are a result of the teleost whole genome (3R) duplication (Taylor et al., 2003). Particularly given that the zebrafish genome retained at least 20% of the gene pairs, which were a product of the 3R duplication (Postlethwait et al., 2000). Subsequent whole genome duplication events in members of the subfamily Cyprininae and Salmonids appear to have generated opsin duplicates in the LWS & SWS2 and RH2 subfamilies respectively (Temple et al., 2008a; Li et al., 2009).

Gene Conversion

Gene conversion in the opsin gene family was first recognized in the human LWS genes. Gene conversion in humans has been found to be a cause of color blindness, whereby different tandem LWS genes are overwritten by pseudogenes (or perfectly good genes) resulting in a loss of function, or generation of two genes with one function, i.e., wavelength sensitivity (Winderickx et al., 1993; Reyniers et al., 1995). The loss of function in humans through gene conversion is a detrimental event and generally complete gene conversion between functional genes reduces sequence diversity through homogenization. However, the gene conversion events I have observed in fish may be beneficial, allowing for the production of greater phenotypic (maximal absorption) diversity than would be possible without this mechanism. The events that have been so far been described in fish are partial gene conversions events, which produce hybrid loci. These hybrid loci can generate opsin haplotype diversity, by combining mutations that have occurred separately during divergence of two duplicates; unique key site haplotypes and consequently novel phenotypes appear to have been generated (Windsor and Owens 2009). An example of this is seen in *Anableps anableps*, where gene conversion between

LWS genes has generated the novel five-key site haplotype SHYAA. Aside from my demonstration of gene conversion in livebearer opsins and a putative case in zebrafish LWS gene conversion has also been suggested to occur in cavefish and may explain intermediate phenotype pigments (Yokoyama & Yokoyama, 1990)

On a practical level an important consideration with regard to gene conversion is that it can complicate the determination of the frequency and timing of gene duplication. Gene conversion results in paralogs that appear on a phylogeny to be more closely related to each other than they really are. Thus duplicates with a high degree of sequence similarity could be the product of either a recent duplication or a full-length gene conversion event. A classic example of this is seen the multi-copy testis-expressed gene families found within pallindromic regions of the human Y chromosome. These genes have a sequence similarity of ~99.97%. This degree of sequence similarity is something that would support the hypothesis of a recent duplication event, however these pallindromic regions have been dated to be ~5 MY old (using an estimated time of divergence for the recent most common ancestor of human and chimpanzee). Thus indicating that gene conversion can indeed be misleading for dating duplications (Rozen et al., 2003). It is only when there is partial gene conversion that detection by sequence identity based methods (e.g. the program GENECONV (Sawyer, 1999)) is feasible or when conserved synteny data for orthologs is available to confirm that high sequence similarity is due to recent duplication. Given that opsin duplicates in the SWS2, RH2 and LWS subclasses are all found in tandem it is surprising that gene conversion has only been identified in LWS opsins. However, since gene conversion requires a high degree of sequence identity (at least 92%) there is a short time frame for gene conversion to occur (Chen et al., 2007). Since many of the duplication events are very ancient, perhaps gene duplicates that were over written by gene conversion in non-LWS subclasses were subsequently lost. Alternatively, gene conversion may have occurred shortly after duplication and the two identical duplicates subsequently diverged. Another explanation for the lack of gene conversion in the other opsin subclass could be due to the limitations of my detection method, which is sequence identity based. Complete gene conversion appears to reset genes to their post duplication state, something that is functionally equivalent to new gene duplication. At this point since there is functionally redundancy between the two

paralogs the gene fates are again loss or retention and subsequent divergence (neo- or sub-functionalization). Partial gene conversion avoids this functional resetting by maintaining some of the sequence level divergence acquired post duplication.

Key site evolution and spectral sensitivity

There are generally two groups of spectral tuning key sites; the first group includes amino acids that individually cause a significant shift in maximal absorption. The second group involves multiple amino acids, which independently cause little or no shift in maximal absorption, but together can induce a large spectral shift. An example of the first group is the F86Y amino acid substitution seen in goldfish SWS1 pigments, which alone shifts maximal absorption by 60 nm (Cowing et al., 2002). An example of the second group is seen in the mouse SWS1 pigment; here substitutions at seven amino acid sites individually cause no shift in maximal absorption, but together result in a 52 nm shift in maximal absorption (Shi et al., 2001).

In this survey I have observed that the correlation between sequence level divergence and phenotypic differences in opsins is not perfect. This is a synthesis of available opsin nucleotide sequences and previously generated spectral sensitivity data (MSP/*in vitro* reconstruction). There are straightforward examples where amino acid substitution at key site explains all of the variance in maximal absorption between two sequences. But there are also examples where there is variation in maximal absorption between species but no diversity among the key sites for that opsin subclass, or vise versa key site variation but no difference in maxima absorption; both scenarios are discussed below.

LWS

An excellent example of a correlation between key site differences and phenotypic difference is seen in the LWS opsin subclass. In the *Danio rerio* LWS duplicates, a 10 nm difference in maximal absorption can be explained by a single substitution, Y261F found in LWS2 but not LWS1 (Asenjo et al., 1994). Another example is found in *Petromyzon marinus* LWS opsins demonstrates a clear link between key site substitution and phenotype. *P. marinus* possesses the most green shifted LWS opsin yet found (535.5 nm). This green shift is attributed to a S164P substitution, which has been demonstrated to result in a 19 nm shift (Davies et al., 2009). Although this example is not a ray-finned

fish, this is an important example because the S164P substitution found in this species is also found six times in ray-finned fish. Unfortunately no direct measurements have been taken in any ray-finned fish species possessing this mutation, so it is unclear whether the same magnitude of shift occurs in any of these species. Overall it appears that most of the variation in the maximal absorption of LWS genes is explained by the characterized five key sites.

SWS1

Some maximal absorption shifts in SWS1 pigments can be explained by key site substitution, like the goldfish SWS1 example given above. However, there are exceptions. For example, among salmonid SWS1 orthologs there are no key site differences; despite the lack of variation at key sites each sequence has a distinct maximal absorption and they range from 361 nm to 370 nm (Cheng et al., 2006). This is up to a 9 nm difference between species, a shift on par with what can be explained by key site substitution. However, it should be noted that there is a small chance this variation could be attributed to chromophore differences.

RH1

There is less variation in spectral sensitivity among RH1 sequences than other subclasses (Figure 3-9). However, there have been forty-nine key site substitutions in this group, so reconciling the divergence at key sites with the lack of phenotypic divergence is difficult. This is most likely because many key sites were either discovered or tested in non-fish vertebrates, so although they play a significant role in those vertebrate groups they are less important for spectral tuning in fish. However, some differences in RH1 maximal absorption are explained by key site variation. For example five surveyed *Oncorhynchus* species exhibit no amino acid variation among the currently characterized key sites, there is only a small amount of phenotypic variation (up to 3 nm). *Salmo salar* in contrast has the reddest shifted RH1 maximal absorption (515 nm) and does have key site level divergence (Y96A and F261Y (+10 nm) substitutions), which can explain this difference in maximal absorption (Chan et al., 1992). Another example of key site substitution correlating to maximal absorption is seen in the *Anguilla anguilla* saltwater RH1 sequences. In this species A292S (-10 nm) and D83N (-6 nm) substitutions explain

much of the difference in maximal absorption between the freshwater RH1 (500 nm) and saltwater RH1 (479 nm) sequences (Nathans, 1990; Janz & Farrens, 2001). These substitutions also explain why RH1S in *Anguilla anguilla* is the most blue shifted maximal absorption RH1 (Figure 3-9).

RH2

Most RH2 maximal absorptions fall within an 88 nm region of the spectrum. The exception is the blue shifted RH2A of *Oryzias latipes* (452 nm). There have been a number of key site substitutions in this gene. Synergistically two of these, A292S and E122Q, induce a relatively large blue shift, 10 nm and 20 nm respectively (Janz & Farrens, 2001; Takahashi & Yokoyama, 2005). Among RH2 genes there is also an example of how there can be variation maximal absorption without key-site differences. In the cichlid *Pseudotropheus acei* there is variation in the maximal absorption of the two genes RH2Aa (534 nm) and RH2Ab (504 nm), however there are no key site amino acid difference between these two sequences. This is also seen in other cichlid species possessing the RH2Aa and RH2Ab sequence pair.

SWS2

Many of the SWS2 key site substitutions seen in fish have not been directly tested through site mutagenesis, so linking substitutions to phenotypic differences is difficult for this subclass. The difference in maximal absorption between SWS2A and SWS2B in *Lucania goodei* has been carefully studied. The maximal absorption SWS2A and SWS2B are 448 nm and 397 nm respectively. This 51 nm difference is the largest characterized difference among SWS2 paralogs. Mutagenesis analysis in this species has found that substitutions at amino acid sites 44, 46, 94, 97, 109, 116, 118, 265, and 292 explain 80% of their spectral difference, individually and synergistically (Yokoyama et al., 2007). Cumulatively, these site substitutions result in a -41 nm spectral shift in SWS2A. It is only with this type of methodical evaluation of key site substitutions that a clear link between phenotype and amino acid sequence can be obtained. Unfortunately, many key site substitutions have not been characterized (i.e. tested via site directed mutagenesis) yet in fish. When information on the magnitude and direction of the spectral shift is lacking, it is difficult to correlate sequence divergence and functional divergence. This is

particularly true in lineages that have experienced multiple substitutions, as these often act in conjunction with each other to amplify the effect of an individual substitution. For example in the SWS1 subclass multiple substitutions have taken place in *Plecoglossus altivelis* SWS1-1 and *Cyprinus carpio* SWS1 sequences, however there is no spectral data available for comparison. Another complicating factor is that there are two types of examples where amino acid identity at key sites does not correspond to the phenotype of maximal absorption. These phenomena may have significant implications for hypothesized instances of convergent evolution. As what may appear to be convergent evolution could, in fact, be amino acid site-specific structural limitations if these sites are not contributing to differences in maximal absorption. As discussed above, in some species there are spectral differences that appear to be independent from currently characterized key sites (examples from Salmonids). Alternatively, there are instances where key site amino acid identity differs between species but there is no difference in maximal absorption. An example of this is seen in the LWS subclass, where *Astyanax fasciatus* LWS red and *Danio rerio* LWS-1 both have a maximal absorption of 548 nm but *Danio rerio* has a S164A substitution that has been predicted to shift maximal absorption by -7 nm. If these types of examples are common, there may be an over-estimation of functional divergence between duplicates, particularly when no direct measurements have been collected. This would be especially relevant when substitutions at what have been termed 'key sites', have only been investigated and characterized in one vertebrate group and have not been demonstrated to have a functional effect in other vertebrate groups. Future studies will no doubt uncover additional key sites that are important; perhaps a subset of these will be demonstrated to only be important for spectral tuning in ray-finned fish. This may explain the disconnect between the key site haplotype and phenotype seen in some species.

Pseudogenization and gene loss

Identification of pseudogenes is often difficult due to the nature of the process of pseudogenization. As the pseudogene sequences over time become unrecognizable through the accumulation of mutations due to lack of functional constraint. However, in my dataset there are six identified pseudogenes (Figure 3-2A). It has been suggested that

generally pseudogenization occurs within the first few million years post duplication, if the gene of interest is not under any selection (Lynch & Conery, 2000). Only duplicates that assume a novel function or subdivision of ancestral function (or when increased copy number is advantageous) are predicted to be retained. Under these predictions the pseudogenization of anciently duplicated genes, which display evidence of neo- or sub-functionalization should be rare.

In the RH2 subclass, the duplication that produced the RH2A and RH2B paralogs is thought to have occurred 229 MY (+/- 29MY) (Spady 2006); however two independent pseudogenization events are thought to have occurred in the family Tetraodontidae within the last 50 MY (the divergence time of *Tetraodon* and *Takifugu* lineages) (Hurley et al., 2007). In this example independently both *Tetraodon nigroviridis* and *Takifugu rubripes* have undergone pseudogenization of RH2B, however other members of this family have retained a functional copy (Neafsey & Hartl, 2005). This convergent pseudogenization in these species could indicate similar selective pressures, or lack there of. Also interesting is that prior to pseudogenization it appears that the RH2B gene was under purifying selection in *Tetraodon nigroviridis* (Neafsey & Hartl, 2005). The RH2 genes in Northern lampfish (*Stenobrachius leucopsarus*) (RH2C, RH2D) and scabbardfish (*Lepidopus fitchi*) (RH2C, RH2D) are more recent duplicates, having been duplicated within the last ~7.5 MY and ~30 MY respectively (Yokoyama & Tada, 2010). Nonetheless each has experienced an independent pseudogenization event and each species has retained three functionally intact RH2 duplicates. Interestingly, the *Stenobrachius leucopsarus* RH2C, pseudogenization event, although early in divergence, was retained for a significant period of time.

The SWS2B pseudogene in *Gasterosteus aculeatus* characterized in this study is quite interesting, in that only exon 2 is detectable by BLASTn, and the other four exons are completely degraded. The reason for the apparent uneven degradation of the sequence and apparent preservation of exon 2 is unknown. As no close relatives have been surveyed there is currently no time frame for this pseudogenization event.

In most examples of gene loss or pseudogenization in ray-finned fish species, the event has occurred post duplication. Thus most ray-finned fish have at least one member of each of the opsin subfamilies. One group of fish that does not follow this trend is the

Antarctic cod icefish, where there has been gene loss without gene duplication (Pointer et al., 2005).

Conclusions

Four major conclusions can be drawn from this survey and analysis: 1. The large opsin repertoires in many ray-finned fish are the product of duplication events spanning the age of the taxon Teleostei, which includes most ray-finned fish. Thus, large opsin repertoires are not specific to livebearers. Gene duplication is most prevalent in the RH2 and LWS opsin subfamilies and with one exception is absent in the SWS1 opsin tree. 2. Through this survey it was also found that tandem duplication produces more opsin gene duplicates in fish than any other mode of duplication. Tandem duplication also appears to facilitate homogenization in some instances, and diversification in other instances via gene conversion. Surprisingly, none of the duplication nodes appear to coincide with '3R', a whole genome duplication event that occurred in the ancestor of teleosts (Taylor et al., 2003; Hoegg et al., 2004). 3. Opsin gene duplication allows for evolutionary change at the amino acid level; however, variation appears to be constrained at many key sites. This conclusion is based upon the observation of a large number of convergent substitutions. However, the correlation between phenotype and amino acid identity at key sites in some cases is clear, while in others it is ambiguous. 4. Pseudogenization events appear to largely occur post duplication in ray-finned fish for sequences in any given opsin subfamily. These events have also been shown to occur after a significant period of retention, outside of the predictions of Lynch and Conery (2000).

The compilation and analysis of this chapter emphasizes how far repertoire characterization has come. However, opsin expression is not nearly as well studied. This is unfortunate, as how an opsin repertoire is used is also of great importance. To understand the functional implications of opsin duplication and divergence the pattern of opsin expression must also be characterized. Chapter 4 is one of the first attempts to characterize the expression pattern of an entire cone opsin repertoire in a fish. *In situ* hybridization is used to determine how, the guppy, utilizes its large opsin repertoire at the level of expression.

Chapter 4
Cone Opsin Expression Patterns Indicate Spatial Specialization of the Guppy Retina

Abstract

The guppy (*Poecilia reticulata*) is a model species for the study of color-based sexual selection. Guppies also possess one of the largest opsin repertoires of any vertebrate yet examined. I used *in situ* hybridization to characterize the expression pattern of the nine cone opsins in the photoreceptor cells of the retina. *In situ* hybridization on retinal cross-sections showed that at least eight of the cone opsins were expressed in the retina and that most had a distinct pattern of expression. The middle-wavelength sensitive RH2 opsins (green) were expressed in cells of the ventral portion of the retina and the long-wavelength sensitive LWS opsins (red and orange) were expressed primarily in the dorsal portion of the retina. This suggests that different portions of the guppy retina may have distinct wavelength sensitivities and discriminatory abilities. Whole mount *in situ* hybridization was used to determine what opsins were expressed in the different cell types of the cone square mosaic.

Introduction

Guppies (*Poecilia reticulata*) are small freshwater fish in the family Poeciliidae. They are native to Trinidad, Surinam, Guyana and Venezuela east of the Andes Mountains (Bailey, 1963). In the last sixty years guppies have become a model species for the study of sexual selection (Haskins & Haskins, 1949; Houde & Endler, 1990; Rodd et al., 2002). Guppies are sexually dimorphic: Males are colorful, with orange carotenoid-based pigments and black melanin-derived spots and stripes (Endler, 1983; Houde & Endler, 1990). They also have iridescent blue and yellow structural colors (Endler, 1983; Houde & Endler, 1990). Females are a largely uniform dull grey-brown color. Males are also smaller than females. A portion of male color is heritable, specifically the size and location of carotenoid derived spots (orange and red) (Winge & Ditlevsen, 1947; Endler, 1983). Diet also plays a role and has been shown to affect the brightness of male carotenoid spots, but not the location, number or size of the spots (Kodric-Brown, 1989).

Male color, especially the carotenoid spots are an important trait for female mate choice; in many guppy populations, females prefer the males with the greatest chroma (color saturation) and orange area (Endler, 1980; Endler, 1983; Houde 1988; Kodric-Brown, 1989; Houde & Endler, 1990; Houde & Torio, 1992; Grether, 2000). Males from low predation habitats tend to be more colorful than those from high predation regions (Endler, 1983). Thus the observed male color pattern at any given location appears to reflect a balance between predation, which selects for inconspicuousness and female mate choice, which favours conspicuousness.

Given the role of coloration in guppy sexual selection, understanding the visual capacity of this fish is important; without information on the visual system it is difficult to evaluate sexual selection hypotheses. Vision starts with light absorption by visual pigments in the photoreceptor cells of the retina. These visual pigments are composed of two parts; a protein (the opsin) and a vitamin A derived chromophore. The opsin protein component is bound to the chromophore via a lysine residue linked to a protonated Schiff base (Palczewski et al., 2000). When the chromophore absorbs light it isomerizes and induces a conformational change in the opsin leading to G-protein mediated signal transduction through an enzyme cascade. Signal transduction ultimately culminates in membrane hyper-polarization and the signal is then transmitted to the brain (via bipolar and ganglion cells). The signal is also modulated by horizontal and amacrine cells). See (Kolb, 2003) for a review.

Guppies have ten visual opsin genes, the products of multiple duplication events (Figure 4-1). There are four LWS (long-wavelength sensitive) genes (A180, S180, S180r and P180), two RH2 (middle-wavelength sensitive) genes, two SWS2 (short-wavelength sensitive) genes, one SWS1 (UV sensitive) gene and one RH1 (dim-light sensitive) gene (Hoffmann et al., 2007; Weadick & Chang, 2007; Ward et al., 2008; Owens et al., 2009). RT-PCR indicates that all ten genes are expressed to some degree in the photoreceptors of the eye. The RH1 subclass is expressed in the rod photoreceptor cells, while the other found subclasses are expressed in the cone photoreceptor cells of the retina.

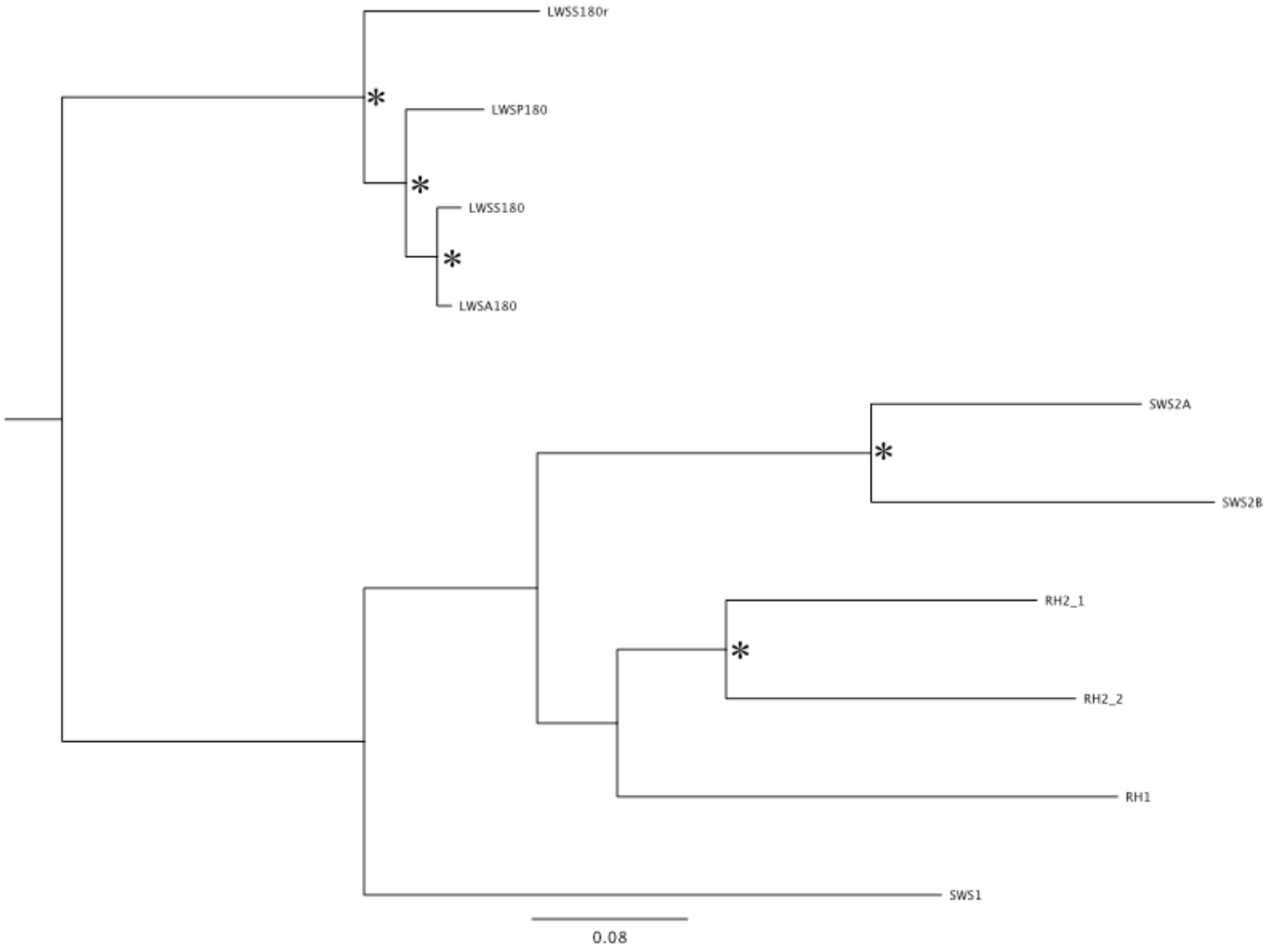


Figure 4-1 Phylogenetic tree of the ten visual opsins in the guppy (*Poecilia reticulata*) repertoire.

Nucleotide sequences were aligned by hand in BioEdit and the tree was generated in from the nucleotide distances by the neighbor-joining method using a Jukes and Cantor model of evolution with 1000 bootstrap replications (Jukes & Cantor, 1969; Felsenstein, 1985; Saitou & Nei, 1987; Hall, 2001; Swofford, 2002). The calibration bar shows distance. Within subfamily opsin duplication nodes are denoted by *. GenBank accession numbers: LWS S180r, EU329457.1; LWS A180, EU329444.1; LWS S180, EU329433.1; LWS P180, EU 329453.1; RH1, DQ912023.1; RH2-1, DQ234859.1; RH2-2, DQ234858.1; SWS1, DQ234861.1; SWS2A, FJ711159.1; SWS2B, DQ234860.1.

Much of the amino acid variation among guppy opsin gene duplicates occurs at key site residues. Key sites are amino acid positions that have been demonstrated through mutagenesis studies to have disproportionate effect on maximal absorption (Yokoyama & Radlwimmer, 1998). For example, despite the LWS opsin duplicates having a high degree of sequence similarity, three of them have a unique amino acid residue at key site 180 (site 164 in the bovine opsin residue numbering scheme) and therefore, unique spectral sensitivities (Yokoyama & Radlwimmer, 1998).

In situ hybridization is the focus of this chapter of my research. I carried out this work because the location of opsin gene expression within the retina must be known in order to evaluate the functional implications of gene duplication and divergence. For example guppies could be monochromatic (truly color-blind) if only one of the nine cone opsins was expressed. In contrast, if all of the genes are expressed uniformly across the surface of the retina guppies could have sensitivity to a broad-spectrum of light and incredible wavelength discrimination abilities. Without knowledge of expression, it is difficult to make predictions of sensitivity and discrimination and therefore difficult to precisely characterize the signals guppies use in sexual selection. qPCR has been used in our lab to evaluate opsin gene expression. However, these data provide an incomplete picture, because they do not reveal how many cells are expressing the gene of interest, or where they are distributed in the retina.

Whole mount in situ was also used to investigate distribution of expression because in fish, the cone photoreceptor cells of the retina are commonly organized to form precise mosaic arrays. In these mosaic arrays cone cells are found in a predictable and regularly spaced arrangement (Lyall, 1957; Engstrom & Ahlbert, 1963; Ahlbert, 1969). Rod photoreceptors in contrast, are distributed in an unordered manor among cones (Walls, 1963). The guppy mosaic is termed a square mosaic, as the unit is composed of a single long (L) centre cone, with four double cones (DB) forming the sides of the square and single short (S) cones at each of the corners (Lyall, 1957; Engstrom & Ahlbert, 1963; Ahlbert, 1969) see Figure 4-2. Double cones as the name implies, have two cells, a smaller accessory cell and a larger principle cell (Cohen, 1972).

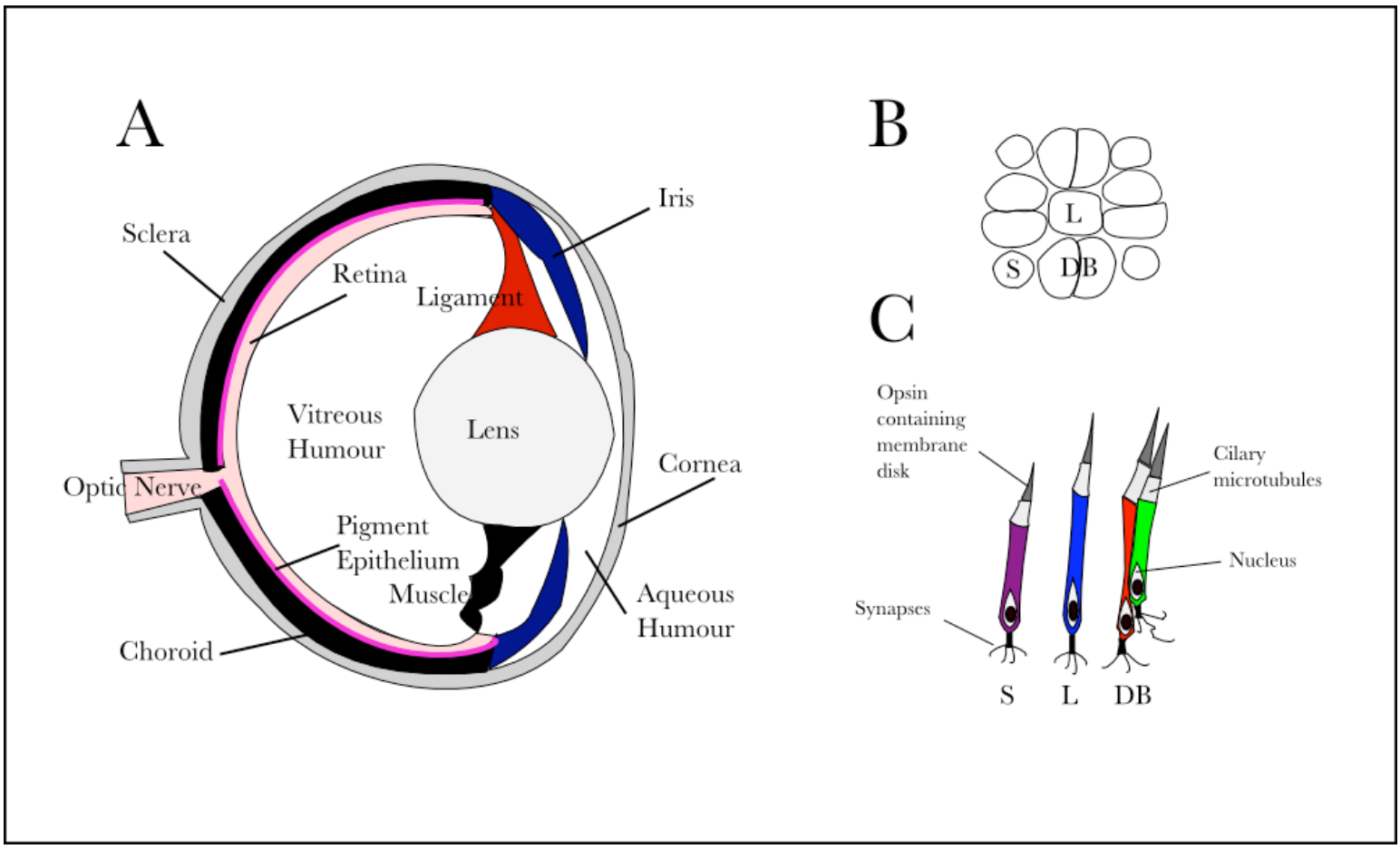


Figure 4-2 Teleost eye and cone morphology

A) Morphology of a teleost eye.

B) The repeating unit of the cone square mosaic. Long single cone, L; Short single cone, S; Double cone, DB.

C) The three cone types of the cone square mosaic. The principle member of the double cone is red, the accessory member is green. Long single cone, L; Short single cone, S; Double cone, DB.

In situ hybridization is a technique used to localize mRNA expression in a tissue. *In situ* hybridization has been used in the context of vision research to localize opsin expression across the surface of the retina. Specifically, labelled RNA probes can be applied to retinal tissue in order to identify the cells expressing different opsins. *In situ* hybridization has most commonly been used to look at opsin expression in the context of development and patterning of the mosaic in both invertebrates and vertebrates (e.g. (Pointer et al., 2005; Takechi & Kawamura, 2005; Sison-Mangus et al., 2006)). *In situ*

hybridization has also identified some interesting opsin expression patterns, including sexually dimorphic expression in Lycaenid butterflies and expression of different opsins in single photoreceptor cells of the Siberian hamster (*Phodopus sungorus*) and pouched mouse (*Saccostomus campestris*) (Lukáts et al., 2002; Sison-Mangus et al., 2006). Few previous studies have used *in situ* hybridization in fish to investigated differential spatial use of opsin duplicates. One example where *in situ* hybridization was used for this purpose was in the zebrafish (*Danio rerio*). In this species LWS and RH2 paralogs were found to have distinct spatial distributions over the surface of the retina (Takechi & Kawamura, 2005). This differential expression might be an adaptation to the aquatic environment, where due to the filtering properties of water the light hitting the dorsal retina differs spectrally from that hitting the ventral retina. However, so far this idea is based upon data from only one species.

Methods

Animal care and tissue preparation

The fish used in this study were from an inbred lab-reared population originally from Cumana Venezuela and were treated in compliance with the protocols of the University of Victoria, Victoria, British Columbia, Canada. The fish were kept on a 14 hr light, 10 hr dark cycle, at 24^{o}C (+/-1). They were fed on flaked fish food (Aquatic ecosystem). The fish were sampled at 11:30 am, at the end of their 14-hr light cycle (after 13.5 hrs of light). Eyes were enucleated from fish after euthanization with Tricaine methanesulfonate (MS222) (Sigma Aldrich, St Louis Missouri USA).

Probe design and synthesis

Total RNA was isolated from two male guppy eyes immediately after euthanazia using Aurum™ Total RNA Fatty and Fibrous Tissue Pack and cDNA was synthesized using BioRad iScript™ Select cDNA Synthesis Kit from total RNA. The cDNA was then used to generate opsin specific sequences. These were obtained using locus specific primers in a reverse transcription polymerase chain reactions (RT-PCR). PCR products were then cloned into pGEM ® T-Easy vectors. Digoxigenin (DIG)-labelled anti-sense and sense RNA probes were subsequently synthesized from the cDNA clones in pGEM® T-Easy

vectors, using a DIG RNA labelling kit (Roche Lewes UK). The cRNA probes were synthesized in run-off transcription reactions using T7 promoters for antisense probes and SP6 promoters for sense probes, on linearized templates. Nine probes were produced: RH2-1, RH2-2, SWS2A, SWS2B, SWS1, LWS generic, LWS S180r, LWS A/S180 and LWS P180. It was not possible to produce unique probes for two of the LWS opsin genes (A180 and S180), so a single probe that is equally complimentary to both genes was employed. See Table 4-1 for a percent difference matrix, where each probe is compared over the same region of sequence (within a subclass).

Table 4-1 Percent nucleotide difference of probe sequence and potential targets

Probe & Length	LWS S/A180 354bp	LWS P180 330bp	LWS S180r 182bp	LWS Gen 548bp	SWS1 799bp	SWS2A 480bp	SWS2B 803bp	RH2-1 827bp	RH2-2 738bp
LWS S180	0%	10.6%	11.3%	6.62%	-	-	-	-	-
LWS P180	10%	0%	14.3%	6.3%	-	-	-	-	-
LWS A180	0.5%	10.6%	11.3%	6.77%	-	-	-	-	-
LWS S180r	12%	16.96%	0%	11.72%	-	-	-	-	-
SWS1	-	-	-	-	0%	-	-	-	-
SWS2A	-	-	-	-	-	0%	26.8%	-	-
SWS2B	-	-	-	-	-	25.8%	0%	-	-
RH2-1	-	-	-	-	-	-	-	0%	26.8%
RH2-2	-	-	-	-	-	-	-	24.3%	0%

Dot blot analysis

The level of probe cross- hybridization, between subfamilies and duplicates was assessed by dot blot analysis. DIG labelled RNA anti-sense probe aliquots were

hybridizaed to the unlabeled sense RNA of each locus, which had been blotted and cross-linked to a nitrocellulose membrane. Prehybridization took place for 30 min at 68 ^{o}C in DIG Easy Hyb. solution (Roche, Lewis UK). Probes diluted in DIG Easy Hyb were then applied to the membrane in excess and hybridized overnight at 68 ^{o}C in the HL-2000 HybriLinker Hybridization oven (UVP, Upland CA USA). The membrane was blocked for two hrs and an anti-DIG-AP antibody (Roche, Lewis UK) was used. The antibody was detected using CSPD chemi-luminescent substrate (Roche, Lewis UK) and the film exposure for all blots was 1 hr.

Cross-section tissue preparation

Six male eyes (three left and three right) and six female eyes (three left and three right) were cross sectioned. These eyes were fixed in 4% (w/v) paraformaldehyde with 5% (w/v) sucrose in 1X PBS overnight at 4^{o}C on a rotator. The eyes were then washed 3 times for 20 minutes in 1X PBS with 5% (w/v) sucrose. The eyes were infiltrated with 1X PBS with 33% (w/v), 50% (w/v), and 66% (w/v) sucrose for 30 minutes each at room temperature, then left to infiltrate overnight at 4^{o}C in 1X PBS with 20 % (w/v) sucrose on a rotator. The eyes were then embedded in OCT medium (Sakura Finetek Europe, Netherlands), (ratio 2:1, 1X PBS/ 20% sucrose (w/v): OCT) and stored at -80^{o}C until sectioning. Sections 8 μm thick were cut nasal-temporally and dorsal-ventrally using a Leica CM1850 UV cryostat at -22 ^{o}C and were stored at -80^{o}C until use for *in situ* hybridization. There were between seven and nine sections per slide for each eye.

Whole mount tissue preparation

The fish used for whole mount (two males and two females per probe) were dark adapted for 20 hours prior to eye removal. Dissection was performed in the dark under a red light. After enucleation the retina was detached from the retinal pigment epithelium and the rest of the eye. The retina was then fixed in 4% (w/v) paraformaldehye with 5% (w/v) sucrose in 1X PBS overnight at 4^{o}C on a rotator.

In situ hybridization

Cross section

Slides were air dried for 1hr, then put through an ethanol hydration series of 2x 100% (w/v), 95% (w/v), 70% (w/v), 50% (w/v) and 2X SSC for 1 min each. The slides were digested using 10mg ml^{-1} Protinase K (Roche Lewes UK) in PBSTw for 4 min, rinsed briefly in DEPC water. The slides were washed with 0.1M TEA for 3 min and acetylated using acetic anhydride in TEA. The slides were subsequently dehydrated in the reversed order used for hydration and allowed to air dry for 2 hrs. DIG-labelled RNA probes were applied to the slides in excess and hybridized overnight at 65 ^{o}C in the HL-HybriLinker Hybridization Oven (UVP, Upland CA USA). The slides were then washed for 0.5 hr in 2X SSC at room temperature, 0.5 hr in 50% (w/v) formamide in 2X SSC at 65 ^{o}C and 0.5hr in 0.2X SSC at 65 ^{o}C. They were rinsed in 1X maleate buffer for 5 min and put into maleate blocking buffer for 2-3 hrs before anti-Dig-AP antibody (Roche Lewes UK) diluted (1:1000) in maleate blocking buffer was applied and incubated overnight at 4 ^{o}C. The slides were washed with 1X maleate buffer for 0.5h and for 10 min in Genius 2 buffer. Finally detection was performed using Nitrobluetetrazoleum (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) prepared in Genius 3 buffer. This was allowed to incubate for 0.5-2 hrs in the dark. The reaction was stopped using alkaline phosphatase substrate wash. Slides were viewed with a Zeiss universal light microscope and images were taken with a SPOT color camera.

Whole mount

After dissection fixed retinas were rinsed three times for 5 min in PBSTw, they were then digested using 12.5 μg/ml protinase K for 15 minutes and rinsed with PBSTw. The retinas were re-fixed in 4% (w/v) paraformaldehye with 5% (w/v) sucrose in 1X PBS for 20 mins and then rinsed three times for 20 min in PBSTw. The retinas were prehybridized in Hauptmann's buffer at 65^{o}C for 2 hours in the hybridization oven. For the hybridization the tissue was placed in an excess of labelled RNA probe and Hauptmann's solution and incubated overnight at 65^{o}C with light agitation. Retinas were subsequently washed two times for 30 mins each in 50% (w/v) Formamide in 2x SSC, one time for 15 min in 2x SSC and 2 times for 30 mins each in 0.2x SSC all at 65^{o}C.

Detection and image capture was by the same method described for cross-sections (above).

Quantification of spatial expression

The number of cells giving a positive signal for each probe was counted for eight sample regions of the retina. Each sample region was 100 μm in length. Six samples were from the peripheral retina and there were also two mid-retina samples taken for each probe.

Results

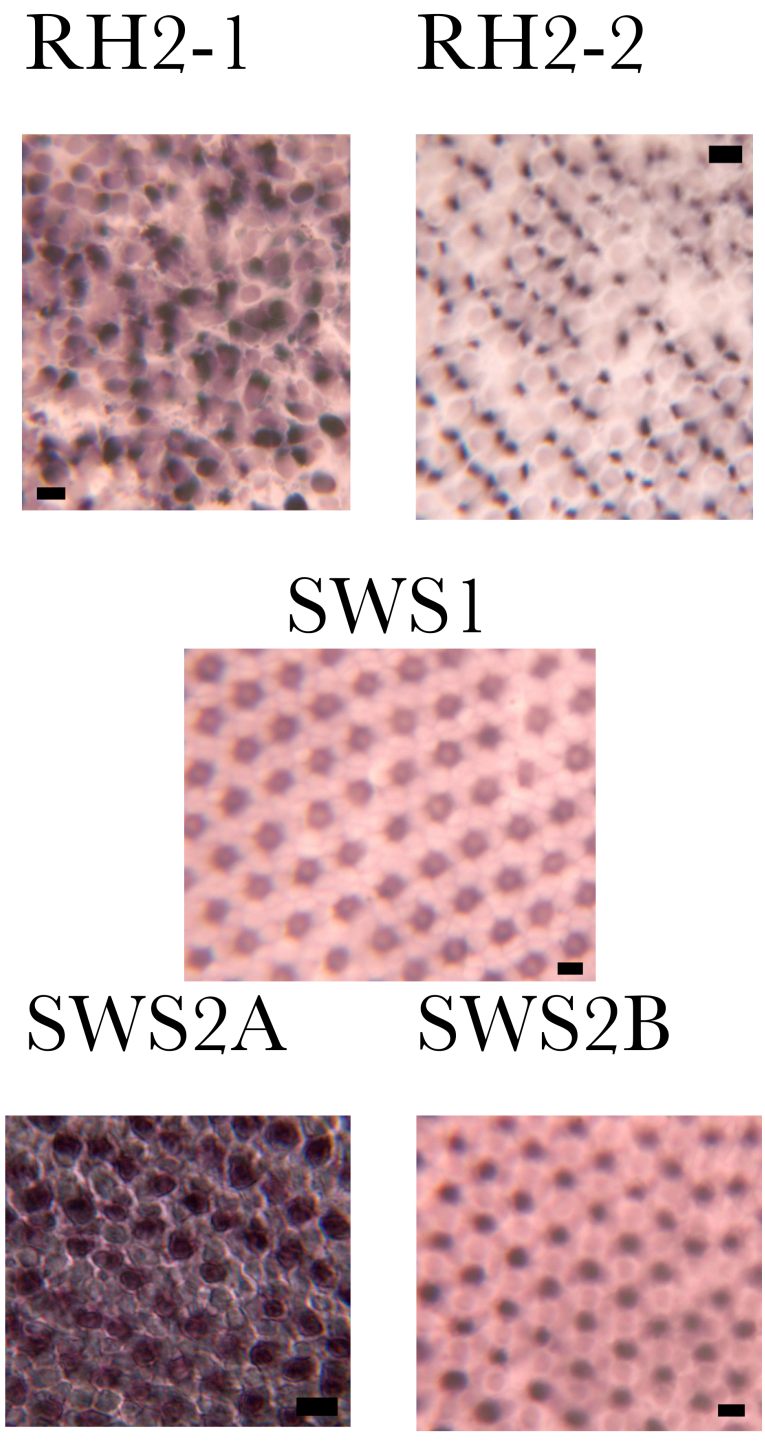
Dot blot analysis

Dot blots indicated that non-specific hybridization was low for SWS1, SWS2 and RH2 genes [see Appendix 5 for figures]. However, some of the LWS opsin probes hybridized to more than one target under northern blotting (dot blot) conditions [see Appendix 5]. Each of the LWS opsin probes appeared to give a distinct pattern of expression when *in situ* hybridization is performed, thus the cross-reactivity seen in northern blotting might not occur during *in situ* hybridization. However, it will be important to keep the possibility of degeneracy in mind when interpreting the results of the four LWS probes.

Whole mount *in situ* hybridization

Whole mount retinal *in situ* hybridization confirmed that guppies have a square mosaic. ISH indicates that short single corner cones express SWS1 (Figure 4-3). The long single centre cones express either SWS2A or SWS2B (Figure 4-3). The double cones can express RH2-1 or RH2-2, in homogeneous (two pigments of the same spectral sensitivity) and heterogeneous (two pigments with different spectral sensitivity) combinations (Figure 4-3).

A



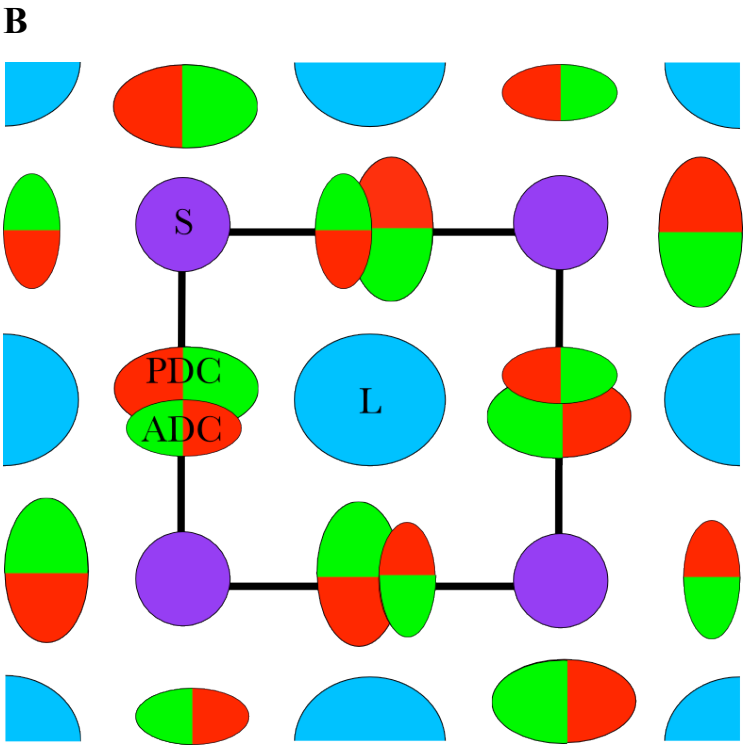


Figure 4-3 Whole mount *in situ* hybridization using SWS1, SWS2 and RH2 opsin gene probes on adult guppy retinas.

A) Whole mount retinas hybridized with RH2-1, RH2-2, SWS1, SWS2A and SWS2B probes. Scale bars represent 10 µm.

B) Summary of whole mount expression. Black square indicates one repeating unit of the mosaic. Cell types are: long single (L), short single (S), Principal component of double cone (PDC), accessory component of double cone (ADC). Color indicates the opsin subclass expressed in each cone type, purple is SWS1, blue SWS2, green is RH2 and red is LWS (this is predicted, but was not tested). The combination of red and green in double cones indicates that they can express both RH2 and LWS in homogenous or heterogeneous pairings.

In situ hybridization using retinal cross-sections

SWS1 expression

ISH indicated that the SWS1 opsin gene is expressed uniformly across the retina, along the dorsal-ventral axis (Figure 4-4B). However, along the nasal-temporal axis SWS1

expression was less dense (fewer cell expressing this gene) in the peripheral nasal retina (Figure 4-4A). When the number of cell expressing this gene was quantified (Appendix 6) this difference was apparent with an average of 1.3 cells in the nasal retina positive in a 100µm compared to 8 cells in the temporal retina. There was no evidence of differential expression of SWS1 between males and females.

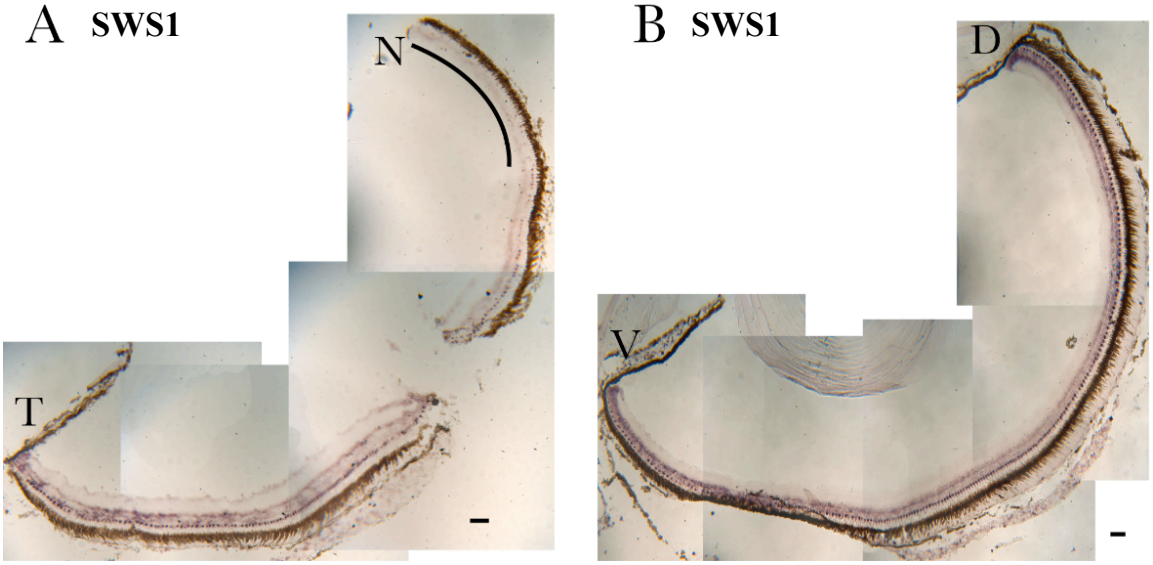


Figure 4-4 Expression of SWS1 in the guppy retina.

A) Nasal-temporal section of a male left eye hybridized with the SWS1 probe. B) Dorsal-ventral section of a female left eye hybridized with the SWS1 probe. D, dorsal; V, ventral; N, nasal; T, temporal. Black line indicates a region of decreased expression. Scale bars, 50 µm.

SWS2 expression

There are two SWS2 genes in the guppy, SWS2A and SWS2B. I designed unique probes for each. SWS2A is expressed uniformly along the nasal-temporal and dorsal-ventral axes (Figure 4-5A & B). There was some indication of variation among individuals for this gene; one of the three males surveyed appeared to have no expression and some individuals had what appeared to be lighter expression (fewer transcripts per cells).

Cells expressing SWS2B were found along the dorsal-ventral and nasal-temporal axes, as seen in Figure 4-5 C & D. The expression of SWS2B was uniform along both axes. This is also exemplified in the similar number of cells indicating expression (Appendix

7). The SWS2B gene did not exhibit any differences in the location of expression among individuals.

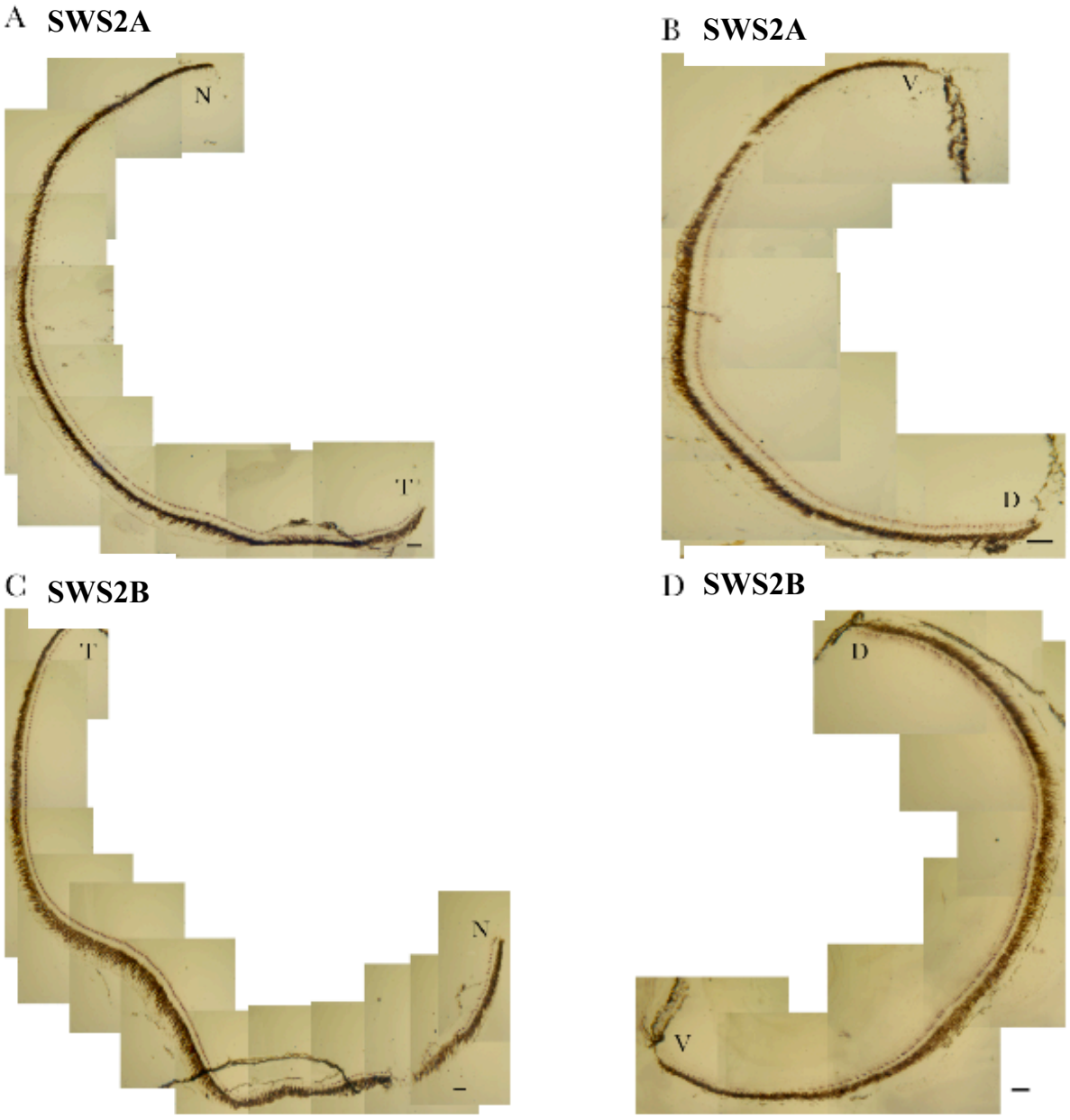


Figure 4-5 Expression of SWS2 gene duplicates in the guppy retina.

A) Nasal-temporal section of a female right eye hybridized with the SWS2A probe. B) Dorsal-ventral section of a male left eye hybridized with the SWS2A probe. C) Nasal-temporal section of a female left eye hybridized with the SWS2B probe. D) Dorsal-ventral section of a male left eye hybridized with the SWS2B probe. D, dorsal; V, ventral; N, nasal; T, temporal. Scale bar 50 µm.

RH2 expression

There are also two RH2 genes in guppies. When using the RH2-1 probe a uniform pattern of expression was detected along the nasal-temporal axis. Along the dorsal-ventral axis it appears there was a slightly lower level of expression in the dorsal region than the ventral (Figure 4-6A & B). Quantification indicated that there were on average 10.3 cells expressing RH2-1 in the ventral retina per 100µm in contrast to 5.3 over the same region in the dorsal retina (Appendix 7). However, there was no difference in the expression pattern of RH2-1 between the sexes.

RH2-2 was found to be largely uniform and equally distributed, across the retina as seen in Figure 4-6C & D and quantified in Appendix 7. However, there appeared to be fewer cells expressing RH2-2 than RH2-1 in the ventral retina (Figure 4-6D & Appendix 6). There was no RH2-2 expression in the distal ventral portion of the male retina and this was also observed in nasal-temporal sections, however this was only seen in two of fish (data not shown).

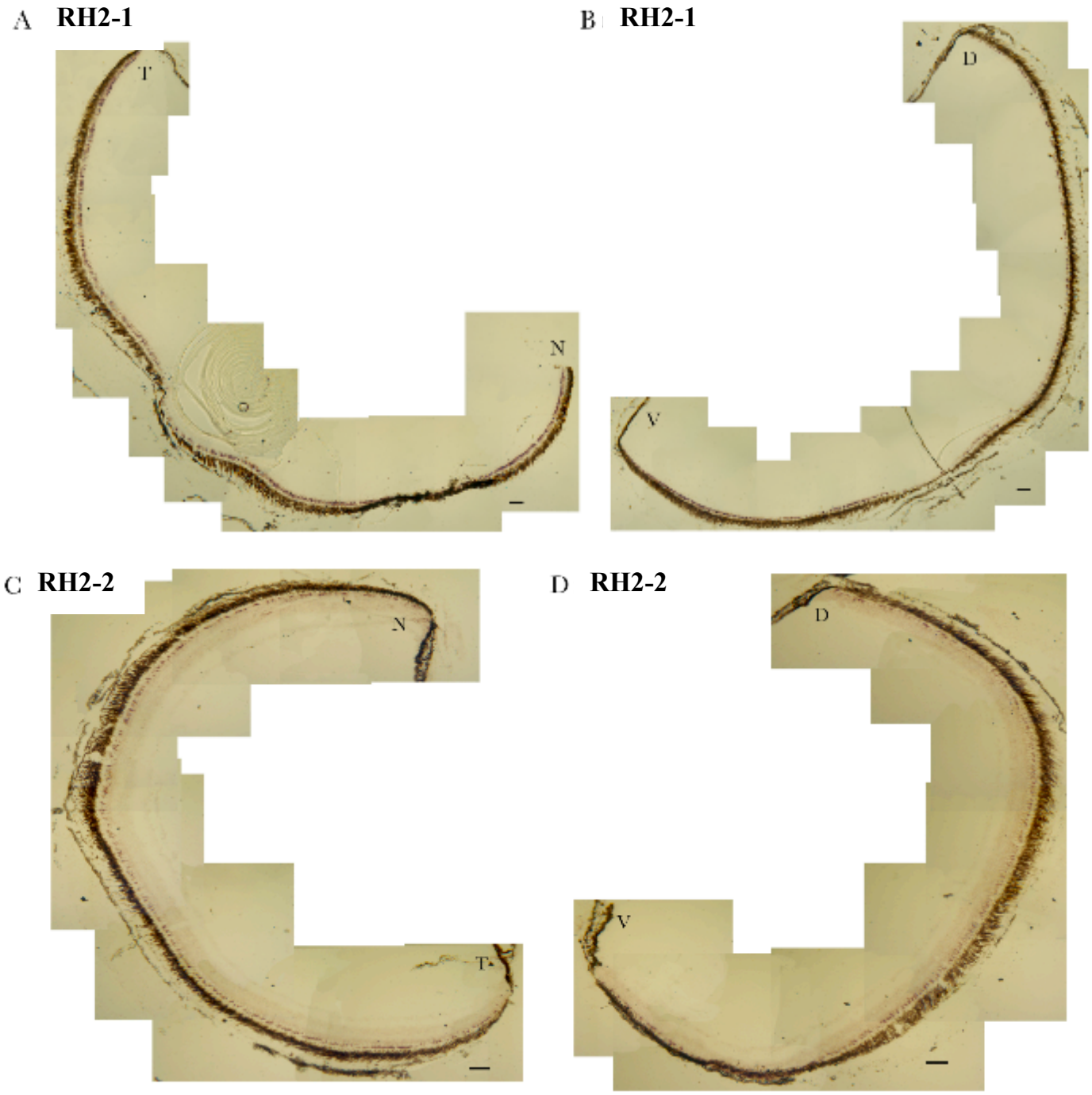


Figure 4-6 Expression of RH2 gene duplicates in the guppy retina.

A) Nasal-temporal section of a female left eye hybridized with the RH2-1 probe. B) Dorsal-ventral section of a female left eye hybridized with the RH2-1 probe. C) Nasal-temporal section of a male right eye hybridized with the RH2-2 probe. D) Dorsal-ventral section of a male right eye hybridized with the RH2-2 probe. D, dorsal; V, ventral; N, nasal; T, temporal. Scale bar 50 µm.

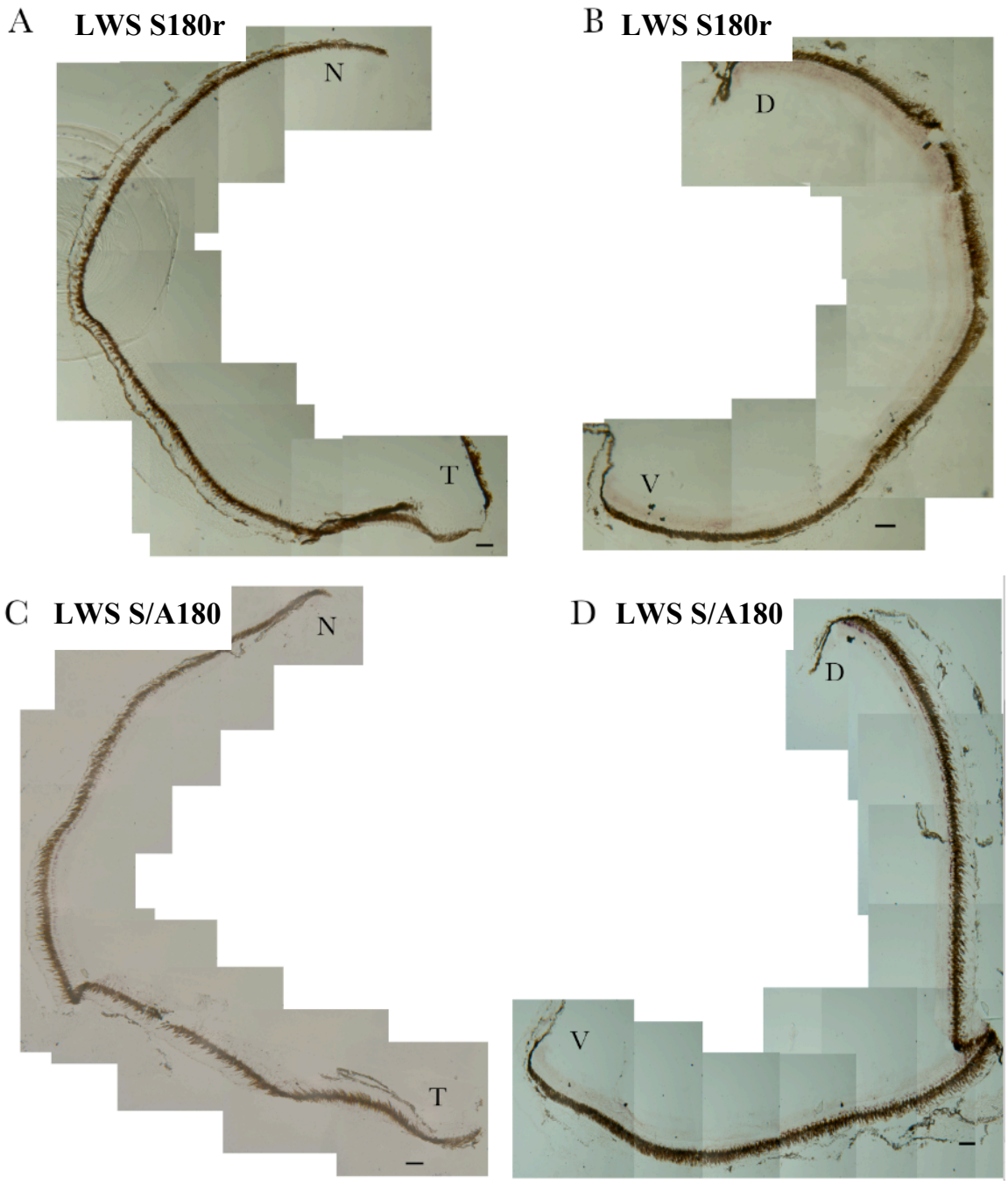
LWS expression

Most cross sections showed no evidence of LWS S180r opsin expression (8 of 12 eyes). However, in one male (both eyes) and one female (both eyes) there were transcript-positive cells in the peripheral dorsal–temporal region of the retina (Figure 4-7B & Appendix 6).

LWS P180 expression was detected along the nasal-temporal axis and largely localized to the mid-dorsal portion of the retina, while absent from the peripheral retina (Figure 4-7E). Along the dorsal axis LWS P180 expression was found to be absent from the bottom region of the ventral retina (Figure 4-7F & Appendix 6). There were no differences in the expression pattern of LWS P180 between sexes.

Along the nasal-temporal axis LWS S180/A180 expression was found in more cells on the nasal side of the retina than the temporal region (Figure 4-7C). Along the dorsal-ventral axis there were more cells expressing LWS S/A180 in the dorsal region of the retina than the ventral (Figure 4-7D & Appendix 6). There also appeared to be differences in the expression pattern of LWS S/A180 between sexes; females had low-density (few cells) expression over ¾ of the retina, with exclusion from the ventral tip, where as males only had expression in the distal dorsal retina (data not shown).

A generic LWS probe sensitive to all four LWS genes revealed an expression pattern that was consistent with the patterns seen for the LWS P180 and LWS S/A180 probes (Figure 4-7G & H). LWS expression along the dorsal-ventral axis is largely absent from the distal ventral region and is found in more cells in the dorsal half of the retina [Appendix 6 & 7]. Along the nasal temporal axis expression was largely confined to the nasal portion of the retina. However, there are a few cells expressing LWS extending from the nasal retina into the mid-temporal retina, this appeared to be largely absent in the distal portion of the temporal retina [Appendix 7].


A
LWS S180r
N
T
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LWS S180r
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LWS S/A180
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LWS S/A180
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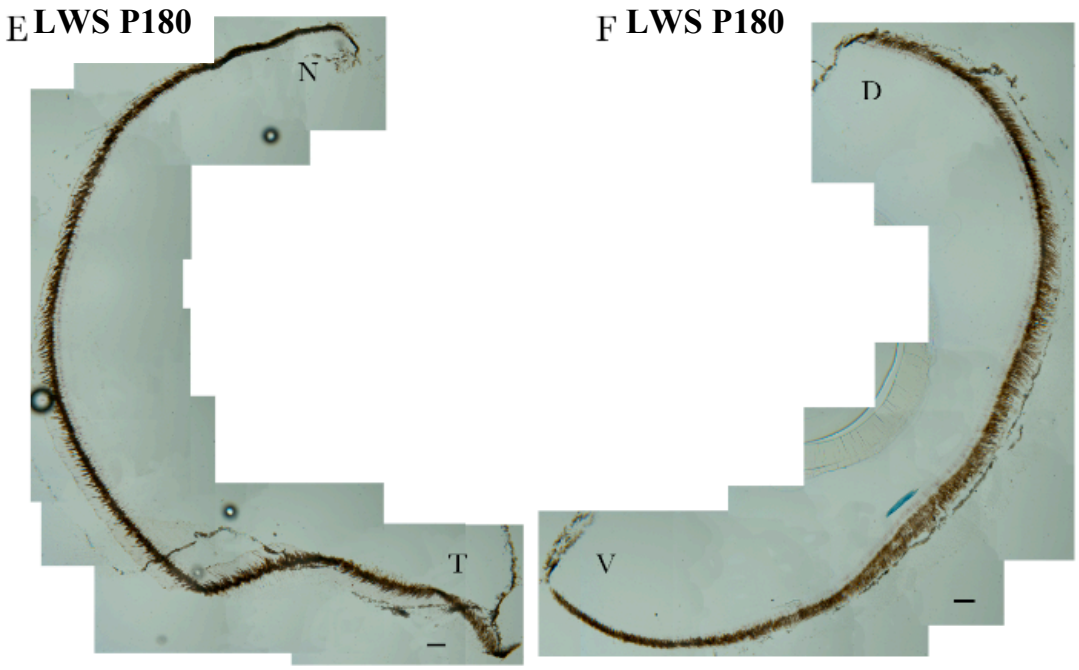


Figure 4-7 Expression of LWS gene duplicates in the adult guppy retina.
(A) Nasal-temporal section of a male right eye hybridized with the LWS S180r probe. (B) Dorsal-ventral section of a male left eye hybridized with the LWS S180r probe. C) Nasal-temporal section of a female right eye hybridized with the LWS S/A180 probe. D) Dorsal-ventral section of a guppy female left eye hybridized with the LWS S/A180 probe. E) Dorsal-ventral section of a female right eye hybridized with the LWS P180 probe. F) Dorsal-ventral section of a male left eye hybridized with the LWS P180 probe. G) Nasal-temporal section of a male left eye hybridized with a generic LWS probe. H) Dorsal-ventral section of a male left eye hybridized with a generic LWS probe. D, dorsal; V, ventral; N, nasal; T, temporal. Scale bars, 50 μm.

Individual differences in LWS expression

There is evidence for individual variation in LWS expression; specifically the strength of signal (number of transcripts per cell) and spatial distribution varied among individuals. Fish that had low or no LWS P180 expression (F1, M1, F3) did express LWS A/S180. Correspondingly, those that had low or no LWS A/S180 (M2) did express strongly LWS P180. There were also individuals that exhibited expression for both

probes (F2 & M3). Both individuals that expressed LWS S180r (M3 & F2) also expressed LWS P180, however LWS S/A180 expression was variable.

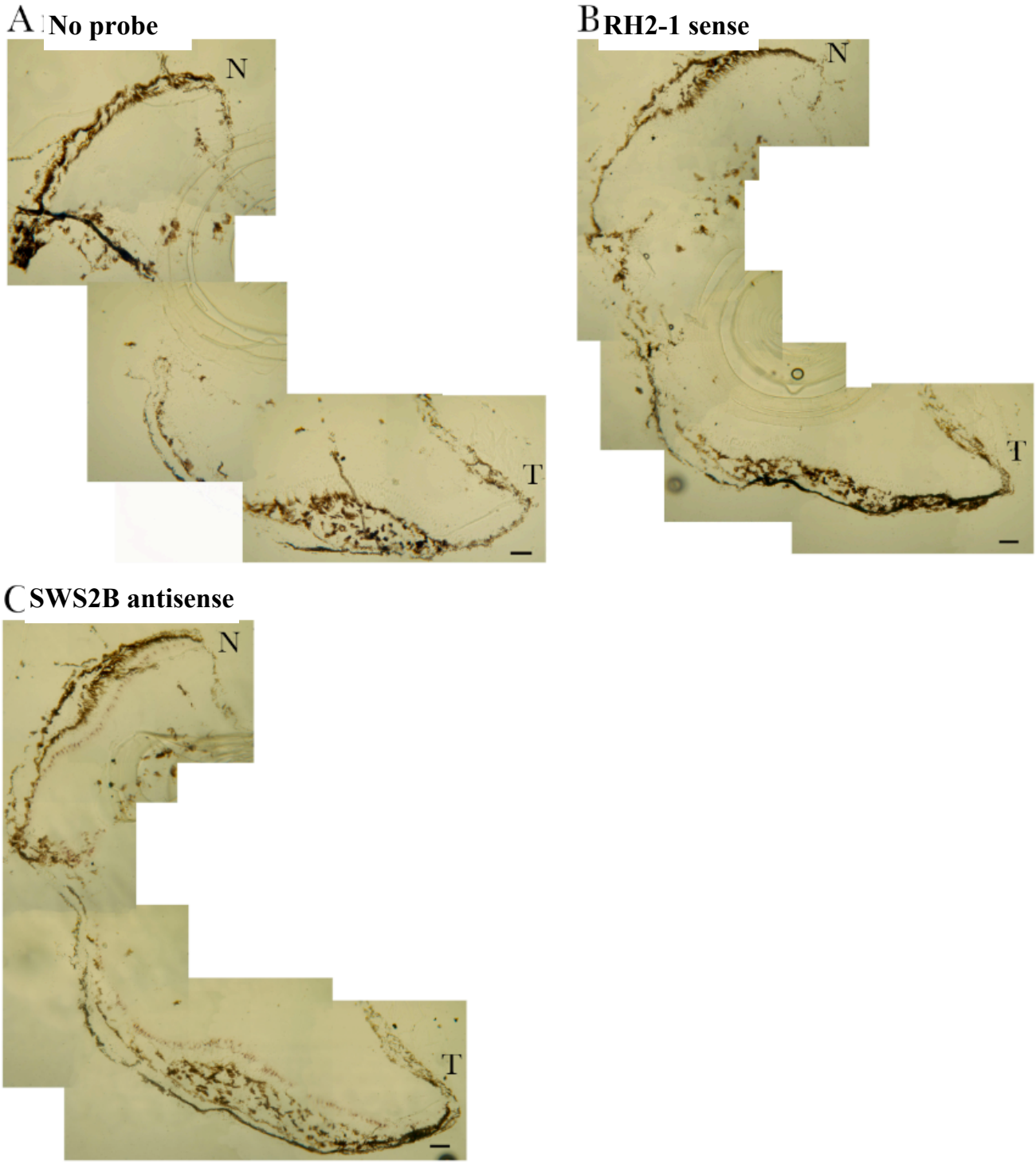


Figure 4-8 Control *in situ* hybridization experiments on male guppy nasal-temporal cross-sections from a right eye.

A) Hybridization using no probe. B) Hybridization using an RH2-1 sense probe. C) Hybridization using an antisense SWS2B probe. D, dorsal; V, ventral; N, nasal; T, temporal. Scale bars, 50 µm.

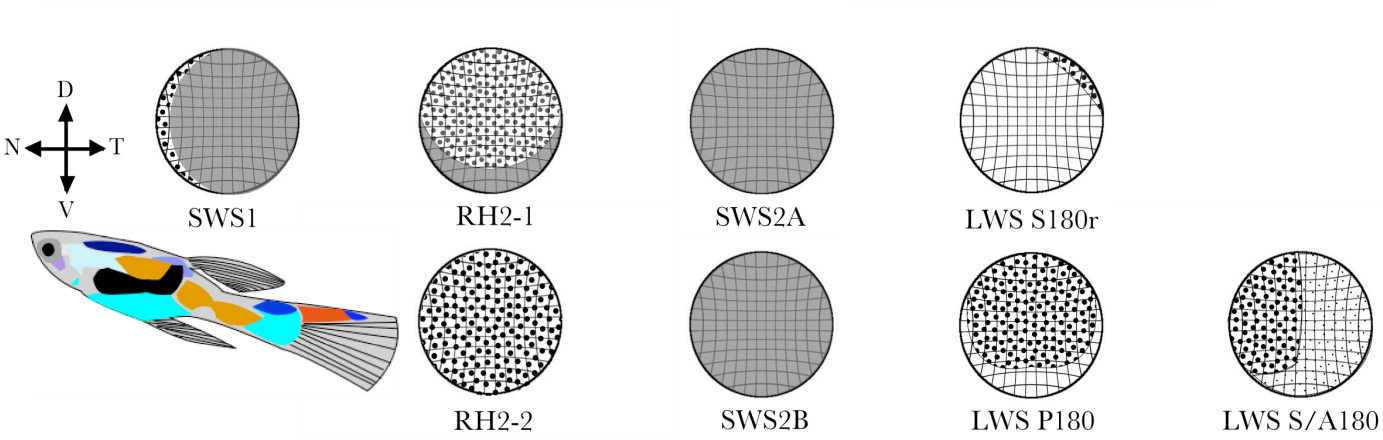


Figure 4-9 Summary schematic of spatial expression patterns of eight* cone opsin genes in the guppy retina, as reconstructed from serial sections through the eye.

Grey shading indicates strong expression, large black dots indicates less dense (fewer cells) expression and small black dots indicates low-level (a small number of cells) expression. D, dorsal; V, ventral; N, nasal; T, temporal.*Indicates that LWS S180 and LWS A180 could not be distinguished.

Lateralization and sexually dimorphic expression

Cross-section and whole mount ISH did not detect a difference in opsin expression between right and left eyes for any of the nine opsin genes surveyed. Thus there was no evidence collected to indicate the presence of asymmetric opsin expression. There was also little evidence for sexually dimorphic expression. The only gene that hinted at sexually dimorphic expression was LWS S/A180, which differed in the spatial

distribution of expression along the dorsal ventral axis. However, no conclusions can be drawn from this observation of variation between males and females, as this was seen only between two male and one female sample. Therefore rather than sex specific expression these differences could fall along the continuum of individual level variation.

Discussion

Guppy opsin sequences indicate that all nine cone opsins are functional, each gene has an intact open reading frame and most have unique key site haplotypes. Furthermore, cDNAs from all nine genes have been isolated from eyes, confirming expression and processing of mRNA. However, it is essential to know where these genes are expressed if one wants to make any connections between guppy color vision, sexual selection and this large opsin gene repertoire. Using *in situ* hybridization I discovered that at least eight of the cone opsin genes are expressed simultaneously in adult male and female guppies. This data now allows us to begin to make predictions about the spectral sensitivity and wavelength discriminatory ability of the guppy.

Spatial distribution

Sequence level divergence has been previously demonstrated for the guppy opsin duplicates (Ward *et al.,* 2008). In each sequence, there have been amino acid substitutions that have lead to a distinct wavelength of maximal absorption (with the exception of LWS S180 and LWS S180r) (Ward *et al.,* 2008). *In situ* hybridization indicates that there are also changes in the cell expressing these genes, particularly paralogs. In the case of RH2-1 and RH2-2, both have a retina-wide distribution of expression but the RH2-1 gene appears to be expressed in more cells in the ventral retina than the RH2-2 gene. There was also evidence for differential expression of LWS paralogs; the LWS S180r gene appears to be restricted to the periphery of the dorsal-temporal retina, while A/S180 is largely limited to the nasal portion of the retina. In contrast the P180 gene appears to have a centralized mid-retina pattern of expression. The SWS2A and SWS2B genes do not appear to follow this trend, as they exhibit a similar distribution over the surface of the retina and appear to be in the same number of cells. This might be a consequence of co-expression given that SWS2 genes are expressed in

the middle long-single cones of the square mosaic and there are fewer of these cones compared to short-single or double cones. Therefore if these genes were not co-expressed one would expect to see fairly sparse distributions for both genes. Future experiments using co-labelling would be able to determine whether both genes localize to the same cells. SWS1 does not have a duplicate, therefore it is not surprising that there are many cells expressing this gene and that it is expressed in a fairly uniformly pattern across the retina (Figure 4-4).

Functional implications

The first insight into the pigments responsible for color vision in the guppy was gleaned using microspectrophotometry (MSP) in 1979 and 1980 (Levine & MacNichol Jr, 1979; Levine et al., 1980). MSP is a technique that determines the absorption spectrum of individual cone cells. Thus before opsin genes were characterized, data was collected on the spectral properties and cellular morphologies of the photoreceptor cells expressing opsin pigments. MacNichol and Levine collected MSP data that indicated the presence of three cone pigments in the guppy, which absorbed at 410 nm, 468 nm and 545 nm. By surveying the dorsal and ventral portions of the retina MacNichol and Levine identified differences in the spectral properties of the cones in these two regions of the retina. They found that there were more green (545 nm) pigments in the ventral portion of the retina than the dorsal. Additionally, it was found that the 545 nm pigment could form homogenous pairings in the double cones or heterogeneous pairings with the 468 nm pigment (Levine & MacNichol Jr, 1979; Levine et al., 1980; Levine & MacNichol, 1982). In 1987, further insight into guppy opsin pigments was generated by MSP when Archer *et al.* discovered that in addition to the previously identified pigments there were also a variety of pigments sensitive to the 529-579 nm (long-wavelength) portion of the spectrum. This range of spectral sensitivity was suggested to be individual level polymorphism in opsin expression (chromophore switching was not believed to be responsible for this observation) (Archer et al., 1987). Archer *et al* also refined the spectral absorptions that MacNichol & Levine predicted (See Table 4-2). In 1990, a third MSP study expanded on the idea of polymorphic LWS sensitivity in the guppy (Archer & Lythgoe, 1990). This 1990 paper confirmed the presence of individual level LWS

polymorphism and again modified the predicted sensitivities presented in the first two studies, it also estimated sensitivity of a ultra-violet (UV) sensitive pigment (summarized in Table 4-2). UV pigments had been neglected in the first two studies due to limitations of the MSP technology. The 1990 study also suggested the idea of co-expression, i.e. that two pigments expressed simultaneously in the same cone could explain three cone sensitivities; Archer and Lythgoe predicted that the cells with 548 nm sensitivity were a product of co-expression of the 533 and 572 pigments, particularly given the observed broad spectral absorption.

Using the expression data generated by this study and a comparison of reconstitution data generated in species related to the guppy (Matsumoto et al., 2006), I have attempted to assign genes to each MSP peak (see Table 4-2). Two genes were difficult to reconcile with the MSP data, SWS2A and LWS A180. SWS2A does not appear to be represented in the MSP studies, however this could be due to co-expression or perhaps this gene was not expressed in the guppy populations surveyed by MSP. The LWS A180 gene based on amino acid sequence is predicted to have a maximal absorption only 7 nm less than LWS S180 (Yokoyama & Radlwimmer, 1998). Therefore, if both genes were expressed in distinct cells, I would expect to find two MSP peaks 7 nm apart. Distinct cone types with spectral sensitivities this close might be mistaken for variation in a single cone type.

Table 4-2 Cone opsin gene identity predictions based on MSP and gene expression

Gene identity prediction based on expression	Levine & MacNichol 1979 MSP	Archer *et al*. 1987 MSP	Archer & Lythgoe 1990 MSP
SWS1	n/a	n/a	~359
SWS2A	-	-	-
SWS2B	420	410	408
RH2-1	545	*	533
RH2-2	485	465	464
LWS S180	-	573	572
LWS S180r	-	573	572
LWS A180	-	*	-
LWS P180	-	*	548

*Predicted to fall within the range of observed peaks collected (529-579 nm)

Using *in situ* hybridization, I found that LWS expression was largely limited to the dorsal portion of the retina while green-sensitive RH2-1 was most highly expressed in the ventral portion of the retina. Interestingly, it appears that perhaps the exclusion of LWS P180 from the ventral retina makes room for RH2-1, which has higher expression in the ventral portion of the retina than the dorsal. This would not be surprising given that these two genes are predicted to have similar phenotypes (Table 4-2) and given that RH2 and LWS genes are both expressed in the same cone type (double cones). This differential pattern of expression was originally predicted by Levine and MacNichol (Levine & MacNichol, 1982). They suggested that this spatial distribution of cones allowed the ventral retina to detect dark objects against the down-welling light, while leaving the dorsal retina with color discriminatory ability and long wavelength sensitivity (Levine & MacNichol, 1982). Given the finding that SWS1, SWS2A, SWS2B, RH2-1, RH2-2 and the LWS paralogs are all expressed in the dorsal retina it is likely that guppies have broad spectral sensitivity in the dorsal portion of the retina. This pattern of expression also indicates that the dorsal retina may have a great capacity for wavelength discrimination. Additionally, it has been suggested that double cones could play a role in wavelength discrimination (Pignatelli et al., 2010), which would support this capacity. However, this is dependant upon the receptive field of the ganglion cells and signal integration. Another functional suggestion for this pattern of expression and sensitivity suggested by Levine and MacNichol was that the mating position of the male slightly below and in front of the female would maximize the spectral sensitivity and discriminatory ability of the female's dorsal retina (Levine & MacNichol, 1982). The pattern of opsin expression exposed by *in situ* hybridization indicates that this remains a realistic possibility.

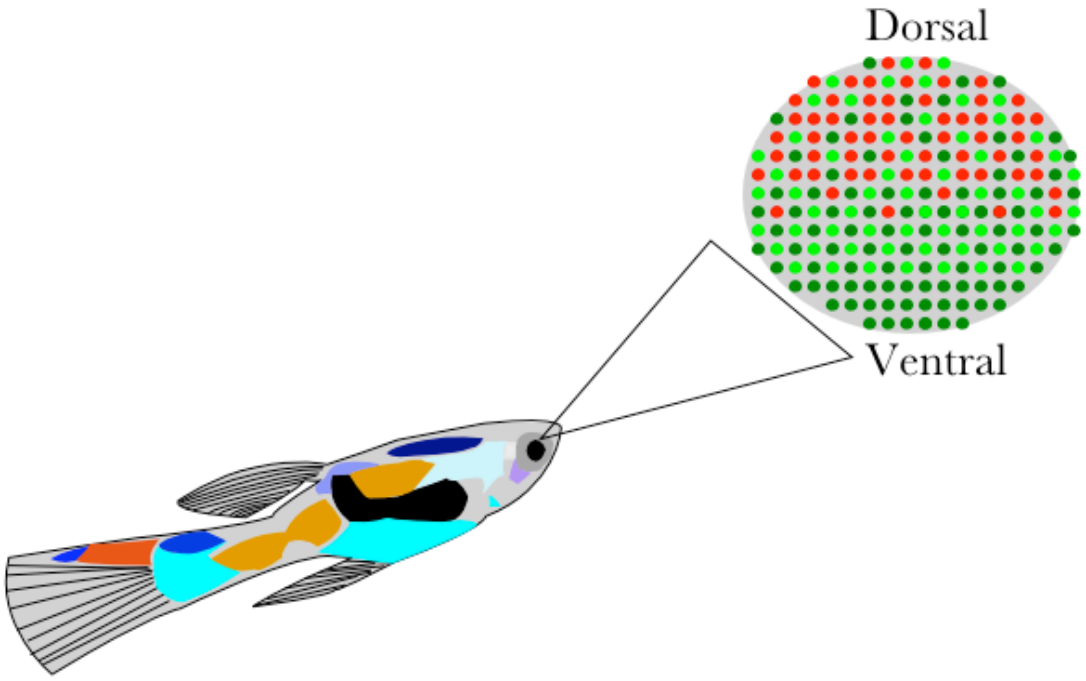


Figure 4-10 Summary of RH2 and LWS opsin expression on the surface of the retina
Composite image of the expression patterns of RH2-1 (Dark green), RH2-2 (Light green) and LWS genes (Red) over the entire retinal surface for male and female guppies.

Differential opsin expression in teleosts

The expression of all cone opsins in a repertoire simultaneously is not a universal finding in fish. For example in the African cichlids that inhabit the clear waters of Lake Malawi express only a portion of their repertoire. These fish express primarily three of their seven opsin genes. Each species expresses two longer-wavelength pigments (a LWS and an RH2) in the double cones and they express only one of SWS1, SWS2A or SWS2B (Parry et al., 2005). In contrast it appears adult guppies expresses all three SWS genes simultaneously. My findings also differ from what is seen in the Nile tilapia (*Oreochromis niloticus*), which expresses all of its opsins, but only over the entire course of development (Spady et al., 2006; Carleton et al., 2008). However, some of these differences in repertoire use could be due to the method of investigation. *In situ* hybridization has not been used in cichlids to investigate opsin repertoire use; rather

qPCR has been the method of study. One drawback of this approach is that although qPCR can give precise measurements of the level of opsin expression it cannot indicate how many cells are expressing the gene of interest. Thus low-level opsin expression could be attributed to either low-quantity (background) expression in many cells, or a small population of cells with high (biologically functional) expression. Therefore, in the future one may find that many fish express entire opsin repertoire but some genes are only expressed in a small population of cells.

The finding of SWS1 expression in adult guppies is also not a universal finding in fish. In many species, it has been found that ultraviolet sensitive cones are often primarily present in young fishes; for example in many salmonids ultraviolet sensitive cones can only be measured in fish up to 1-2 years old (Bowmaker & Kunz, 1987). However, it has been suggested that UV cones are regained upon sexual maturity (Beaudet et al., 1997); there has also been evidence presented that opsin expression in the single cones of salmonid fish switch from expression SWS1 in juveniles to SWS2 in adults (Cheng & Flamarique, 2007). This finding of ontogenetic reorganization of the retina was suggested to occur in most vertebrates. However, my finding of simultaneous expression of SWS1 and SWS2 genes in adult fish does not support this hypothesis, at least in guppies.

Mice had previously been shown to exhibit spatial variation in the expression of short- and middle-wavelength sensitive opsins (Applebury et al., 2000). However, Zebrafish (*Danio rerio*) provided the first evidence of differential expression of cone opsin duplicates in fish (Takechi & Kawamura, 2005). Expression patterns of duplicates were demonstrated to vary temporally and spatially, when *in situ* hybridization was used to characterize opsin gene expression. LWS opsin duplicates localize differentially in the zebrafish, with shorter-wavelength sensitive LWS-2 in the central and dorsal regions of the retina. Whereas the longer-wavelength sensitive LWS-1 localizes to the ventral and peripheral areas. The four RH2 genes are also not used homogeneously across the surface of the retina, rather in dorsal-ventral and medial lateral gradients the shortest wavelength RH2 (RH2-1 and RH2-2) is found predominantly in the dorsal retina and the longest wavelength RH2 (RH2-3 and RH2-4) copies in the ventral and peripheral retinal areas (Takechi & Kawamura, 2005). Takechi and Kawamura suggested that this heterogeneous pattern of expression could be an adaptation to the heterogeneous light environment

experience by fish. As the spectral properties of the up welling light from the water's depths differs from that of the down welling sunlight. The same could be true in guppies.

Individual level differences in LWS

The degree of LWS gene expression appears to vary between individuals. These *in situ* hybridization observations are consistent with the MSP studies that have also shown among-individual variation in cone cell diversity (Archer et al., 1987; Archer & Lythgoe, 1990). The finding of variation in LWS expression among individuals has important implications with regard to mate choice. Given that orange coloration in males is an important cue for females (Endler, 1980; Endler, 1983; Houde, 1988; Houde & Endler, 1990) variation in the perception of male color could alter female male preference for that trait. Specifically, variation in the perception of male color means that one female could potentially find a male particularly attractive based on her expression profile, while another female with a distinct profile would not. If this variation is heritable then there could be significant implications with regard to the co-evolution of male color and female preference for the color, particularly Fisherian run away sexual selection, as this process requires that female preference varies within a population and that it is heritable (Lande, 1981). This process will be discussed further in Chapter 5. However, to rule out the role random stochastic factors in this variation (i.e. experimental variation) a larger sample size will be required in the future to confirm the degree of variation.

Lateralization

Laterality is the asymmetry of brain functions (Reviewed in Vallortigara et al., 1999). Previous studies have indicated that some species of fish, including members of the family Poeciliidae (of which guppies are members), display asymmetric eye use when performing certain tasks, or viewing particular stimuli (Bisazza et al., 1997; Bisazza et al., 1998). However, there were no differences in opsin expression between left and right eyes. This could however be attributed to the qualitative nature of the technique of *in situ* hybridization. Perhaps a more quantitative and sensitive technique such as qPCR would reveal differences in the opsin expression of left vs. right eyes. Alternatively, these lateralized behaviours could be attributed to differences in neuronal processing i.e. in the brain rather than at the level of the receptors.

Sexually dimorphic expression

Sexually dimorphic opsin expression patterns have been identified in lycaenid butterflies (Sison-Mangus et al., 2006), however this phenomenon has not been demonstrated in many fish species. There was a hint of sex-specific differences in the expression pattern of LWS S/A180. However, given the small sample size of three males and three females, and that individual level variation appear to be present it was difficult to definitively conclude that these difference were indicative of sexually dimorphic expression rather than general individual variation. Using MSP, Archer *et al.* 1987 also noted evidence for sexually dimorphic expression of LWS cones, they saw differences in the distribution of cones sensitive to long-wavelengths (Archer et al., 1987). However, like in this study, the sample size in the Archer *et al.* 1987 study was too small to make definitive conclusions.

LWS-S180r

LWS S180r exhibited the most restricted expression profile, limited to the peripheral dorsal-temporal retina. This may indicate a developmental role for this gene. Alternatively, given that expression appeared to either be low or non-existent perhaps LWS S180r is primarily used at a different life stage. Differential opsin gene expression during ontogeny has been demonstrated in a diversity of fishes including cichlids (Carleton et al., 2008), flounder (Mader & Cameron, 2004), salmon (Cheng & Flamarique, 2007) and zebrafish (Takechi & Kawamura, 2005). The sampling of previously surveyed species is phylogenetically diverse; suggesting the occurrence of ontogenetically modified expression may be common in fish. Another alternative is that LWS S180r has an extra-ocular function. Extra-ocular expression of visual opsins has been demonstrated in the skin of neon tetra and even humans (Kasai & Oshima, 2006; Tsutsumi et al., 2009).

Mosaic

Based on whole mount *in situ* hybridization, this study shows that the cones of the guppy are organized into a square mosaic, with cell-specific expression similar to the patterns found in halibut (*Hippoglossus hippoglossus*), goldfish (*Carassius auratus*) and medaka (*Oryzias latipes*) (Hisatomi et al., 1996; Stenkamp et al., 1996; Hisatomi et al.,

1997; Helvik et al., 2001). Unlike these past findings, in this study within subfamily duplicates were taken into account. Within subfamily paralogs are expressed in the same cone type (single short, single long or double). However, one drawback of this study is that since co-labelling was not undertaken it is unclear whether there is co-expression of any of the paralogs.

Summary & Conclusion

In situ hybridization demonstrates that guppies express at least eight cone opsins (assuming only one of LWS S180 or A180 is expressed), suggesting guppies may have to potential to have better wavelength discrimination than humans. However, this prediction is dependent on neuronal signal integration and the receptive field of ganglion cells. The generally broad spatial expression patterns of genes from each opsin subclass are predicted to provide guppies with broad-spectral sensitivity. The results indicate that subtype differentiation of opsins in absorption and expression pattern may be another way for fish to achieve finer spatial grading of spectral sensitivity in the retina. This differentiation may allow the guppy to optimize the dorsal retina for color discrimination, which may directly aid mate selection. The ventral retina, with fewer cone types, may specialize in detecting dark objects against the down-welling light, perhaps aiding aerial predator avoidance (This will be discussed further in Chapter 5). Individual variation in the expression of LWS genes may have functional implications with regard to wavelength sensitivity and discriminatory ability and thus could affect mate preference. However, further study is necessary to confirm, the degree of individual variation, as well as, the functional effect of such variation. This work sets the stage for behavioural studies, using these methods sensitivity and discrimination can be measured in live fish.

Chapter 5
Opsin Gene Repertoire in the Guppy (*Poecilia reticulata*) More Than Meets the Eye.

Summary

Guppies have long been considered a model species for the study of sexual selection (Haskins & Haskins, 1949). Laboratory based studies have indicated that when females are presented with two males, females tend to spend more time with males which possess the greatest color saturation and orange area (e.g. (Houde, 1987)); this type of proximity-data is a good proxy for mate choice and male reproductive success. However, in the wild conspicuous coloration is believed to be a trade-off; while enhancing courtship success, it likely increases conspicuousness to predators. This hypothesis is based upon the observation that male guppies are especially colorful in low-predation environments (Endler, 1983). Additionally, transplant experiments have demonstrated that male color increases when predators are absent and female preference has been suggested to drive this change (Endler, 1983). However, little is known about the color vision capacity of guppies or their predators.

The first step in understanding color-based sexual selection from the perspective of the guppy was to characterize its opsin repertoire. PCR based analysis indicated that guppies and other members of Poeciliidae, such a swordtails (*Xiphophorus* sp.), had surprisingly large opsin gene repertoires (nine or ten genes) Hoffmann et al., 2007; Ward et al., 2008; Watson et al., 2010). Given the presence of color vision based sexual selection in this lineage, these large repertoires were suggested to have evolved for mate discrimination (Archer et al., 1987; Hoffmann et al., 2007). However, the characterization of the opsin repertoires of *Jenynsia onca* and *Anableps anableps*, non-colorful members of Poeciliidae's sister family Anablepidae, also revealed a large opsin repertoire of nine genes and ten genes respectively (Chapter 2, (Owens et al., 2009; Windsor & Owens, 2009)). This showed that opsin gene duplication pre-dates color based sexual selection on a phylogenetic tree, suggesting that it is unlikely that the ten-opsin repertoire of the guppy evolved exclusively for color-base sexual selection. Furthermore, when the phylogenetic perspective of opsin repertoire size was expanded to include all surveyed

ray-finned fish it was determined that large opsin repertoires are fairly common (Chapter 3). It was also determined that these large opsin repertoires have largely been produced through multiple tandem duplication events, which span the age of the taxon Teleostei. Thus the goal of my *in situ* hybridization project (Chapter 4) was to determine how the guppy utilized its opsin repertoire, in hopes of further understanding the basis of female mate preference and the evolution of ornate male coloration in this species. Using *in situ* hybridization, I found that adult guppies use at least eight of the cone opsin genes in their repertoire, albeit not equally. Each gene appears to have a slightly different spatial distribution of expression. There also appears to among-individual variation in LWS opsin expression.

Implications for interpreting models of sexual selection

The origin of color-based sexual selection, in guppies and in other species is a subject of much debate. The *in situ* hybridization results reported in Chapter 4 can be used to further evaluate hypotheses regarding this subject. Evolution of male nuptial color in *Pundamilia* cichlids appears to be driven by changes in spectral sensitivity mediated by opsins (Seehausen et al., 2008). Thus similar theories have been proposed to explain the evolution of male color in the guppy. For example, Rodd *et al.* (2002) proposed that a pre-existing bias model of sexual selection might apply. Rodd *et al.* suggested that male guppies might be exploiting a pre-existing sensitivity to long wavelengths (e.g. oranges and reds), because these are the colors of preferred food items (fruits) (Rodd et al., 2002). The number of cone cells expressing a given opsin gene can provide an indication of the spectral sensitivity of the fish. Rodd *et al.*'s hypothesis predicts that a large proportion of cone cells would be tuned to the colors of preferred food items (long-wavelengths). My finding of LWS opsin expression largely confined to the dorsal retina and in fewer cells than SWS1, SWS2 or RH2 (middle-wavelength sensitive) opsins predicts that females (and males) are less sensitive to long-wavelengths of light than medium- or short-wavelengths. Additionally, the ventral retina, which is used for viewing objects at the water's surface, such as food items, appears to be tuned to middle wavelength light (green) and would have less wavelength discriminatory ability than the dorsal retina. Thus, the ISH findings do not support the pre-existing bias model of sexual selection suggested by Rodd *et al* (2002).

Alternative models that have been suggested to explain the apparent co-evolution of male coloration and female preference (Houde & Endler, 1990) include Fisherian runaway sexual selection and good genes models. In Fisherian models of runaway selection, female preference evolves for a male trait that is favoured by natural selection (Fisher & Bennett, 1999). This preference evolves, because offspring of the males with the trait have higher fitness than those lacking it. This is because males with the trait have an advantage with regard to natural and sexual selection (they mate with females lacking preference at the same frequency as males that lack the trait and they mate with all of the females that possess the preference allele). The trait and the preference increase in frequency in the population, even when the trait has been developed to the point where the advantage the trait affords to natural selection has ceased (or even become detrimental). This is why this process is often referred to as runaway sexual selection. Fisherian models would not have a requirement of heightened sensitivity to male color, unlike pre-existing bias models. Rather Fisherian models would only require that females have the visual ability to discriminate between males of different colors and patterns, as color based preference could not exist without this ability. Given that we find male and female guppies express at least eight cone opsins in the dorsal retina, provided that the neuronal infrastructure of the guppy can interpret the multiple signals generated by cone cells expressing these distinct pigments, it is likely that these fish have excellent wavelength discriminatory ability. Based on these observed patterns of opsin expression in the guppy, Fisherian models remain plausible models to explain the co-evolution of female preference and male color. However, behavioral tests will be required to confirm this prediction of wavelength discriminatory ability. Another important thing to note with regard to run-away sexual selection is that it can lead to detrimental exaggeration of the male secondary trait (color) and in theory the female preference for it (visual capacity). Therefore, a signature of this process in the guppy could be that wavelength discrimination has evolved at the cost of visual acuity/resolution. This, too, could be investigated through behavioral tests.

Another possible model, which can be evaluated using the *in situ* hybridization data collected, is a good genes model of sexual selection. Good genes models suggest that female preferences for males possessing traits indicative of genes that enhance viability

are favored by evolution (Zahavi 1975; Hamilton & Zuk 1982). Carotenoid spots in male guppies are thought to be honest signals as, the color saturation of carotenoid spots is dependent on carotenoid ingestion and is thought to be correlated to overall quality (Kodric-Brown, 1989; Grether, 2000). I find that there are fewer cones expressing LWS genes than middle-wavelength or short-wavelength sensitive genes. This observation may explain why brighter, more highly saturated orange coloration in males is preferred by females in some populations (Endler, 1980; Endler, 1983; Houde, 1988; Kodric-Brown, 1989; Houde & Endler, 1990; Houde & Torio, 1992; Grether, 2000). Hypothetically, if females have low sensitivity to long-wavelength light then male color may be required to be bright and saturated in order for females to detect/evaluate that coloration. Given that carotenoid derived coloration is thought to be an honest signal, perhaps only the fittest males will be those able to produce and maintain orange coloration that is saturated and bright enough to stimulate females. This hypothesis is supported by mate choice experiments, which have found that females respond most strongly to males that possess a more-than-average amount of orange (Houde, 1987). Houde also found that there is a maximum level of orange coloration, above which there is no further increase in female mate preference (Houde, 1987), this could correlate to low sensitivity to long-wavelength light (i.e. once the orange color is bright enough to stimulate the LWS cones, there is no gain for being more orange).

An important caveat of this work is that the fish studied were reared in broad-spectrum light and that environmental plasticity to different spectral conditions will need to be taken into account to thoroughly evaluate hypotheses of sexual selection (i.e. the effect of experimental conditions on expression). Additionally, the background upon which these courtship interactions take place should also be taken into account; field tests of spectral conditions are imperative to further understanding of these interactions.

Spatial sub-functionalization of the retina.

The patterns of expression described in chapter 4 indicate that there may be sub-functionalization of the guppy retina. The dorsal retina expresses at least eight of the cone opsins, whereas the ventral retina expresses five and has a high density of cone cells expressing middle-wavelength sensitive opsins (green light). Previously collect MSP data first suggested these patterns of sensitivity (Levine & MacNichol Jr, 1979; Levine et al.,

1980; Levine & MacNichol, 1982). The dorsal sensitivity and discriminatory ability has been suggested to correlate to the courtship displays of the male below and in front of the female, projecting onto the dorsal retina (Levine & MacNichol, 1982). Where as the ventral retina might be used to detecting dark objects against the down-welling light (Levine & MacNichol, 1982). The theory behind this is that in order to maximize the contrast of objects against a background, fish should have both a pigment matched to the background and one offset from the background (i.e. where maximal absorption does not match the background and is of either a shorter or longer wavelength); a visual pigment matched to background would maximize the contrast for viewing dark objects and an offset pigment would maximize contrast when viewing bright objects (McFarland & Munz, 1975). With regard to the guppy, the prevalence of middle-wavelength genes for upward viewing fits the observation that many guppy environments are streams with thick rainforest cover, where the predominant wavelengths are middle-wavelengths (500-600nm) (Endler, 1993). Thus the short-wavelength (SWS2) pigments in the ventral retina would act as offset pigments and the middle-wavelength (RH2 pigments) would be matched to the background light.

Future directions

Another component of the sexual selection research in guppies pertinent to opsin expression involves the observation that female preference for male color varies among populations. Assuming color is the key target of female mate choice, it is possible that population level variation in female preference is driven by variation in opsin gene expression. Opsin expression data from other populations will be required to test this hypothesis in guppies. While I have focused on only one population, this study has set a baseline for comparison in future studies of opsin expression and has also provided the molecular tools to investigate a diversity of questions. Questions remain that can be resolved using *in situ* hybridization about how the rearing environment affects expression of opsins. Environmentally influenced or determined expression patterns have been previously investigated in the bluefin killifish (*Lucania goodei*) and the black bream (*Acanthopagrus butcheri*) (Fuller et al., 2005; Shand et al., 2008). These studies have demonstrated that some expression patterns are contingent on the local environmental

factors during fish rearing such as water color and the wavelengths of ambient light. However, it remains to be determined how common a phenomenon this is and whether those species had a genetic predisposition for environmentally driven modulation of opsin expression. Additionally, given that so many teleosts have demonstrated differential use of their opsin repertoire over ontogeny (e.g zebrafish, salmon, cichlids) (Takechi & Kawamura, 2005; Carleton et al., 2008; Temple et al., 2008b), future studies examining opsin expression over guppy ontogeny may reveal that developmental processes have also played a role in shaping the expression patterns of opsins in the guppy. In the future *in situ* hybridization should also be used to characterize the opsin expression pattern of guppy relatives such as *Jenynsia onca*, which will give insight into how phylogenetically labile heterogenous opsin gene expression is. *Jenynsia onca* would be a particularly interesting comparison due to the similarity of its repertoire to the guppy and its lack of sexually dimorphic coloration. This comparison could help to determine whether limited LWS expression plays a role in female mate choice; it could also help date the evolution of these expression patterns.

Another pivotal future direction of this work is behavioral tests. Without such studies it will be difficult to further interpret hypotheses of sexual selection and determine the guppy's visual capacity for color vision. Particularly, in terms of what effect heterogeneous opsin expression has on wavelength discrimination, which is contingent on the photoreceptor receptive field of ganglion cells. Optomotor response tests exploit an innate behavior to orient to a pattern of moving stripes; they have previously been used to test wavelength sensitivity in guppies, but can also be used to test wavelength discrimination (Anstis et al., 1998). Wavelength discrimination tests can also be used, whereby a fish is taught to associate a given wavelength of light with a reward (most often food) and is then tested to see if it can distinguish between distinct wavelengths of light (one associated with food and the other not) (Kelber et al., 2003). An example of this is seen in goldfish, where they were trained to discriminate between the three primary colors (red, blue and green) (Muntz & Cronly-Dillon, 1966). Lastly, the finding that LWS opsin expression appears to vary among individuals, something supported by previous MSP studies (Archer et al., 1987) merits future examination (greater replication) and needs to be explored using behavioral tests. These tests will be integral in the

assessment of the affect of variation at the functional level. As individual variation in LWS expression could affect the perception of male color and thus the evolution of female preference for that color.

Conclusions

Relatives of the guppy have similarly sized opsin repertoires. The opsin repertoire of the guppy contains one opsin (LWS A180) not found in the sister group of Poeciliidae, Anablepidae. Gene conversion has played a role in the divergence and diversification of LWS genes in livebearers. Tandem duplication appears to be the most prevalent mechanism of opsin duplication and these duplication events span the age of Teleostei. *In situ* hybridization experiments suggests that guppies may have spatially sub-functionalized retinas, which may allow them to simultaneously evaluate mates below them and dark objects above them. The observed patterns of opsin expression allow us to begin to evaluate models of sexual selection in this species from the perspective of opsin sensitivity. Future behavioral tests can now be designed with the expression data in mind. These tests will be integral in evaluating the wavelength sensitivity and discriminatory ability of the guppy.

Literature Cited

Ahlbert, I. B. (1969). The organization of the cone cells in the retinae of four teleosts with different feeding habits (*Perca fluviatilis L., Lucioperca lucioperca L., Acerina cernua L.* & *Coregonus albula L.*). Archive for Zoologic, 22, 445-481.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. Journal of Molecular Biology, 215, 403-410.

Applebury, M. L. (1994). Relationships of G-protein-coupled receptors. A survey with the photoreceptor opsin subfamily. Society of General Physiologists Series, 49, 235-248.

Applebury, M. L., Antoch, M. P., Baxter, L. C., Chun, L. L. Y., Falk, J. D. Farhangfar, F., Kage, K., Krzystolik, M. G., Lyass, L. A., & Tobbins, J. T. (2000). The murine cone photoreceptor: a single cone type expresses both S and M opsins with retinal spatial patterning. Neuron, 27, 513-523.

Archer, S. N., & Lythgoe, J. N. (1990). The visual pigment basis for cone polymorphism in the guppy, *Poecilia reticulata*. Vision Research, 30, 225-233.

Archer, S. N., Endler, J. A., Lythgoe, J. N., & Partridge, J. C. (1987). Visual pigment polymorphism in the guppy *Poecilia reticulata*. Vision Research, 27, 1243-1252.

Arrese, C. A., Beazley, L. D., Ferguson, M. C., Oddy, A., & Hunt, D. M. (2006). Spectral tuning of the long wavelength-sensitive cone pigment in four Australian marsupials. Gene, 381, 13-17.

Asenjo, A. B., Rim, J., & Oprian, D. D. (1994). Molecular determinants of human red/green color discrimination. Neuron, 12, 1131-1138.

Babu, K. R., Dukkipati, A., Birge, R. R., & Knox, B. E. (2001). Regulation of phototransduction in short-wavelength cone visual pigments via the retinylidene Schiff base counterion. Biochemistry, 40, 13760-13766.

Bailes, H. J., Davies, W. L., Trezise, A. E., & Collin, S. P. (2007). Visual pigments in a living fossil, the Australian lungfish *Neoceratodus forsteri*. BMC Evolutionary Biology, 7:200.

Bailey, R. M. (1963). The poeciliid fishes (Cyprinodontiformes): their structure, zoogeography, and systematics. Bulletin of the American Museum of Natural History 126: 1-176.

Beaudet, L., Novales Flamarique, I., & Hawryshyn, C. W. (1997). Cone photoreceptor topography in the retina of sexually mature Pacific salmonid fishes. The Journal of Comparative Neurology, 383, 49-59.

Bisazza, A., Facchin, L., Pignatti, R., & Vallortigara, G. (1998). Lateralization of detour behaviour in poeciliid fish: the effect of species, gender and sexual motivation. Behavioural Brain Research, 91, 157-164.

Bisazza, A., Pignatti, R., & Vallortigara, G. (1997). Laterality in detour behaviour: interspecific variation in poeciliid fish. Animal behaviour, 54, 1273-1281.

Bloedel, P. (1955). Hunting methods of fish-eating bats, particularly *Noctilio leporinus*. Journal of Mammalogy, 36, 390-399.

Bowmaker, J. K., & Hunt, D. M. (2006). Evolution of vertebrate visual pigments. Current Biology : CB, 16, R484-R489.

Bowmaker, J. K., & Knowles, A. (1977). The visual pigments and oil droplets of the chicken, *Gallus gallus*. Vision Research, 17, 755-764.

Bowmaker, J. K., & Kunz, Y. W. (1987). Ultraviolet receptors, tetrachromatic colour vision and retinal mosaics in the brown trout (*Salmo trutta*): age-dependent changes. Vision Research, 27, 2101-2108.

Breden, F., & Stoner, G. (1987). Male predation risk determines female preference in the Trinidad guppy. Nature. 329: 831-833.

Brigati, D. J., Myerson, D., Leary, J. J., Spalholz, B., Travis, S. Z., Fong, C. K., Hsiung, G. D., & Ward, D. C. (1983). Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. Virology, 126, 32-50.

Brosius, J. (1999). RNAs from all categories generate retrosequences that may be exapted as novel genes or regulatory elements. Gene, 238, 115-134.

Carleton, K. L., & Kocher, T. D. (2001). Cone opsin genes of african cichlid fishes: tuning spectral sensitivity by differential gene expression. Molecular Biology and Evolution, 18, 1540-1550.

Carleton, K. L., Parry, J. W., Bowmaker, J. K., Hunt, D. M., & Seehausen, O. (2005). Colour vision and speciation in Lake Victoria cichlids of the genus *Pundamilia*. Molecular Ecology, 14, 4341-4353.

Carleton, K. L., Spady, T. C., Streelman, J. T., Kidd, M. R., McFarland, W. N., & Loew, E. R. (2008). Visual sensitivities tuned by heterochronic shifts in opsin gene expression. BMC Biology, 6:22.

Chan, T., Lee, M., & Sakmar, T. P. (1992). Introduction of hydroxyl-bearing amino acids causes bathochromic spectral shifts in rhodopsin. Amino acid substitutions responsible for red-green color pigment spectral tuning. The Journal of Biological Chemistry, 267, 9478-9480.

Chen, J. M., Cooper, D. N., Chuzhanova, N., Férec, C., & Patrinos, G. P. (2007). Gene conversion: mechanisms, evolution and human disease. Nature Reviews Genetics, 8, 762-775.

Cheng, C. L., & Flamarique, I. N. (2007). Chromatic organization of cone photoreceptors in the retina of rainbow trout: single cones irreversibly switch from UV (SWS1) to blue (SWS2) light sensitive opsin during natural development. The Journal of Experimental Biology, 210, 4123-4135.

Cheng, C. L., Flamarique, I. N., Hárosi, F. I., Rickers-Haunerland, J., & Haunerland, N. H. (2006). Photoreceptor layer of salmonid fishes: transformation and loss of single cones in juvenile fish. The Journal of Comparative Neurology, 495, 213-235.

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

Chinen, A., Matsumoto, Y., & Kawamura, S. (2005a). Reconstitution of ancestral green visual pigments of zebrafish and molecular mechanism of their spectral differentiation. Molecular Biology and Evolution, 22, 1001-1010.

Chinen, A., Matsumoto, Y., & Kawamura, S. (2005b). Spectral differentiation of blue opsins between phylogenetically close but ecologically distant goldfish and zebrafish. The Journal of Biological Chemistry, 280, 9460-9466.

Cohen, A. (1972). Rods and cones. In: M. Fuortes (Eds.), Handbook of Sensory Physiology, Physiol. Receptor Organs. Berlin: Springer-Verlag. VII, 63-110.

Collin, S. P., & Trezise, A. E. (2004). The origins of colour vision in vertebrates. Clinical & experimental optometry: Journal of the Australian Optometrical Association, 87, 217-223.

Cottrill, P. B., Davies, W. L., Semo, M., Bowmaker, J. K., Hunt, D. M., & Jeffery, G. (2009). Developmental dynamics of cone photoreceptors in the eel. BMC Developmental Biology, 9, 71.

Cowing, J. A., Poopalasundaram, S., Wilkie, S. E., Robinson, P. R., Bowmaker, J. K., & Hunt, D. M. (2002). The molecular mechanism for the spectral shifts between vertebrate ultraviolet-and violet-sensitive cone visual pigments. Biochemical Journal, 367, 129-135.

Davies, W. L., Carvalho, L. S., Cowing, J. A., Beazley, L. D., Hunt, D. M., & Arrese, C. A. (2007). Visual pigments of the platypus: A novel route to mammalian colour vision. Current Biology, 17, R161-R163.

Davies, W. L., Collin, S. P., & Hunt, D. M. (2009). Adaptive gene loss reflects differences in the visual ecology of basal vertebrates. Molecular Biology and Evolution, 26, 1803-1809.

Davies, W. L., Cowing, J. A., Carvalho, L. S., Potter, I. C., Trezise, A. E., Hunt, D. M., & Collin, S. P. (2007). Functional characterization, tuning, and regulation of visual pigment gene expression in an anadromous lamprey. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 21, 2713-2724.

Eakin, R. M., & Westfall, J. A. (1962). Fine Structure Of Photoreceptors In the Hydromedusan, *Polyorchis penicillatus*. Proceedings of the National Academy of Sciences of the United States of America, 48, 826-833.

Eakin, R.M. (1965). Evolution of photoreceptors. Cold Spring Harbour Symposia on Quantitative Biology 30: 363-370.

Ebrey, T. G., & Takahashi, Y. (2002). Photobiology of Retinal Proteins. Photobiology for the 21st Century. Coohill T, Valenzeno D (Eds.). Overland Park, Kansas: Valdenmar. 101-133.

Endler, J. A. (1978). A predator's view of animal color patterns. Evol. Biol, 11, 319-364.

Endler, J. A. (1980). Natural selection on color patterns in *Poecilia reticulata*. Evolution; International Journal of Organic Evolution, 34, 76-91.

Endler, J. A. (1983). Natural and sexual selection on color patterns in poeciliid fishes. Environmental Biology of Fishes, 9, 173-190.

Endler, J. A. (1993). The color of light in forests and its implications. Ecological Monographs, 63, 2-27.

Engstrom, K., & Ahlbert, I. B. (1963). Cone types and cone arrangement in the retina of some flatfishes. Acta Zoologica, 44, 119-129.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. Journal of Molecular Evolution, 17, 368-376.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution; International Journal of Organic Evolution, 39, 783-791.

Fisher, S. R. A., & Bennett, J. H. (1999). The genetical theory of natural selection: a complete variorum edition. Oxford University Press, USA.

Forster, A. C., McInnes, J. L., Skingle, D. C., & Symons, R. H. (1985). Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. Nucleic Acids Research, 13, 745-761.

Fox, D. L. (1979). Biochromy, natural coloration of living things. Berkeley: University of California Press.

Fuller, R. C., Carleton, K. L., Fadool, J. M., Spady, T. C., & Travis, J. (2005). Genetic and environmental variation in the visual properties of bluefin killifish, *Lucania goodei*. Journal of Evolutionary Biology, 18, 516-523.

Gall, J. G., & Pardue, M. L. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proceedings of the National Academy of Sciences of the United States of America, 63, 378-383.

Gerhard, D. S., Kawasaki, E. S., Bancroft, F. C., & Szabo, P. (1981). Localization of a unique gene by direct hybridization *in situ*. Proceedings of the National Academy of Sciences of the United States of America, 78, 3755-3759.

Geuskens, M., & May, E. (1974). Ultrastructural localization of SV40 viral DNA in cells, during lytic infection, by *in situ* molecular hybridization. Exp Cell Res, 87, 175-185.

Godin, J. G. J., & McDonough, H. E. (2003). Predator preference for brightly colored males in the guppy: a viability cost for a sexually selected trait. Behavioral Ecology, 14, 194-200.

Gojobori, J., & Innan, H. (2009). Potential of fish opsin gene duplications to evolve new adaptive functions. Trends in Genetics, 25, 198-202.

Gowans, E. J., Burrell, C. J., Jilbert, A. R., & Marmion, B. P. (1981). Detection of hepatitis B virus DNA sequences in infected hepatocytes by *in situ* cytohybridisation. Journal of Medical Virology, 8, 67-78.

Grether, G. F. (2000). Carotenoid limitation and mate preference evolution: a test of the indicator hypothesis in guppies (*Poecilia reticulata*). Evolution; International Journal of Organic Evolution, 54, 1712-1724.

Grether, G. F., Hudon, J., & Millie, D. F. (1999). Carotenoid limitation of sexual coloration along an environmental gradient in guppies. Proceedings of the Royal Society B: Biological Sciences, 266, 1317-1322.

Hall, T. (2001). BioEdit version 5.0. 6. North Carolina State University, Department of Microbiology, Raleigh, North Carolina.

Hart, N. S., Lisney, T. J., & Collin, S. P. (2006). Cone photoreceptor oil droplet pigmentation is affected by ambient light intensity. The Journal of Experimental Biology, 209, 4776-4787.

Haskins, C. P., & Haskins, E. F. (1949). The role of sexual selection as an isolating mechanism in three species of poeciliid fishes. Evolution; International Journal of Organic Evolution, 3, 160-169.

Haskins, C. P., Haskins, E. F., McLaughlin, J. J. A., & Hewitt, R. E. (1961). Polymorphism and population structure in *Lebistes reticulatus*, an ecological study. Vertebrate Speciation (Ed. W.F. Blair), University of Texas Press, Austin, 320-395.

Hárosi, F. I. (1994). An analysis of two spectral properties of vertebrate visual pigments. Vision Research, 34, 1359-1367.

Helvik, J. V., Drivenes, O., Naess, T. H., Fjose, A., & Seo, H. C. (2001). Molecular cloning and characterization of five opsin genes from the marine flatfish Atlantic halibut (*Hippoglossus hippoglossus*). Visual Neuroscience, 18, 767-780.

Herrington, C. S., Burns, J., Graham, A. K., Evans, M., & McGee, J. O. (1989). Interphase cytogenetics using biotin and digoxigenin labelled probes I: relative sensitivity of both reporter molecules for detection of HPV16 in CaSki cells. Journal of Clincal Pathology, 42, 592-600.

Hisatomi, O., Satoh, T., & Tokunaga, F. (1997). The primary structure and distribution of killifish visual pigments. Vision Research, 37, 3089-3096.

Hisatomi, O., Satoh, T., Barthel, L. K., Stenkamp, D. L., Raymond, P. A., & Tokunaga, F. (1996). Molecular cloning and characterization of the putative ultraviolet-sensitive visual pigment of goldfish. Vision Research, 36, 933-939.

Hoegg, S., Brinkmann, H., Taylor, J. S., & Meyer, A. (2004). Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. Journal of Molecular Evolution, 59, 190-203.

Hoffmann, M., Tripathi, N., Henz, S. R., Lindholm, A. K., Weigel, D., Breden, F., & Dreyer, C. (2007). Opsin gene duplication and diversification in the guppy, a model for sexual selection. Proceedings of the Royal Society of London Series B: Biological Sciences, 274, 33-42.

Hofmann, C. M., & Carleton, K. L. (2009). Gene duplication and differential gene expression play an important role in the diversification of visual pigments in fish. Integrative and Comparative Biology, 49, 630-643.

Houde, A. E. (1987). Mate choice based upon naturally occurring color-pattern variation in a guppy population. Evolution; International Journal of Organic Evolution, 41, 1-10.

Houde, A. E. (1988). Genetic difference in female choice between two guppy populations. Animal Behaviour, 36, 510-516.

Houde, A. E. (1992). Sex-linked heritability of a sexually selected character in a natural population of *Poecilia reticulata* (Pisces: Poeciliidae)(guppies). Heredity, 69, 229-235.

Houde, A. E., & Endler, J. A. (1990). Correlated Evolution of Female Mating Preferences and Male Color Patterns in the Guppy *Poecilia reticulata*. Science, 248, 1405-1408.

Houde, A. E., & Torio, A. J. (1992). Effect of parasitic infection on male color pattern and female choice in guppies. Behavioral Ecology, 3, 346.

Hrbek, T., Seckinger, J., & Meyer, A. (2007). A phylogenetic and biogeographic perspective on the evolution of poeciliid fishes. Molecular Phylogenetics and Evolution, 43, 986-998.

Hu, X., & Worton, R. G. (1992). Partial gene duplication as a cause of human disease. Human Mutation, 1, 3-12.

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 of the Royal Society of London Series B: Biological Sciences, 274, 489-498.

Ibbotson, R. E., Hunt, D. M., Bowmaker, J. K., & Mollon, J. D. (1992). Sequence divergence and copy number of the middle- and long-wave photopigment genes in Old World monkeys. Proceedings of the Royal Society of London Series B: Biological Sciences, 247, 145-154.

Jacobs, G. H. (1993). The distribution and nature of colour vision among the mammals. Biological Reviews of the Cambridge Philosophical Society, 68, 413-471.

Jacobs, G. H., & Rowe, M. P. (2004). Evolution of vertebrate colour vision. Clinical and Experimental Optometry, 87, 206-216.

Jacobs, G. H., Deegan, J. F., & Neitz, J. (1998). Photopigment basis for dichromatic color vision in cows, goats, and sheep. Visual Neuroscience, 15, 581-584.

Jacobs, G. H., Neitz, M., Deegan, J. F., & Neitz, J. (1996). Trichromatic colour vision in New World monkeys. Nature, 382, 156-158.

Janz, J. M., & Farrens, D. L. (2001). Engineering a functional blue-wavelength-shifted rhodopsin mutant. Biochemistry, 40, 7219-7227.

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.

Jukes, T. H., & Cantor, C. R. (1969). Evolution of protein molecules. Mammalian Protein Metabolism, 3, 21-132.

Kasai, A., & Oshima, N. (2006). Light-sensitive motile iridophores and visual pigments in the neon tetra, Paracheirodon innesi. Zoological Science, 23, 815-819.

Kawamura, S., & Yokoyama, S. (1993). Molecular characterization of the red visual pigment gene of the American chameleon (*Anolis carolinensis*). FEBS Letters, 323, 247-251.

Kawamura, S., & Yokoyama, S. (1995). Paralogous origin of the rhodopsinlike opsin genes in lizards. Journal of Molecular Evolution, 40, 594-600.

Kawamura, S., & Yokoyama, S. (1996). Phylogenetic relationships among short wavelength-sensitive opsins of American chameleon (*Anolis carolinensis*) and other vertebrates. Vision research, 36, 2797-2804.

Kelber, A., Vorobyev, M., & Osorio, D. (2003). Animal colour vision--behavioural tests and physiological concepts. Biological reviews of the Cambridge Philosophical Society, 78, 81-118.

Kodric-Brown, A. (1989). Dietary carotenoids and male mating success in the guppy: an environmental component to female choice. Behavioral Ecology and Sociobiology, 25, 393-401.

Kolakowski, L. F. (1994). GCRDb: a G-protein-coupled receptor database. Receptors & Channels, 2, 1-7.

Kolb, H. (2003). How the Retina Works Much of the construction of an image takes place in the retina itself through the use of specialized neural circuits. American Scientist, 91, 28-35.

Kunz, Y. W., Wildenburg, G., Goodrich, L., & Callaghan, E. (1994). The fate of ultraviolet receptors in the retina of the Atlantic salmon (*Salmo salar*). Vision research, 34, 1375-1383.

Lamb, T. D. (2009). Evolution of vertebrate retinal photoreception. Philosophical transactions of the Royal Society of London. Series B: Biological Sciences, 364, 2911-2924.

Lande, R. (1981). Models of speciation by sexual selection on polygenic traits. Proceedings of the National Academy of Sciences of the United States of America, 78, 3721-3725.

Langer-Safer, P. R., Levine, M., & Ward, D. C. (1982). Immunological method for mapping genes on *Drosophila* polytene chromosomes. Proceedings of the National Academy of Sciences of the United States of America, 79, 4381-4385.

Larhammar, D., Nordström, K., & Larsson, T. A. (2009). Evolution of vertebrate rod and cone phototransduction genes. Philosophical transactions of the Royal Society of London Series B: Biological Sciences, 364, 2867-2880.

Levine, J. S., & MacNichol Jr, E. F. (1979). Visual pigments in teleost fishes: Effects of habitat, microhabitat, and behavior on visual system evolution. Sensory Process, 3, 95-131.

Levine, J. S., & MacNichol, E. F. (1982). Color vision in fishes. Scientific American, 246, 140-149.

Levine, J. S., Lobel, P. S., & MacNichol, E. f. F. (1980). Visual Communication in Fishes. (Ed. Ali M. A), Environmental physiology of fishes, New York: Plenum Press, 447-475.

Li, W. H. (1993). Unbiased estimation of the rates of synonymous and nonsynonymous substitution. Journal of Molecular Evolution, 36, 96-99.

Li, Z., Gan, X., & He, S. (2009). Distinct evolutionary patterns between two duplicated color vision genes within cyprinid fishes. Journal of Molecular Evolution, 69, 346-359.

Liao, D. (1999). Concerted evolution: molecular mechanism and biological implications. American Journal of Human Genetics, 64, 24-30.

Lucinda, P. H. F., Reis, R. E., & Quevedo, R. (2002). *Jenynsia onca*, a new species of anablepid fish (Teleostei: Cyprinodontiformes) from southern Brazil and its phylogenetic position. Ichthyological Exploration of Freshwaters, 13, 33-40.

Lukáts, A., Dkhissi-Benyahya, O., Szepessy, Z., Röhlich, P., Vígh, B., Bennett, N. C., Cooper, H. M., & Szél, A. (2002). Visual pigment coexpression in all cones of two rodents, the Siberian hamster, and the pouched mouse. Investigative Ophthalmology & Visual Science, 43, 2468-2473.

Lyall, A. H. (1957). Cone arrangements in teleost retinae. Journal of Cell Science, 3, 189.

Lynch, M., & Conery, J. S. (2000). The evolutionary fate and consequences of duplicate genes. Science, 290, 1151.

Mader, M. M., & Cameron, D. A. (2004). Photoreceptor differentiation during retinal development, growth, and regeneration in a metamorphic vertebrate. The Journal of neuroscience : the official journal of the Society for Neuroscience, 24, 11463-11472.

Marques, A. C., Dupanloup, I., Vinckenbosch, N., Reymond, A., & Kaessmann, H. (2005). Emergence of young human genes after a burst of retroposition in primates. PLoS biology, 3, e357.

Martin, V. J. (2002). Photoreceptors of cnidarians. Canadian Journal of Zoology, 80, 1703-1722.

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

McFarland, W. N., & Munz, F. W. (1975). Part III: The evolution of photopic visual pigments in fishes. Vision Research, 15, 1071-1080.

Minamoto, T., & Shimizu, I. (2005). Molecular cloning of cone opsin genes and their expression in the retina of a smelt, Ayu (*Plecoglossus altivelis*, Teleostei). Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology, 140, 197-205.

Muntz, W. R. A., & Cronly-Dillon, J. R. (1966). Colour discrimination in goldfish. Animal Behaviour, 14, 351-355.

Nathans, J. (1990). Determinants of visual pigment absorbance: role of charged amino acids in the putative transmembrane segments. Biochemistry, 29, 937-942.

Nathans, J., Piantanida, T. P., Eddy, R. L., Shows, T. B., & Hogness, D. S. (1986a). Molecular genetics of inherited variation in human color vision. Science, 232, 203-210.

Nathans, J., Thomas, D., & Hogness, D. S. (1986b). Molecular genetics of human color vision: the genes encoding blue, green, and red pigments. Science, 232, 193-202.

Neafsey, D. E., & Hartl, D. L. (2005). Convergent loss of an anciently duplicated, functionally divergent RH2 opsin gene in the fugu and Tetraodon pufferfish lineages. Gene, 350, 161-171.

Near, T. J., & Miyab, M. (2009). Ray-finned fishes (Actinopterygii). The Time Tree of Life, 328.

Nelson, J. S. (2006). Fishes of the world. New York, NY: Wiley.

Nicoletto, P. F. (1991). The relationship between male ornamentation and swimming performance in the guppy, *Poecilia reticulata.* Behavioral Ecology and Sociobiology, 28, 365-370.

Niimura, Y., & Nei, M. (2003). Evolution of olfactory receptor genes in the human genome. Proceedings of the National Academy of Sciences of the United States of America, 100, 12235-12240.

Ohno, S. (1970). Evolution by gene duplication. Berlin: Springer.

Okamura, K., & Nakai, K. (2008). Retrotransposition as a source of new promoters. Molecular Biology and Evolution, 25, 1231-1238.

Okano, T., Kojima, D., Fukada, Y., Shichida, Y., & Yoshizawa, T. (1992). Primary structures of chicken cone visual pigments: vertebrate rhodopsins have evolved out of cone visual pigments. Proceedings of the National Academy of Sciences of the United States of America, 89, 5932-5936.

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., Trong, I. L., Teller, D. C., Okada, T., Stenkamp, R. E., & others (2000). Crystal structure of rhodopsin: AG protein-coupled receptor. Science, 289, 739-745.

Parry, J. W., & Bowmaker, J. K. (2000). Visual pigment reconstitution in intact goldfish retina using synthetic retinaldehyde isomers. Vision Research, 40, 2241-2247.

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 : CB, 15, 1734-1739.

Parry, J. W., Poopalasundaram, S., Bowmaker, J. K., & Hunt, D. M. (2004). A novel amino acid substitution is responsible for spectral tuning in a rodent violet-sensitive visual pigment. Biochemistry, 43, 8014-8020.

Partridge, J. C. (1989). The visual ecology of avian cone oil droplets. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 165, 415-426.

Pignatelli, V., Champ, C., Marshall, J., & Vorobyev, M. (2010). Double cones are used for colour discimination in the reef fish, *Rhinecanthus aculeatus*.

Pointer, M. A., Cheng, C. H., Bowmaker, J. K., Parry, J. W., Soto, N., Jeffery, G., Cowing, J. A., & Hunt, D. M. (2005). Adaptations to an extreme environment: retinal organisation and spectral properties of photoreceptors in Antarctic notothenioid fish. The Journal of experimental biology, 208, 2363-2376.

Posada, D., & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. Bioinformatics (Oxford, England), 14, 817-818.

Postlethwait, J. H., Woods, I. G., Ngo-Hazelett, P., Yan, Y. L., Kelly, P. D., Chu, F., Huang, H., Hill-Force, A., & Talbot, W. S. (2000). Zebrafish comparative genomics and the origins of vertebrate chromosomes. Genome research, 10, 1890-1902.

Reyniers, E., Van Thienen, M. N., Meire, F., De Boulle, K., Devries, K., Kestelijn, P., & Willems, P. J. (1995). Gene conversion between red and defective green opsin gene in blue cone monochromacy. Genomics, 29, 323-328.

Reznick, D., & Endler, J. A. (1982). The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). Evolution; International Journal of Organic Evolution, 36, 160-177.

Robertson, H. M. (1998). Two large families of chemoreceptor genes in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae* reveal extensive gene duplication, diversification, movement, and intron loss. Genome Research, 8, 449-463.

Rodd, F. H., Hughes, K. A., Grether, G. F., & Baril, C. T. (2002). A possible non-sexual origin of mate preference: are male guppies mimicking fruit? Proceedings of the Royal Society of London Series B: Biological Sciences, 269, 475-481.

Rozen, S., Skaletsky, H., Marszalek, J. D., Minx, P. J., Cordum, H. S., Waterston, R. H., Wilson, R. K., & Page, D. C. (2003). Abundant gene conversion between arms of palindromes in human and ape Y chromosomes. Nature, 423, 873-876.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4, 406-425.

Sawyer, S. A. (1999). GENECONV: a computer package for the statistical detection of gene conversion. Department of Mathematics, Washington University, St. Louis, Missouri.

Seehausen, O., Terai, Y., Magalhaes, I. S., Carleton, K. L., Mrosso, H. D., Miyagi, R., van der Sluijs, I., Schneider, M. V., Maan, M. E., Tachida, H., Imai, H., & Okada, N. (2008). Speciation through sensory drive in cichlid fish. Nature, 455, 620-626.

Seghers, B. H. (1974). Geographic variation in the responses of guppies (*Poecilia reticulata*) to aerial predators. Oecologia, 14, 93-98.

Shand, J., Davies, W. L., Thomas, N., Balmer, L., Cowing, J. A., Pointer, M., Carvalho, L. S., Trezise, A. E. O., Collin, S. P., Beazley, L. D., & others (2008). The influence of ontogeny and light environment on the expression of visual pigment opsins in the retina of the black bream, *Acanthopagrus butcheri.* Journal of Experimental Biology, 211, 1495-1503.

Shand, J., Partridge, J. C., Archer, S. N., Potts, G. W., & Lythgoe, J. N. (1988). Spectral absorbance changes in the violet/blue sensitive cones of the juvenile pollack, Pollachius pollachius. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 163, 699-703.

Shi, Y., Radlwimmer, F. B., & Yokoyama, S. (2001). Molecular genetics and the evolution of ultraviolet vision in vertebrates. Proceedings of the National Academy of Sciences of the United States of America, 98, 11731-11736.

Simerly, R. B., Chang, C., Muramatsu, M., & Swanson, L. W. (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an *in situ* hybridization study. The Journal of comparative neurology, 294, 76-95.

Sison-Mangus, M. P., Bernard, G. D., Lampel, J., & Briscoe, A. D. (2006). Beauty in the eye of the beholder: the two blue opsins of lycaenid butterflies and the opsin gene-driven evolution of sexually dimorphic eyes. The Journal of experimental biology, 209, 3079-3090.

Soares, M. B., Schon, E., Henderson, A., Karathanasis, S. K., Cate, R., Zeitlin, S., Chirgwin, J., & Efstratiadis, A. (1985). RNA-mediated gene duplication: the rat preproinsulin I gene is a functional retroposon. Mol Cell Biol, 5, 2090-2103.

Spady, T.C. (2006). Cichlids as a model for the evolution of visual sensitivity. Thesis University of New Hampshire, Durham New Hampshire.

Spady, T. C., Parry, J. W., Robinson, P. R., Hunt, D. M., Bowmaker, J. K., & Carleton, K. L. (2006). Evolution of the cichlid visual palette through ontogenetic subfunctionalization of the opsin gene arrays. Molecular Biology and Evolution, 23, 1538-1547.

Stenkamp, D. L., Hisatomi, O., Barthel, L. K., Tokunaga, F., & Raymond, P. A. (1996). Temporal expression of rod and cone opsins in embryonic goldfish retina predicts the spatial organization of the cone mosaic. Investigative Ophthalmology & Visual Science, 37, 363-376.

Stoner, G., & Breden, F. (1988). Phenotypic differentiation in female preference related to geographic variation in male predation risk in the Trinidad guppy (*Poecilia reticulata*). Behavioral Ecology and Sociobiology, 22, 285-291.

Strachan, J., Chang, L. Y., Wakefield, M. J., Graves, J. A., & Deeb, S. S. (2004). Cone visual pigments of the Australian marsupials, the stripe-faced and fat-tailed dunnarts: sequence and inferred spectral properties. Visual Neuroscience, 21, 223-229.

Sturtevant, A. H. (1925). The Effects of Unequal Crossing over at the Bar Locus in Drosophila. Genetics, 10, 117-147.

Sun, H., Macke, J. P., & Nathans, J. (1997). Mechanisms of spectral tuning in the mouse green cone pigment. Proceedings of the National Academy of Sciences of the United States of America, 94, 8860-8865.

Swofford, D. L. (2002). PAUP*, version 4b10 [computer program]. Sunderland (Massachusetts): Sinauer Associates.

Tada, T., Altun, A., & Yokoyama, S. (2009). Evolutionary replacement of UV vision by violet vision in fish. Proceedings of the National Academy of Sciences of the United States of America, 106, 17457-17462.

Takahashi, Y., & Ebrey, T. G. (2003). Molecular basis of spectral tuning in the newt short wavelength sensitive visual pigment. Biochemistry, 42, 6025-6034.

Takahashi, Y., & Yokoyama, S. (2005). Genetic basis of spectral tuning in the violet-sensitive visual pigment of African clawed frog, *Xenopus laevis*. Genetics, 171, 1153-1160.

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.

Takenaka, N., & Yokoyama, S. (2007). Mechanisms of spectral tuning in the RH2 pigments of Tokay gecko and American chameleon. Gene, 399, 26-32.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24, 1596-1599.

Taylor, J. S., & Raes, J. (2004). Duplication and divergence: the evolution of new genes and old ideas. Annual Review of Genetics, 38, 615-643.

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.

Temple, S. E., Plate, E. M., Ramsden, S., Haimberger, T. J., Roth, W. M., & Hawryshyn, C. W. (2006). Seasonal cycle in vitamin A1/A2-based visual pigment composition during the life history of coho salmon (*Oncorhynchus kisutch*). Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology, 192, 301-313.

Temple, S. E., Ramsden, S. D., Haimberger, T. J., Veldhoen, K. M., Veldhoen, N. J., Carter, N. L., Roth, W. M., & Hawryshyn, C. W. (2008a). Effects of exogenous thyroid hormones on visual pigment composition in coho salmon (*Oncorhynchus kisutch*). The Journal of Experimental Biology, 211, 2134-2143.

Temple, S. E., Veldhoen, K. M., Phelan, J. T., Veldhoen, N. J., & Hawryshyn, C. W. (2008b). Ontogenetic changes in photoreceptor opsin gene expression in coho salmon (*Oncorhynchus kisutch,* Walbaum). The Journal of Experimental Biology, 211, 3879-3888.

Templeton, C. N., & Shriner, W.M. (2004). Multiple selection pressures influence Trinidadian guppy (*Poecilia reticulata*) antipredator behavior. Behavioral Ecology, 15(4), 673-678.

Tsutsumi, M., Ikeyama, K., Denda, S., Nakanishi, J., Fuziwara, S., Aoki, H., & Denda, M. (2009). Expressions of rod and cone photoreceptor-like proteins in human epidermis. Experimental Dermatology, 18, 567-570.

Vallortigara, G., Rogers, L. J., & Bisazza, A. (1999). Possible evolutionary origins of cognitive brain lateralization. Brain Research Review, 30, 164-175.

Vihtelic, T. S., Doro, C. J., & Hyde, D. R. (1999). Cloning and characterization of six zebrafish photoreceptor opsin cDNAs and immunolocalization of their corresponding proteins. Visual Neuroscience, 16, 571-585.

Wakefield, M. J., Anderson, M., Chang, E., Wei, K. J., Kaul, R., Graves, J. A., Grützner, F., & Deeb, S. S. (2008). Cone visual pigments of monotremes: filling the phylogenetic gap. Visual Neuroscience, 25, 257-264.

Walls, G. L. (1963). The vertebrate eye. Hafner, New York,

Wang, D., Oakley, T., Mower, J., Shimmin, L. C., Yim, S., Honeycutt, R. L., Tsao, H., & Li, W. H. (2004). Molecular evolution of bat color vision genes. Molecular Biology and Evolution, 21, 295-302.

Wang, F. Y., Chung, W. S., Yan, H. Y., & Tzeng, C. S. (2008). Adaptive evolution of cone opsin genes in two colorful cyprinids, *Opsariichthys pachycephalus* and *Candidia barbatus*. Vision Research, 48, 1695-1704.

Wang, F. Y., Yan, H. Y., Chen, J. S., Wang, T. Y., & Wang, D. (2009). Adaptation of visual spectra and opsin genes in seabreams. Vision Research, 49, 1860-1868.

Wang, Z., Dong, X., Ding, G., & Li, Y. (2010). Comparing the retention mechanisms of tandem duplicates and retrogenes in human and mouse genomes. Genetics Selection Evolution, 42:24.

Ward, M. N., Churcher, A. M., Dick, K. J., Laver, C. R., Owens, G. L., Polack, M. D., Ward, P. R., Breden, F., & Taylor, J. S. (2008). The molecular basis of color vision in colorful fish: four long wave-sensitive (LWS) opsins in guppies (*Poecilia reticulata*) are defined by amino acid substitutions at key functional sites. BMC Evolutionary Biology, 8:210.

Watson, C. T., Lubieniecki, K. P., Loew, E., Davidson, W. S., & Breden, F. (2010). Genomic organization of duplicated short wave-sensitive and long wave-sensitive

opsin genes in the green swordtail, *Xiphophorus helleri*. BMC Evolutionary Biology, 10: 87.

Weadick, C. J., & Chang, B. S. (2007). Long-wavelength sensitive visual pigments of the guppy (*Poecilia reticulata*): six opsins expressed in a single individual. BMC Evolutionary Biology, 7 Suppl 1, Spp11.

Winderickx, J., Battisti, L., Hibiya, Y., Motulsky, A. G., & Deeb, S. S. (1993). Haplotype diversity in the human red and green opsin genes: evidence for frequent sequence exchange in exon 3. Human Molecular Genetics, 2, 1413-1421.

Windsor, D. J., & Owens, G. L. (2009). The opsin repertoire of *Jenynsia onca*: a new perspective on gene duplication and divergence in livebearers. BMC Research Notes, 2:159.

Winge, O., & Ditlevsen, E. (1947). Colour inheritance and sex determination in Lebistes. Heredity, 1, 65-83.

Worth, C. B. (1967). A naturalist in Trinidad. Lippincott Company, Philadelphia Pennsylvania.

Yokoyama, R., & Yokoyama, S. (1990). Convergent evolution of the red- and green-like visual pigment genes in fish, *Astyanax fasciatus*, and human. Proceedings of the National Academy of Sciences of the United States of America, 87, 9315-9318.

Yokoyama, S. (1994). Gene duplications and evolution of the short wavelength-sensitive visual pigments in vertebrates. Molecular Biology and Evolution, 11, 32-39.

Yokoyama, S. (1995). Amino acid replacements and wavelength absorption of visual pigments in vertebrates. Molecular Biology and Evolution, 12, 53-56.

Yokoyama, S. (2000). Molecular evolution of vertebrate visual pigments. Progress in Retinal and Eye Research, 19, 385-420.

Yokoyama, S. (2002). Molecular evolution of color vision in vertebrates. Gene, 300, 69-78.

Yokoyama, S. (2008). Evolution of dim-light and color vision pigments. Annual Review of Genomics and Human Genetics, 9, 259-282.

Yokoyama, S., & Radlwimmer, F. B. (1998). The "five-sites" rule and the evolution of red and green color vision in mammals. Molecular Biology and Evolution, 15, 560-567.

Yokoyama, S., & Radlwimmer, F. B. (1999). The molecular genetics of red and green color vision in mammals. Genetics, 153, 919-932.

Yokoyama, S., & Tada, T. (2000). Adaptive evolution of the African and Indonesian coelacanths to deep-sea environments. Gene, 261, 35-42.

Yokoyama, S., & Tada, T. (2003). The spectral tuning in the short wavelength-sensitive type 2 pigments. Gene, 306, 91-98.

Yokoyama, S., & Tada, T. (2010). Evolutionary dynamics of rhodopsin type 2 opsins in vertebrates. Molecular Biology and Evolution, 27, 133-141.

Yokoyama, S., Tada, T., Zhang, H., & Britt, L. (2008). Elucidation of phenotypic adaptations: Molecular analyses of dim-light vision proteins in vertebrates. Proceedings of the National Academy of Sciences of the United States of America, 105, 13480-13485.

Yokoyama, S., Takenaka, N., & Blow, N. (2007). A novel spectral tuning in the short wavelength-sensitive (SWS1 and SWS2) pigments of bluefin killifish (*Lucania goodei*). Gene, 396, 196-202.

Yokoyama, S., Zhang, H., Radlwimmer, F. B., & Blow, N. S. (1999). Adaptive evolution of color vision of the Comoran coelacanth (*Latimeria chalumnae*). Proceedings of the National Academy of Sciences of the United States of America, 96, 6279-6284.

Zahavi, A. (1975). Mate selection--a selection for a handicap. Journal of Theoretical Biology, 53, 205-214.

Appendix 1
Accession Numbers Used in *Jenynsia onca* Phylogenetic Analysis (Chapter 2).

Scientific names	Gene name	Accession number
J. onca	LWS S180	GQ221677
	LWS P180	GQ221676
	LWS S180r	GQ221671
	SWS1	GQ221672
	SWS2A	GQ221673
	SWS2B	GQ221674
	RH2-1	GQ221668
	RH2-2	GQ221669
	RH1	GQ221670
A. anableps	LWS S180α	FJ11154
	LWS S180β	FJ11158
	LWS S180γ	FJ11157
	LWS S180r	FJ11155
	SWS1	FJ11153
	SWS2A	FJ11152
	SWS2B	FJ11151
	RH2-1	FJ11149
	RH2-2	FJ11150
	RH1	FJ11156
P. reticulata	LWS S180	EU329434
	LWS A180	EU329442
	LWS P180	EU329456
	LWS S180r	EU329457
	SWS1	DQ234861
	SWS2A	FJ11159
	SWS2B	DQ234860
	RH2-1	DQ234859
	RH2-2	DQ234858
	RH1	DQ912024
X. pygmaeus	LWS S180	EU329481
	LWS P180	EU329478
	LWS S180r	EU329479
L. goodei	LWS-1	AY296740
	LWS-2	AY296741
	SWS1	AY296735
	SWS2A	AY296737
	SWS2B	AY296736

	RH2-1	AY296739
	RH1	AY296737
O. latipes	LWS-1	AB223051
	LWS-2	AB223052
	SWS1	AB223058
	SWS2A	AB223056
	SWS2B	AB223057
	RH2a	AB223053
	RH2b	AB223054
	RH2c	AB223055
	RH1	AB180742
D. rerio	LWS-1	NM131175
	LWS-2	NM001002443
	SWS1	BC060894
	SWS2	NM131192
	RH2-1	NM131253
	RH2-2	NM182891
	RH2-3	NM182892
	RH2-4	NM131254
	RH1	BC05288
P. bifurca	S180	EU329460
	P180	EU329465
P. picta	S180	EU329473
	P180	EU329476
P. parae	S180	EU329468
	P180	EU329470
T. gracilis	S180	EU329482

Appendix 2
Amino Acid Key Site Haplotypes and LWS Maximal Absorption Predictions for *Jenynsia onca* and Relatives.

Key site haplotypes for *J. onca*, *A. anableps*, *P. reticulata* and *L. goodie* [Accession numbers are listed in Appendix 1]. Amino acid site numbering is based on human LWS (Nathans et al., 1986b). Maximal absorption values estimated from mutational analysis of other LWS opsins (Yokoyama, 2008; Davies et al., 2009). An 'X' indicates that the sequence obtained in our PCR survey did not encompass the respective site.

Species	Gene	Amino acid key site					Predicted λ_{max}
		180	197	277	285	308	
Consensus		S	H	Y	T	A	
J. onca	LWS S180	-	-	-	-	-	560
	LWS P180	P	-	F	A	-	513
	LWS S180r	-	-	-	-	-	560
A. Anableps	LWS S180 α/β	-	-	-	-	-	560
	LWS S180 γ	-	-	-	A	-	545
	LWS S180r	-	-	-	-	-	560
P. reticulata	LWS S180	-	-	-	-	-	560
	LWS P180	P	-	F	A	-	513
	LWS S180r	-	-	-	-	-	560
	LWS A180	A	-	-	-	-	558
L. goodei	LWS-1	-	-	-	-	-	560
	LWS-2	-	-	-	-	-	560

Species	Gene	Amino acid key site										
		99	112	118	138	199	210	211	269	277	305	308	333
Consensus		D	Y	Y	E	M	R	A	M	F	T	A	M
J. onca	RH1	X	-	-	-	-	-	-	-	-	-	-	-
A. anableps	RH1	-	-	-	-	-	-	-	-	-	-	-	-
P. reticulata	RH1	-	-	-	-	-	-	-	-	-	-	-	-
L. goodei	RH1	-	-	-	-	-	-	-	-	-	-	-	-

Species	Gene	Amino acid key site									Gene	Amino acid key site								
		65	68	99	102	113	138	180	223	308		65	68	99	102	113	138	180	223	306
Consensus		C	T	G	M	S	E	A	M	A		I	L	G	M	S	Q	A	L	A
J. onca	RH2-1	-	-	-	-	-	-	-	-	-	RH2-2	V	-	-	-	-	-	-	-	-
A. anableps	RH2-1	X	-	-	-	-	-	-	-	-	RH2-2	-	-	-	-	-	-	-	-	-
P. reticulata	RH2-1	-	-	-	-	-	-	-	-	-	RH2-2	-	F	-	-	-	-	-	-	-
L. goodei	RH2-1	-	-	-	-	-	-	-	-	-										

Species	Gene	Amino acid key site												
		62	65	68	102	106	107	109	113	125	129	130	132	134
Consensus		F	F	T	F	S	V	Q	A	L	E	A	M	A
J. onca	SWS1	X	X	X	-	-	-	-	-	-	-	-	-	-
A. anableps	SWS1	-	-	-	-	-	-	-	-	-	-	-	-	-
P. reticulata	SWS1	-	-	-	-	-	-	-	-	-	-	-	-	-
L. goodei	SWS1	-	-	-	-	-	-	-	S	-	-	-	-	-

Species	Gene	Amino acid key site									Gene	Amino acid key site								
		60	62	107	110	125	132	134	138	277		60	62	107	110	125	132	134	138	277
Consensus		T	F	S	A	A	V	T	M	F		M	F	S	C	G	T	S	M	F
J. onca	SWS2A	-	-	-	-	-	-	-	-	-	SWS2B	X	X	-	-	-	-	-	-	X
A. anableps	SWS2A	-	-	-	-	-	-	-	-	-	SWS2B	X	X	-	-	-	-	-	-	X
P. reticulata	SWS2A	-	-	-	T	-	-	-	-	-	SWS2B	-	-	-	-	-	-	-	-	-
L. goodei	SWS2A	-	L	-	-	-	I	-	-	-	SWS2B	-	-	-	-	-	-	G	-	-

Appendix 3
Accession Numbers Used in Ray-finned Fish Opsin Phylogenetic Analysis (Chapter 3).

Scientific Name	SWS1	SWS2	RH1 (Errlo)	RH2	LWS
Actinopterygians					
Acanthopagrus berda	X	X	*	*	*
Acanthopagrus butcheri	DQ354579.1	X	DQ354577.1	EU090914.1, EU090913.1	DQ354578.1
Acanthopagrus schlegeli	X	X	*	X	*
Acipenser sp.	X	X	AF137206.1	X	X
Amia calva	X	X	AF137208.1	X	X
Anableps anableps	FJ711153.1	FJ711151.1, HM627004	FJ711149.1	FJ711149.1, FJ711150.1	FJ711158.1, FJ711157.1, FJ711155.1, FJ711154.1
Anguilla anguilla	X	FJ515779.1	L78007.1, L78008.1	FJ515778.1	X
Anguilla japonica	X	X	AJ249203.1, AJ249202.1	X	X
Astatotilapia burtoni	X	AY660538.1	AF315354.1	AY660539.1	AY660540.1
Astyanax fasciatus	X	AH007939.1	U12328.1	X	M90075.1, U12025.1, U12024.1
Batrachocottus nikolskii	X	AJ430474.1	U97268.1	X	X
Candidia barbatus	EU410458.1	EU410459.1	EU919559.1	EU410461.1, EU410460.1	EU410457.1
Carassius auratus	D85863.1	L11864.1	L11863.1	L11866.1, L11865.1	L11867.1, GQ168789.1
Chrysiptera parasema	X	X	X	X	DQ073800.1
Conger conger	X	X	S82619.1	X	X
Cottocomephorus inermis	X	AJ430479.1	U97266.1	X	X
Cottus kessleri	X	AJ430484.1	L42953.1	X	X
Cyprinus carpio	AB113669.1	AB113668.1	S74449.1, Z71999.1	AB110603.1, AB110602.2	AB055656.1
Danio rerio	BC060894.1	NM_131192.1	BC045288.1, XM_001336009.2 (NM_131212.2)	NM_131253.2, NM_182891.2, NM_182892.2, NM_131254.1	NM_001002443.1, NM_131175.1
Dissostichus mawsoni	AY927651.1	X	DQ498794.1	AY771352.1	X
Gadus morhua	X	AF385822.1	AF385832.1	AF385824.1	X

Gasterosteus aculeatus	BT028514.1	BT027452.1	BT028623.1	BT027598.1, ENSGACT00000001859	BT027981.1
Girella punctata	X	AB158256.1	X	X	AB158261.1
Gymnodraco acuticeps	AY927656.1	X	X	AY771355.1	X
Hippoglossus hippoglossus	AF156264.1	AF316497.1	AF156265.1	AF156263.1	AF316498.1
Ictalurus punctatus	X	X	AF028016.1	X	X
Jenynsia onca	X	GQ221673.1	GQ221670.1	GQ221668.1, GQ221669.1	GQ221677.1, GQ221675.1, GQ221671.1
Jordanella floridae	X	X	X	X	HM627007, HM627009
Lepidopus fitchi	FJ443126.1	X	EU407252.1, EU407253.1	GQ414752.1, GQ414753.1, GQ414754.1, GQ414755.1	X
Limnocottus eurystomus	X	AJ430469.1	X	X	X
Lucania goodei	AY296735.1	AY296737.1, AY296736.1	AY296738.1	AY296739.1	AY296740.1, AY296741.1
Melanochromis vermivorus	DQ088643.1	DQ088637.1, DQ088640.1	GQ422472.1	DQ088634.1, DQ088646.1, DQ088631	DQ088628.1
Melanotaenia australis	X	FJ940705.1	FJ940704.1	FJ940703.1	FJ940702.1
Metriaclima zebra	DQ088648.1	AF247118.1, AF247114.1	AY775114.1	DQ088650.1, DQ088651.1, DQ088652	AF247126.1
Mullus surmuletus	X	X	Y18666.1	Y18680.1	X
Nannostomus beckfordi	X	X	X	X	HM627006
Notothenia angustata	AY927654.1	X	DQ498787.1	AY771354.1	X
Oncorhynchus gorbuscha	AY214153.1	AY214154.1	AY214151.1	AY214152.1	AY214150.1
Oncorhynchus keta	AY214143.1	AY214144.1	AY214141.1	AY214142.1	AY214140.1
Oncorhynchus kisutch	AY214148.1	AY214149.1	AY214146.1	AY214147.1, DQ309027	AY214145.1
Oncorhynchus mykiss	NM_001124321.1	NM_001124322.1	NM_001124319.1	NM_001124323.1	NM_001124320.1
Oncorhynchus nerka	AY214158.1	AY214159.1	AY214156.1	AY214157.1	AY214155.1
Oncorhynchus tshawytscha	AY214138.1	AY214139.1	AY214136.1	AY214137.1	AY214135.1
Oreochromis niloticus	AF191221.1	AF247116.1, AF247120.1	AB084938.1	DQ235683.1, DQ235681.1, DQ235682.1	AF247128.1

Oryzias latipes	AB223058.1	AB223056.1, AB223057.1	AB180742.1, (ENSORLG00000010979)	AB223053.1, AB223054.1. AB223055.1	AB223052.1, AB223051.1
Pagetopsis macropterus	AY927655.1	X	EU637990.1	X	X
Pagothenia borchgrevinki	AY927653.1	X	DQ498791.1	AY771353.1	X
Pagrus major	X	X	*	*	*
Paracheirodon innesi	X	X	AB245433.1	X	AB239250.1, AB239249.1, AB545021.1
Plecoglossus altivelis	AB098706.1, AB098705.1	X	AB074484.1 (AB089247.1)	AB098703.1 AB098704.1	AB098702.1, AB107771.1
Poecilia reticulata	DQ234861.1	FJ711159.1, DQ234860.1	DQ912024.1	DQ234859.1, DQ234858.1	EU329457.1, EU329453.1, HM627005, EU329433.1
Polyodon spathula	X	X	AF369050.1	X	X
Pomatoschistus minutus	X	X	FN430594.1	Y18679.1	X
Pseudopleuronectes americanus	X	AY631038.1	AY631036.1	AY631037.1	AY631039.1
Pseudotropheus acei	DQ088642.1	DQ088636.1, DQ088639.1	GQ422475.1	DQ088645.1, DQ088633, DQ088630	DQ088627.1
Pundamilia pundamilia	AY673729.1	AY673719.1, AY673709.1	AY673739.1	AY673699.1	AB448425.1
Salmo salar	NM_001123708.1	NM_001123706.1	AF201470 (NM_001123536.1)	NM_001123707.1	NM_001123705.1
Scopelarchus analis	X	X	EF517404.1, EF517405.1	EF517406.1	X
Scophthalmus maximus	AF385825.1	X	X	AF385828.1, AF385827.1	AF385826.1
Sinocyclocheilus anophthalmus	X	GQ168759.1	EU606007.1	X	GQ168786.1 GQ168787.1
Sinocyclocheilus jii	X	GQ168748.1, GQ168749.1	EU605989.1	X	GQ168768.1
Stenobrachius leucopsarus	FJ443127.1	X	EU407251.1	GQ414753.1, GQ414754.1, GQ414756.1	X
Takifugu rubripes	X	AY598947.1	NM_001078631.1 (NM_001033849.1)	NM_001033712.1	AY598942.1
Tetraodon nigroviridus	X	AY598948.1	AJ293018.1, (ENSTNIG00000017925)	AY598944.1	AY598943.1
Thunnus orientalis	X	AB290450.1	AB290449.1	AB290451.1	X
Tramitichromis	DQ088644.1	DQ088638.1	GQ422473.1	DQ088635.1,	DQ088629.1

intermedius		, DQ088641.1		DQ088647, DQ088632.1	
Trematomus loennbergii	AY927652.1	AY771356.1	X	X	X
Xiphophorus birchmanni	EU825689.1	EU825682.1	X	X	EU825688.1
Xiphophorus malinche	EU825684.1	EU825683.1	X	X	EU825686.1
Xiphophorus pygmaeus	X	X	X	X	EU329480.1, EU329478.1
Xiphopohorus helleri	X	GQ999832	X	X	GQ999832, GQ999833
Zacco pachycephalus	EU410468.1	EU410467.1	EU919560.1	EU410463.1, EU410464.1, EU410465.1	EU410466.1
Sarcopterygians					
Latimeria chalumnae	X	X	AH007712.1	AH007713.1	X
Neoceratodus forsteri	EF526298.1	EF526299.1	EF526295.1	EF526296.1	EF526297.1
Agnathans					
Geotria australis	AY366495.1	AY366492.1	AY366493.1	AY366494.1	AY366491.1
Lethenteron japonicum	X	X	AB116382.1	X	AB116381.1
Petromyzon marinus	X	X	AH005459	X	EU571209.1

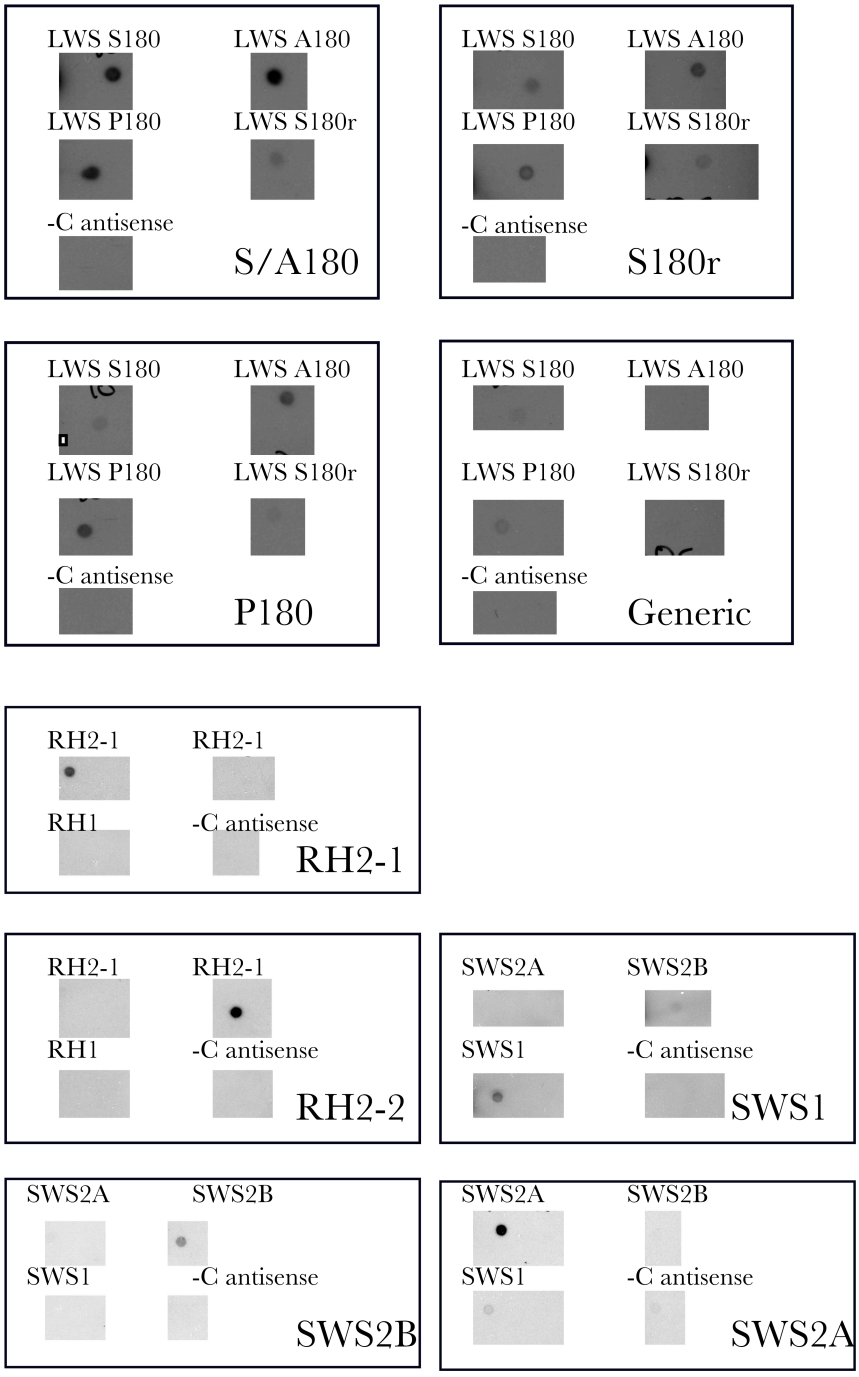
Appendix 4
Maximal absorption Values for Ray-finned Fish Obtained From MSP and *in vitro* Reconstruction.

Family	Species	SWS 1	SWS2	RH1	RH2	LWS	Reference
Adrianichthyidae	*Oryzias latipes*[1]	356	439 & 405	502	452, 519 & 492	561 & 562	(Matsumoto et al., 2006)
Anguillidae	*Anguillla anguillla*[1]	-	445	-	524	-	(Cottrill et al., 2009)
Anguillidae	*Anguillla japonica*[1]	-	-	500 & 479	-	-	(Yokoyama et al., 2008)
Cichlidae	*Pundamilia pundamilia*[2]	-	455	506	529	563	(Carleton et al., 2005)
Cichlidae	*Melanochromis vermivorus*[2]	-	418	-	534, 504 & 485	566	(Parry et al., 2005)
Cichlidae	*Pseudotropheus acei*[2]	378	452 & 415	-	534, 505 & 482	556	(Parry et al., 2005)
Cichlidae	*Metriaclima* zebra[2]	368	423[1]	-	533, 519[1] & 488	-	(Parry et al., 2005)
Cichlidae	*Oreochromis niloticus*[1]	360	456 & 425	-	528, 517 & 472	560	(Spady et al., 2006)
Cichlidae	*Tramitichromis intermedius*[2]	-	455	-	532	569	(Parry et al., 2005)
Cyprinidae	*Danio rerio*[2]	362	417	501	467, 476, 488 & 505	558 & 548	(Chinen et al., 2003)
Cyprinidae	*Carassius auratus*[1]	370	447	502	515	565	(Parry & Bowmaker, 2000)
Cyprinidae	*Zacco pachycephalus*[2]	376	413	505	486	567	(Wang et al., 2008)
Cyprinidae	*Candidia barbatus*[2]	374	423	504	500	564	(Wang et al., 2008)
Fundulidae	*Lucania goodei*[2]	359	455 & 405	504	539	573	(Yokoyama et al., 2008)

Myctophidae	*Stenobrachius leucopsarus*[1]	371	-	-	-	-	(Tada et al., 2009)
Nototheniidae	*Dissostichus mawsoni*[2]	369[1]	415	500	490	-	(Pointer et al., 2005)
Pleuronectidae	*Pseudopleuronectes americanus*[2]	-	461	509	529	547	(Mader & Cameron, 2004)
Salmonidae	*Oncorhynchus kisutch*[2]	366	434	505	517	566	(Cheng et al., 2006)
Salmonidae	*Oncorhynchus tshawytscha*[2]	362	434	508	518	563	(Cheng et al., 2006)
Salmonidae	*Oncorhynchus gorbuscha*[2]	370	436	506	494	568	(Cheng et al., 2006)
Salmonidae	*Oncorhynchus keta*[2]	364	432	505	508	563	(Cheng et al., 2006)
Salmonidae	*Salmo salar*[2]	361	435	515	518	578	(Cheng et al., 2006)
Sparidae	*Acanthopagrus butcheri*[2]	-	475 & 425	-	535& 555	570	(Shand et al., 1988; Shand et al., 2008)
Sparidae	*Acanthopagrus schlegeli*[2]	-	471	499	528	563	(Wang et al., 2009)
Sparidae	*Acanthopagrus berda*[2]	-	472	501	-	566	(Wang et al., 2009)
Sparidae	*Pagrus major*[2]	-	460	491	525	-	(Wang et al., 2009)
Trichiuridae	*Lepidopus fitchi*[1]	423	-	507 & 481	-	-	(Tada et al., 2009; Yokoyama, 2008)

*Superscript 1 indicates the method of determining maximal absorption was *in vitro* reconstitution using 11-cis-retinal
Superscript 2 indicates the method of determining maximal absorption was MSP.

Appendix 5
Dot-blot Analysis of *in situ* Hybridization Probes and Gene Targets

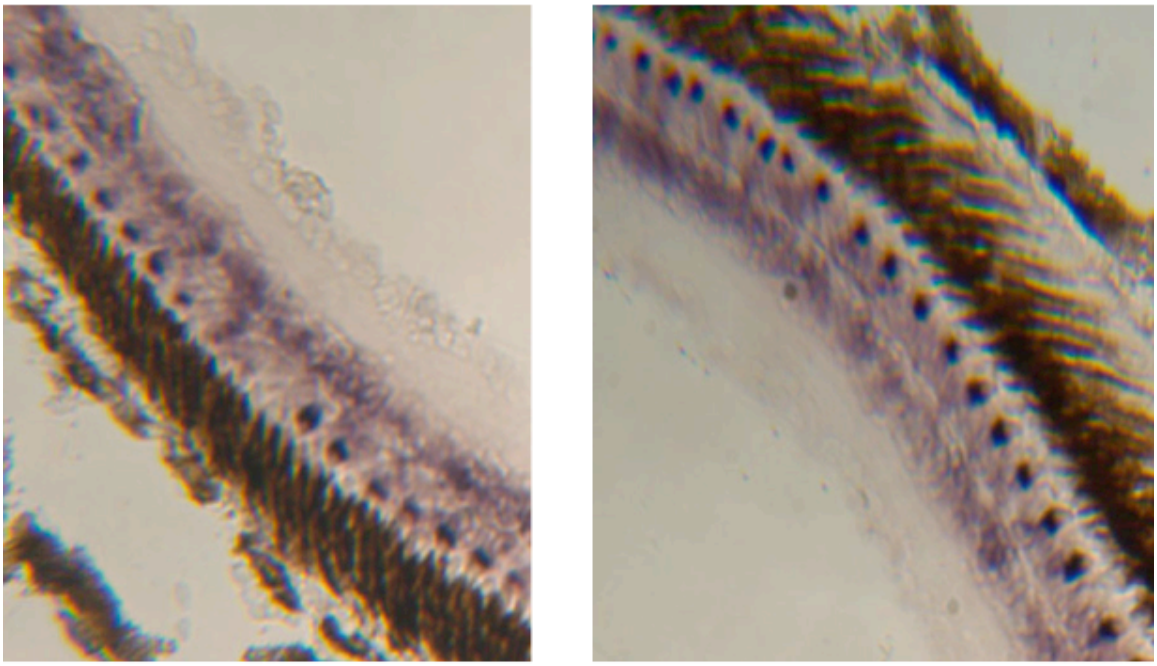


*Gene target is labelled above each membrane portion. The probe used on each blot is indicated in the bottom right corner.

Appendix 6 Magnification of Cross-sectional *in situ* Hybridization Along the Dorsal-ventral Axis

Ventral

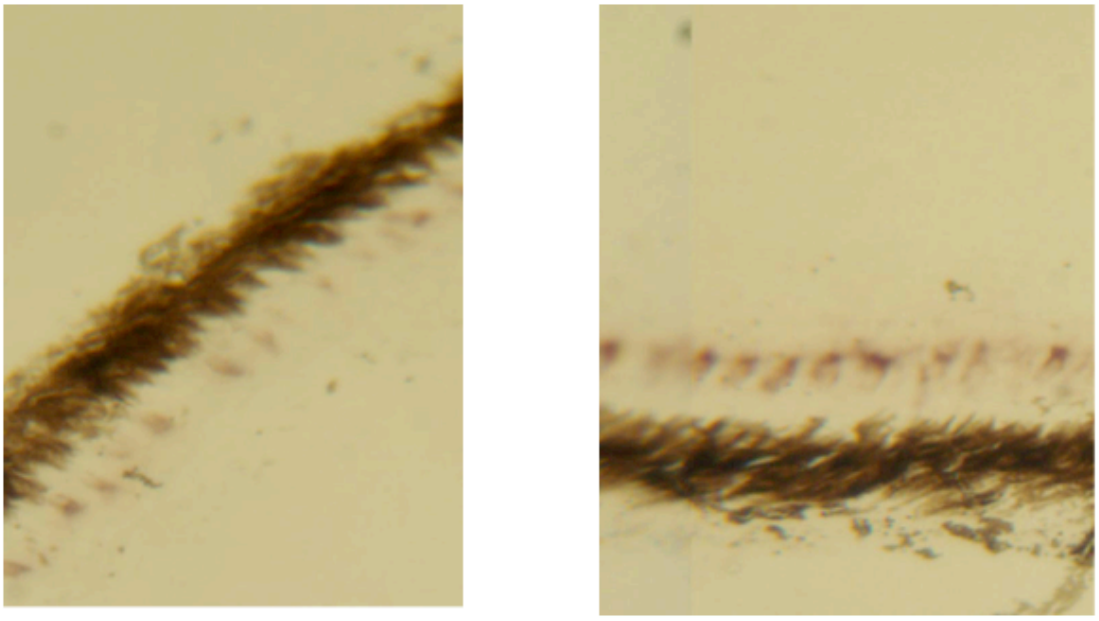
Dorsal



SWS1

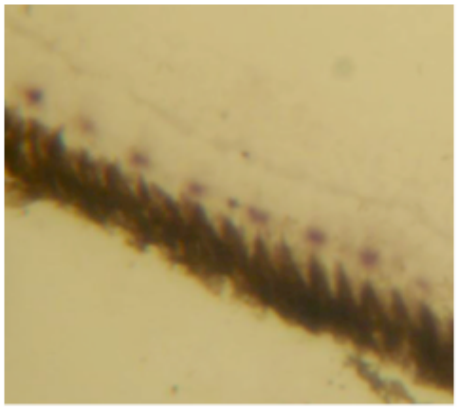
Ventral

Dorsal

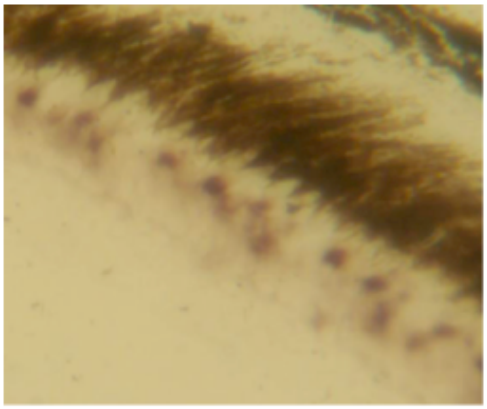


SWS2A

Ventral

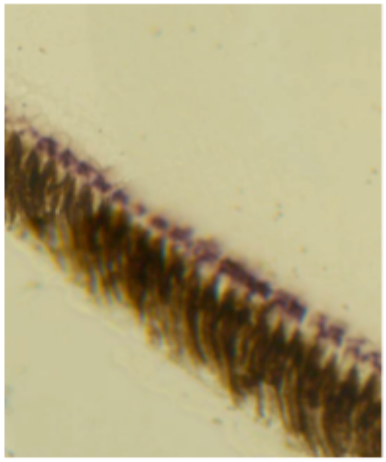


Dorsal

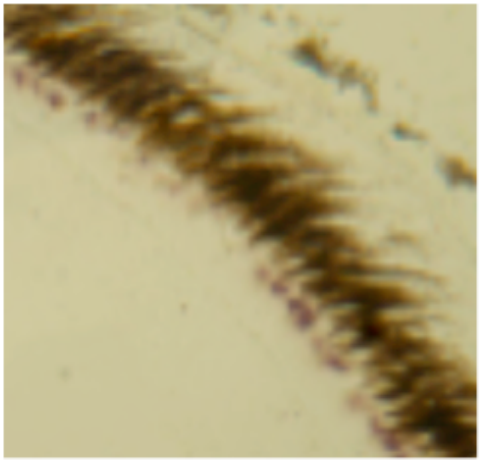


SWS2B

Ventral

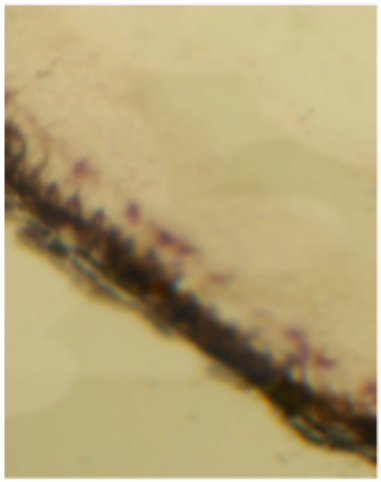


Dorsal

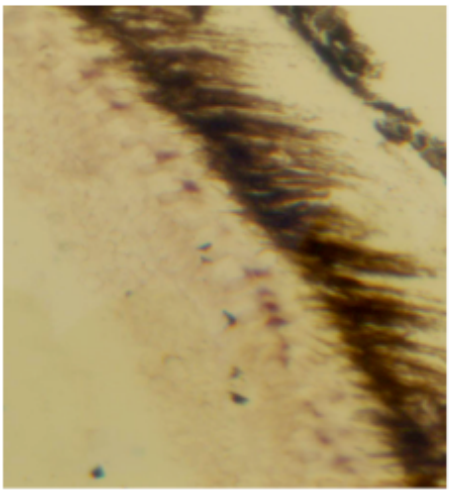


RH2-1

Ventral

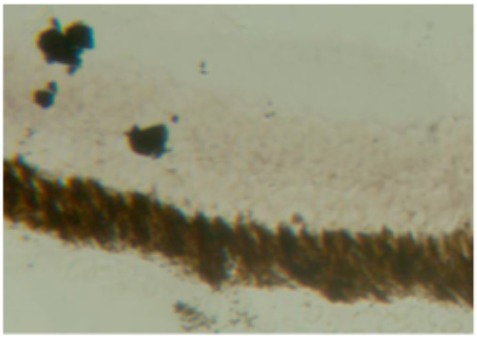


Dorsal

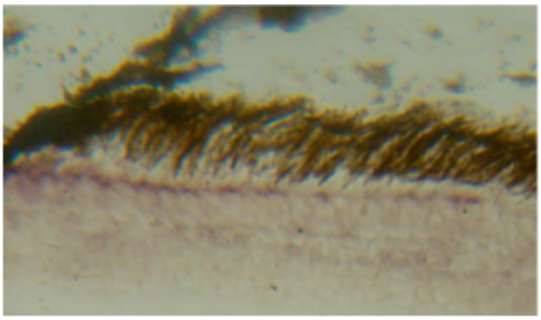


RH2-2

Ventral

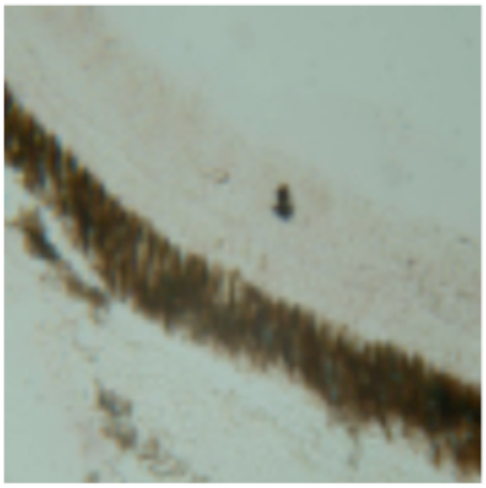


Dorsal

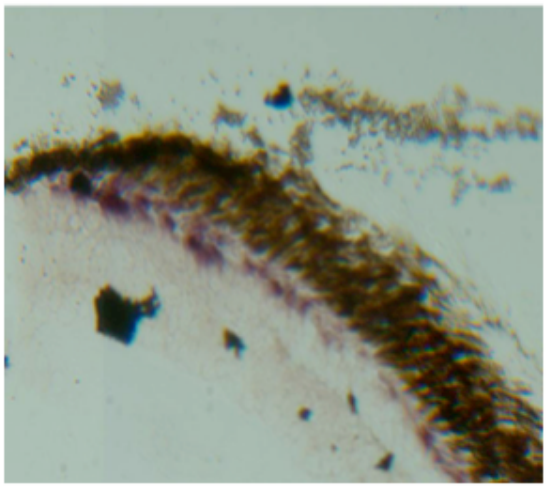


LWS S180r

Ventral



Dorsal



LWS S/A180

Ventral

Dorsal

LWS P180

Appendix 7
Spatial Quantification of Opsin Expression Generated by *in situ* Hybridization.

Gene	Region	0-100μm	100-200μm	200-300μm	100μm mid-retina
SWS1	N	0	0	4	-
	T	5	9	10	-
	D	15	10	10	-
	V	12	9	11	-
SWS2A	N	7	3	6	7
	T	10	10	10	8
	D	6	6	6	8
	V	4	3	3	6
SWS2B	N	11	10	9	11
	T	9	10	10	10
	D	8	8	9	8
	V	5	6	6	9
RH2-1	N	10	10	8	7
	T	5	6	7	9
	D	5	4	7	6
	V	8	12	11	7
RH2-2	N	5	5	3	8
	T	2	7	5	8
	D	4	3	4	5
	V	2	4	4	5
LWS S180r	N	0	0	0	0
	T	0	0	0	0
	D	6	4	3	0
	V	0	0	0	0
LWS S/A180	N	0	0	0	4

	T	0	0	0	2
	D	6	7	8	5
	V	0	2	0	5
LWS P180	N	0	0	0	4
	T	0	0	0	4
	D	4	4	5	6
	V	0	0	1	6