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Cichlids as a model for the evolution of visual sensitivity

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CICHLIDS AS A MODEL FOR THE EVOLUTION OF VISUAL SENSITIVITY

BY

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ABSTRACT

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The cichlid fishes of East Africa are the most ecologically diverse radiation of recent vertebrates. These highly visual fish live in habitats ranging from turbid rivers to clear lakes. They have evolved to exploit an astounding array of foraging strategies. The combination of phenotypic diversity and varied environmental conditions makes the cichlid system ideal for the examination of the relationship between ecology and the evolution of visual sensitivity. In this dissertation, I explore several aspects of this relationship. In Chapter 1, I compare the opsin gene sequences from 17 African cichlid species that have evolved in either clear or turbid light environments. I identify statistical evidence of molecular adaptation. I also find evidence of differences in the relative rates of substitution across clear and turbid lineages. When patterns of amino acid substitution are compared to possible tuning sites, only the ultraviolet sensitive SWS1 class have patterns of substitution that are consistent with photic environment- driven evolution. In Chapter 2, I determine the peak absorbances of in vitro expressed pigments for all seven Nile tilapia cone opsin genes and chart opsin expression across ontogeny. Each gene is found to encode a distinct photopigment. Despite the expression of a limited subset of opsin genes in adults, each opsin was found to be expressed at some point during ontogeny. In Chapter 3, I use MSP and real-time RT-PCR to characterize the differences

in visual sensitivity among one of Lake Malawi's most species rich genera, *Labidochromis*. This study suggests that visual sensitivity is quite labile and can change during the evolution of closely related species. In chapter 4, I examine the distribution of retained opsin gene duplicates among all fish opsins sequenced to date. Duplicates are found differentially across opsin classes. Overall, the majority of retained gene duplicates began to accumulate around the time of the radiation of higher teleosts. Finally in Chapter 5, I highlight ways in which the cichlid system might be especially useful in relating ecology and vision. This includes identifying how visual sensitivity has been shaped by specific foraging strategies and how this affects the long-term evolution of the cone opsin family.

INTRODUCTION

The radiation of cichlid fish in the African Great Lakes (Tanganyika, Malawi and Victoria) is unparalleled among modern vertebrates. Well over a thousand species have evolved since the lacustrine cichlids diverged from the Nile tilapia ten million years ago (MYA) (Kocher et al. 1995). The Tanganyikan radiation is the oldest, with some lineages appearing eight MYA (Snoeks et al. 1994). The Malawi and Victoria radiations are much younger, having emerged 1 and 0.5 MYA, respectively (Meyer et al. 1990; Nagl et al. 2000; Kornfield and Smith, 2000; Genner et al. 2005). The Tanganyikan radiation is also unique in that it is polyphyletic, being the product of colonizations by multiple lineages (Salzburger et al. 2002). The Malawi and Victoria radiations are thought to have each evolved from a unique common ancestor (Kocher et al. 1995; Meyer et al. 1990). The ancestors of lacustrine cichlids are thought to have been non-specialist, riverine species. From these generalized ancestors, the species of the lakes have evolved to exploit a wide spectrum of ecological niches, as well as a variety of habitats (Barlow 2000).

Vision is critical to East African cichlids. The numerous piscivores, plankivores, scale-biters, molluscivores, herbivores, and generalist alike all rely on vision to detect food and avoid predators (Fryer and Iles, 1972). Cichlids also rely heavily on vision in mating. The males of many species employ bright colors to attract mates. Male color patterns have been shown to be crucial in mate recognition and choice in cichlids (Seehausen 1997). Thus the evolution of the visual sensitivity is likely an important force in the evolution of these fishes.

Visual sensitivity is determined by visual pigments. Visual pigments serve as photosensory molecules in the outer segments of photoreceptor cells, within the retina. Visual pigments are composed of a vitamin A derived chromophore bound to an opsin protein. The chromophore is housed in a pocket in the center of the opsin protein. It is the photo-isomerization of the chromophore that initiates the photo-excitation signal cascade. The absorbance properties of a visual pigment are primarily determined by electrostatic interactions between the chromophore and the amino acid residues within the chromophore-binding pocket (Nathans 1990; Sakmar et al. 1989; Zhukovsky and Oprian 1989).

Fish visual sensitivities are correlated with environmental light conditions (Bowmaker 1995). Lakes Tanganyika and Malawi are among the world's clearest and most transmissive fresh water systems (Fryer and Iles 1972; Muntz 1976; Carleton et al. in press). In situ measurements have confirmed the broad spectral transmissiveness and the richness of the ambient spectra available in shallower habitats of Lake Malawi (Spady et al. unpublished data). This is a stark contrast to the turbidity associated with Lake Victoria (Fryer and Iles 1972; Seehausen et al. 1997) and the surrounding rivers. Turbidity shifts the maximum transmission toward the long wavelength region by filtering the shortest wavelengths. The end result is that the spectral breadth of light available for vision is limited in turbid habitats. Relative to the riverine environment of the ancestral cichlid (and Lake Victoria), the spectrum of light available for vision in Lakes Tanganyika and Malawi is likely to be much broader. This broadening of the spectral content of the visual environment to include the shorter wavelengths (including the uv spectral regions) has increased the sensory possibilities of the vision of fishes in

the clear lakes. The dramatic difference in photic environment across the East African cichlid flocks lends itself to the determination of the extent to which photic environment drives opsin gene evolution.

East African cichlids have been shown to have seven cone opsin genes, which correspond to red (LWS), long wavelength green (Rh2a α), short wavelength green (Rh2a β), blue-green (Rh2b), blue (SWS2a), violet (SWS2b), and ultraviolet (SWS1) pigment sensitivities (Parry et al. 2005). Individual species express varying subsets of the seven cone opsin genes (Levine and MacNichol 1979; Fernald and Liebman 1980; van der Meer and Bowmaker 1995; Carleton et al. 2000, 2001, 2005; Parry et al. 2005; Jordan et al. in press). Differential gene expression, therefore, can have a large effect on how these fishes perceive their world. Most of the observed variation seems to occur between ecologically divergent genera. However, it was unknown whether differential gene expression occurs within genera or between sister taxa. Work in killifish has shown quantitative differences in gene expression across populations living in different photic environments (turbid vs. clear) (Fuller et al. 2004). This work suggests that at the very least there may be quantitative differences in gene expression across cichlid populations. However, it is unknown at what phylogenetic scale qualitative differences (e.g., genes turned on in one group but off in another group) occur. If these opsin expression differences occur between sister taxa, they may suggest that differential opsin expression is important in speciation. Further, it is unclear what role foraging behavior plays in visual system differentiation either at the immediate level of opsin gene expression or the more long-term level of gene retention and loss.

This dissertation probes the relationship between visual sensitivity and ecology, through the use of the cichlid system. Chapter 1 determines if dramatic differences in photic environment have driven the molecular evolution of cichlid opsin genes. In Chapter 2, the peak absorbances of all known East African cichlid cone opsin gene classes were characterized. Expression across ontogeny was then examined to determine how these species have been able to retain so many cone opsin genes when many are not expressed in the adult life stage. Chapter 3 describes experiments to determine if differences in opsin gene expression can occur within a genus. Chapter 4 looks at the distribution of cone opsin gene duplication and retention across fishes. The final chapter highlights the need for further work in the cichlids to better understand the evolutionary connection between visual sensitivity and ecology.

CHAPTER I

ADAPTIVE MOLECULAR EVOLUTION IN THE OPSIN GENES OF RAPIDLY SPECIATING CICHLID SPECIES

Abstract

Cichlid fish inhabit a diverse range of environments that vary in the spectral content of light available for vision. These differences should result in adaptive selective pressure on the genes involved in visual sensitivity, the opsin genes. This study examines the evidence for differential adaptive molecular evolution in East African cichlid opsin genes due to gross differences in environmental light conditions. First, I characterize the selective regime experienced by cichlid opsin genes using a likelihood ratio test format, comparing likelihood models with different constraints on the relative rates of amino acid substitution, across sites. Second, I compare turbid and clear lineages to determine if there is evidence of differences in relative rates of substitution. Third, I present evidence of functional diversification and its relationship to photic environment among cichlid opsin genes. I report statistical evidence of positive selection in all cichlid opsin genes, except short wavelength-sensitive 1 and short wavelength-sensitive 2b. In all genes predicted to be under positive selection, except short wavelength-sensitive 2a, I find differences in selective pressure between turbid and clear lineages.

Potential spectral tuning sites are variable among all cichlid opsin genes. However, patterns of substitution consistent with photic environment-driven evolution of opsin genes are observed only for short wavelength-sensitive 1 opsin genes. This study identifies a number of promising candidate tuning sites for future study by site-directed mutagenesis. This work also begins to demonstrate the molecular evolutionary dynamics of cichlid visual sensitivity and its relationship to photic environment.

Introduction

Visual ecologists have long observed a correlation between the photic environment and visual sensitivity (Bowmaker 1995). Some of the most dramatic examples have been found in fish rod photoreceptors. Deep-sea fish rod spectral sensitivities are shortwave shifted, relative to shallow dwelling species, to match the ambient spectra of the deep-sea environment (Partridge et. al. 1988, Crescitelli 1991). Adaptation to depth has also been observed in the rods of freshwater teleosts. Among the cottoids of Lake Baikal, the world's deepest lake, rhodopsin's (Rh1) absorption maxima decreases as depth increases (Hunt et. al. 1996). Muntz (1976) compared a shallow and deeper living pair of closely related cichlid species of the genus *Lethrinops*. Again, the deep-water species had shortwave shifted rod sensitivity.

There are also several examples of differences in cone spectral sensitivity associated with disparities in photic environment. Both deep-dwelling Lake Baikal cottoids and coelacanths show a marked shortwave shift in cone spectral sensitivities (Bowmaker et al. 1994; Yokoyama et al. 1999). Lutjanid fishes, of the Great Barrier Reef, demonstrate the interaction between water clarity and cone spectral sensitivity, with fish in clearer habitats having shortwave shifted visual sensitivities (Lythgoe et al. 1994).

Visual pigments determine spectral sensitivity and are spectrally distinct photosensory molecules in the outer segments of retinal photoreceptor cells. Visual pigments are composed of a vitamin A-derived chromophore bound to an opsin protein. Photoisomerization of the chromophore initiates the transduction cascade culminating in a neural response. Interactions between the chromophore and the amino acid residues of the opsin protein determine the absorbance properties of a visual pigment (Sakmar et al.

1989; Zhukovsky and Oprian 1989; Nathans 1990a: Nathans 1990b; Sakmar et al. 2002; Yokoyama 2002).

Spectral sensitivity of cichlid fishes can be tuned using four nonexclusive mechanisms. First, the lenses of some cichlids contain inert short wavelength-absorbing carotenoid pigments (Thorpe, Douglas, and Truscott 1993). The presence of ocular pigments seems to be independent of photic environment, with nonpigmented species occurring in fish of both turbid and clear habitats (Thorpe, Douglas, and Truscott 1993).

Second, Carleton and Kocher (2001) have shown that cichlids use differential cone opsin expression to modulate visual sensitivity. Cichlids have six opsin genes (five cone opsins and one rod opsin): long wavelength-sensitive (LWS), rhodopsin-like (Rh2), short wavelength-sensitive 2b (SWS2b), short wavelength-sensitive 2a (SWS2a), short wavelength-sensitive 1 (SWS1), and rod opsin, rhodopsin (Rh1). Individual species express varying subsets of the five cone opsin genes. For example, the ambush predator *Dimidiochromis compressiceps* expresses LWS, Rh2, and SWS2a genes. In contrast, the planktivorous *Metriaclima zebra* expresses Rh2, SWS2b, and SWS1, a radically different subset of opsin genes.

Third, chromophore usage can vary among cichlids (vitamin A1 or A2 derived). Visual pigments based on a vitamin A2-derived chromophore have long wavelength-shifted absorbance maxima, relative to those based on an A1-derived chromophore (Partridge and Cummings 1999). Fish that inhabit turbid environments more commonly use A2 or A1/A2 mixtures (Bowmaker 1995), although chromophore thermal stability may also shape usage (Partridge and Cummings 1999). Cichlids from the turbid waters of Lake Victoria utilize A1/A2 mixtures (van der Meer and Bowmaker 1995); however

clear-water Lake Malawi cichlids use only A1 chromophores (Carleton, Harosi, and Kocher 2000; Jordan et al. unpublished data).

Finally, amino acid substitutions in the opsin protein can alter visual sensitivity (summarized in Yokoyama 2002, and Takahashi and Ebrey 2003). The effects of individual substitutions are highly variable, ranging from 0 to 75nm. Further the effects of individual substitutions depend upon the amino acid background of the opsin protein.

There is mounting evidence for molecular adaptation to photic environment, via amino acid substitutions in opsin proteins, in East African cichlids. Recently, Sugawara, Terai, and Okada (2002) found evidence of functional divergence in Tanganyikan cichlid Rh1 opsin genes. They found an A292S substitution in several Tanganyikan lineages (bovine rhodopsin numbering will be used exclusively in this paper). In mammalian LWS visual pigments, an A292S substitution causes a –18 nm spectral shift, and can be expected to have a similar effect in cichlid Rh1 visual pigments. Interestingly, all three species with the A292S substitution are deep-water species, further supporting the relationship of spectral sensitivity to depth. Further, Terai et al. (2002) report high variation of the LWS gene in Lake Victoria cichlids and highlight several potential functionally important substitutions (reviewed in Carleton and Kocher 2003). Terai et al. (2002) contend that similarities between ancestral and modern photic environments maintained ancestral variation in the Lake Victoria LWS gene.

The present study looks for evidence of adaptive molecular evolution among closely related cichlid species that inhabit dramatically different photic environments. I then test for differences in relative rates of evolution between turbid and clear water lineages. Finally, I identify amino acid substitutions that are likely to be involved in the

adaptive/functional differentiation of cichlid opsins. Cichlids endemic to clear lakes Tanganyika and Malawi are contrasted against cichlids endemic to more turbid environments in Lake Victoria and the Nile River. Due to geologic and climatic conditions, Lake Tanganyika and Lake Malawi are among the clearest freshwater systems in the world (Muntz 1976) and provide a stark contrast to the generally more turbid environments of Lake Victoria (Seehausen, van Alphen, and Witte 1997) and the Nile River. Since turbidity directly limits the transmission of the shortest wavelengths of the visible spectrum, the spectrum of ambient light is shifted toward the long wavelength region. Thus the spectral breadth of light available for vision is restricted in turbid habitats.

We use Codon-based Maximum Likelihood methods (CodeML; Yang et al. 2000), as implemented in Phylogenetic Analysis by Maximum Likelihood (PAML; Yang 1997) to look for evidence of adaptive molecular evolution. Comparisons of nonsynonymous (dN) and synonymous substitution rates (dS), $dN/dS = \omega$, are used to infer the selective regime experienced by a gene (Graur and Li 2000). When $\omega = 1$ a neutral mode of evolution is indicated. $\omega < 1$ indicates purifying selection. $\omega > 1$ indicates positive selection. PAML methods account for the different functional and structural constraints experienced by individual sites/domains of a protein by allowing for heterogeneous ω across sites (ie. Yang and Swanson 2002). PAML has been used to detect positive selection among fertilization proteins (Civetta 2003; Swanson, Nielson, and Yang 2003), lysozymes (Yang 1998; Yang and Nielsen 2002), tumor suppressors (Yang and Nielsen 2002), dopamine receptors (Ding et. al. 2002) and among insect opsins (Briscoe 2001).

Methods

PCR and Sequence Analysis

We sequenced SWS1, SWS2a, SWS2b, Rh2, LWS, and Rh1 cichlid opsin genes. Opsin coding sequences were obtained for 17 East African cichlid species (Table 1.1). Cone opsin gene sequences from 5 species and rod opsin gene sequences from 2 of those species were obtained from Genbank (Carleton, Harosi, and Kocher 2000; Carleton and Kocher 2001; Carleton et al. in prep). All other rod opsin sequences and the cone opsin sequences from the remaining 12 species are new additions to the sequence database. The species studied represent lineages from the Nile River and Lake Victoria, both turbid, and Lakes Malawi and Lake Tanganyika, both clear. Retinal tissue was used to extract opsin mRNA, whenever possible. Retinas were homogenized and RNA extracted with Trizol (Invitrogen). Retinal RNA preparations were then reverse transcribed with a poly T primer and Superscript II Reverse Transcriptase (Invitrogen). Genomic DNA was extracted as well and used to determine opsin coding sequences when necessary. Opsin coding sequences were PCR amplified using sequence-specific primers as previously described by Carleton and Kocher (2001) for each of the 6 opsin classes found in cichlids. Dynazyme Ext. (MJ Research), a polymerase mixture containing a high fidelity polymerase with 3'-5' proofreading activity, was used to amplify all sequencing templates. Opsin PCR-products were sequenced using previously described primers and DYEnamic™ ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Inc.) (Carleton and Kocher 2001). Sequences were aligned using Sequencher 4.1.2 (Gene Codes Corporation). Whenever possible, the complete opsin coding sequence was used. In cases where complete sequences were not obtained, at least 97% of the continuous

coding sequence from each opsin gene (6) was used. Regions of missing sequence, made up of small stretches in the 3' and/or 5' part of the coding sequence, were not used in the analysis. The regions used in the analysis always included all transmembrane (TM) and inter-TM regions, which control the spectral absorbance of visual pigments.

Gene trees were generated from opsin coding sequences. Using PAUP (Swofford 2002), aligned sequences were used to calculate bootstrap trees (100 replicates, 50% majority rule). Bootstrap topologies were then used as a constraint in maximum likelihood estimation of gamma parameters. Maximum likelihood estimates of gamma parameters and Tamura-Nei distances were then used to generate unrooted neighbor-joining (NJ) (Saitou and Nei 1987) tree topologies (Figure 1.1). Additionally, a putative species tree was constructed based on published cichlid phylogenies (Kocher et al. 1995, Streelman et al. 1998; Albertson et al. 1999).

Maximum Likelihood Analysis

A total of seven tree topologies were used by PAML to examine relative rates of substitution. Each opsin gene was tested with all gene trees and a putative species tree. Nested models were compared using a likelihood ratio test (LRT) as described in Yang et al. (2000) and Yang and Nielsen (2002). The LRT statistic was calculated as twice the difference in maximum likelihood values ($2\Delta\ell$) between nested models. The significance of the LRT statistic was determined using a χ^2 distribution. The standard degrees of freedom (DF) were used for each analysis (i.e., Yang and Nielsen 2002). Several site-specific likelihood models were used to fit the data. These models allow for ω heterogeneity among sites, except in the case of the one rate model. In all models, synonymous rates of substitution are assumed to be invariant across sites. Three LRT's

were carried out using site-specific models (Figure 1.2). 1) The comparison of M0 (one rate) and M3 (discrete) was used to test for rate heterogeneity among amino acid sites (Figure 1.2a). M0 (one rate) averages the rates of substitution across all sites. M3 (discrete) assumes rate variation, by allowing for a discrete number of rate categories (3 used here). 2) The comparison of M1 (neutral) and M2 (selection) was used to test for selection (Figure 1.2b). M1 (neutral) allows for 2 rate classes, one with $\omega = 0$ and the other with $\omega = 1$. M2 is a variant of M1 and adds an additional unconstrained rate class where ω can be greater than 1. The stringent nature of M2 allows for either a class of sites under weak purifying selection or positive selection. M2 likelihood optimization can be affected by local optima (Yang et al. 2000; Anisimova et al. 2001; Wong et al. 2004). To alleviate this issue , starting ω values both above and below 1 were used. 3) The comparison of M7 (beta) and M8 (beta&ω) was used to test for positive selection (Figure 1.2c). These models allow for more continuous variation in substitution rates across sites. In both M7 and M8, there are 10 rate classes, of a fixed proportion of site, constrained to $\omega \leq 1$, where the shape of the distribution is defined by an additional parameter, beta. In M8 (beta&ω), an additional rate class is unconstrained. Again, to compensate for local optima effects, starting ω values both above and below 1 were used.

Yang and Nielsen's Model B (2002) was used to test for differences between clear and turbid water lineages. Model B is a derivative of M3 (discrete), which simultaneously allows for site and branch heterogeneity in relative substitution rates. First, both turbid lineages were simultaneously compared to the clear water lineages. Each turbid lineage was then tested individually with the other turbid lineage removed

from the analysis. M3 (discrete) served as the null hypothesis for all Model B comparisons.

Site Predictions

Sites that are likely to be involved in the functional differentiation of cichlid opsin genes are determined using two methods. First, PAML uses an empirical Bayes approach to identify amino acid sites that are likely to have been under positive selection. Second, previously published structural and functional studies are used to predict functionally important substitutions. All variable sites were mapped onto the rhodopsin crystal structure (1L9H; Palczewski et al. 2000; Teller et al. 2001). Functionally relevant sites are typically in the chromophore binding pocket and often involve a change in amino acid polarity. While variation at a binding pocket site does not prove changes in spectral sensitivity, nearly all known spectral tuning sites are in the retinal binding pocket (Yokoyama 2002; Takahashi and Ebrey 2003).

Results

Opsin Sequences

Consistent with the close phylogenetic relationships of the species sampled, most nucleotide sites were invariant in pair-wise comparisons. In total, however, approximately 5-10% of sites were variable, out of an average 1035 bp of sequence for each gene. The least variation was seen amongst SWS2a and Rh1, with 4.7% and 5.1% variable nucleotide sites, respectively. SWS2b, Rh2, and LWS had intermediate proportions of variable nucleotide sites, at 6.8%, 7.5%, and 7.8%, respectively. SWS1 had the highest variation, with 10.5% of nucleotide sites being variable. Interestingly, *N. brichardi* and *T. duboisi* have accumulated frameshift mutations in SWS1 (90) and SWS2a (253), and SWS2b (97), respectively. However, when translated without frameshift mutations, I do not observe an excess of substitutions for any of the pseudogenes, which suggests that the nonfunctionalization has been fairly recent. Further, both loci were homozygous in the individuals sequenced. This suggests that these differences may be fixed or at least at high frequency.

Phylogenetic Reconstructions

The small number of substitutions among the 17 species used in this analysis minimized the need for multiple hit corrections of sequence divergence. Several substitution models were implemented and each gave very similar divergence estimates (data not shown). The Tamura-Nei model was chosen for subsequent analyses.

In their simulation study, Anisimova et al. (2001) detected positive selection using the LRT for tree lengths as small as 0.11 (nucleotide substitutions per codon along the tree). They documented the conservative nature of the LRT at low tree lengths, but

also showed that this can be remedied by increasing the number of sequences analyzed. Tree lengths varied among the genes analyzed in this study. The SWS1 tree had the largest tree length of 0.39. The SWS2a tree had the smallest tree length of 0.16. SWS2b, Rh1, Rh2, and LWS had intermediate tree lengths of 0.24, 0.23, 0.28, and 0.29, respectively.

Overall tree topologies were largely conserved among unrooted gene trees and were consistent with published East African cichlid phylogenies based on mitochondrial, microsatellite, and nuclear markers (Kocher et al. 1995, Streelman et al. 1998; Albertson et al. 1999) (Figure 1.1). Among the Lake Malawi species in particular, phylogenetic relationships were highly variable. Rock and sand dwellers were usually intermingled except in the Rh1 tree. The lack of perfect agreement among cichlid opsin gene trees is consistent with observations by other investigators that ancestral polymorphisms have not completely sorted among these species (Moran and Kornfield 1993). Because of this, the phylogenetic relationships reconstructed from single gene loci may not reflect relationships at other loci or at the organismal level (Pamilo and Nei 1988; Streelman et al. 1998; Albertson et al. 1999; Kocher 2003). It is for this reason that several gene trees are used in this study and I do not rely solely on a consensus tree or putative species tree, as other researchers have done (Sugawara, Terai, and Okada 2002).

Yang et al. (2000) and Ford (2001) have asserted that mild uncertainty in tree topology only has a limited effect on the LRT. However to test for the effects of tree topology, I tested each gene with the corresponding gene tree as well as the other 5 noncorresponding gene trees, and the species tree. When comparing the maximum likelihood scores for these seven trees, the corresponding gene tree always gave the best

likelihood scores (Table 1.2). Trees most similar to the corresponding gene tree usually were better performers.

Maximum Likelihood Analysis

LRT's based on poorly fitting trees were more likely to be significant (Table 1.2). In general, LRT's based on corresponding gene trees were the most conservative. Since corresponding gene trees provide the most rigorous examination of the data, they will be the focus of the remainder of the results and the discussion.

LRT results were variable among cichlid opsin genes. All (M0-M3) rate heterogeneity LRT's were significant ($p<0.05$), except for SWS2b (Table 1.2). This indicates that relative rates of substitution are variable among sites in all opsin classes, except for SWS2b. Both (M1-M2) and (M7-M8) LRT's were significant ($p<0.05$) for all remaining opsins, except SWS1. LRT results, ω estimates (Table 1.3), and the prediction of sites that have evolved under positive selection (discussed below) suggest that a portion of sites in SWS2a, Rh2, LWS, and Rh1 cichlid opsin genes have evolved under positive selection.

(M3 - Model B) branch-site variable LRT's detected significant differences ($p<0.05$) in the relative rates of substitution between turbid and clear water species for SWS1, Rh2, LWS and Rh1 genes (Table 1.4a). When Lake Victoria (Table 1.4b) and Nile River (Table 1.4c) lineages were tested individually, the Lake Victoria lineage was never indicated to have a significantly different relative rate of substitution from the clear water species, except for Rh1. In contrast, the Nile lineage was detected to be significantly different from clear water lineages in all opsin genes except SWS2a and Rh1.

Site Predictions

Based on the x-ray crystallographic studies of bovine rhodopsin, nearly all sites that modify the spectral properties of visual pigments are in the vicinity of/oriented toward the chromophore and make up the chromophore binding pocket. Sites that are oriented toward the chromophore roughly comprise the set of possible tuning sites. Possible spectral tuning sites show trans-specific variation in all opsin classes (Table 1.5). The effects of substitution at many of these possible tuning sites have been characterized through mutagenesis studies (reviewed in Takahashi and Ebrey 2003; Yokoyama and Tada 2003). Those sites shown through mutagenesis studies to control opsin tuning comprise known tuning sites. With the exception of the Rh1 class, known tuning sites are trans-specifically variable in all opsin classes (Table 1.5).

PAML predicted amino acid sites ($p \leq 0.05$) to be under positive selection for all opsin classes where LRT's were significant (Table 1.5). I report the summation of PAML predictions from all models where sites are predicted. In the case of Rh2 and LWS M3, all nonsynonymous substitutions are predicted to be under positive selection. Since this is an unreasonable result, the predictions of these specific models are not included unless other models support them. Rh1 M8 predicts all but two nonsynonymous substitutions as having been under positive selection. I include these results since not all sites are predicted. Although most predictions (81%) fell within transmembrane domains, a much smaller proportion (29%) of those site predictions are in the chromophore binding pocket. A total of 31 possible spectral tuning sites are variable within the data. Further, many possible spectral tuning sites are not predicted to have been under positive selection (65%). Some sites within the chromophore binding pocket

have been previously characterized through mutagenesis studies and make up all known spectral tuning sites. A total of three known tuning sites are also PAML predictions. An additional eight known tuning sites are variable among cichlid opsins. Finally, PAML identifies eight possible tuning sites that have not been previously characterized by mutagenesis methods.

Discussion

This study examines the evidence for adaptive molecular evolution in cichlid opsin genes in response to gross differences in environmental light conditions. One might be tempted to conclude that the cichlid opsin gene with the least nucleotide divergence (SWS2a) has experienced the least divergent selection, relative to other opsin genes. However, nucleotide divergence alone is a poor comparative estimator of selective pressure as there are other factors that can be responsible for the observed variation in nucleotide sequence divergence, namely differences in the background rates of evolution, as determined by the rates of synonymous substitution. Variation, among genes, in the rates of synonymous substitution has been documented in previous studies (e.g., Senchina et al.2003). I also observed variation in synonymous substitution rates among the cichlid opsins, further supporting previous findings that the relative selective regime is independent of the absolute number of nucleotide substitutions. Codon usage bias, GC content, and genomic location have all been proposed as possible causes of rate variation among genes (Wolf et al. 1989; Zhang et al. 2002; Senchina et al. 2003). Interestingly, in cichlids, SWS2a, SWS2b, and LWS are located in a tandem array with 4.5 and 6 Kb, respectively, of intervening sequence (Carleton and Kocher 2001). Given the close physical proximity of these 3 genes, it is of note that they still have very different rates of substitution, as can be extrapolated from the proportion of variable sites (SWS2a - 0.047, SWS2b - 0.068, and LWS - 0.078).

LRT Evidence for Positive Selection among Cichlid Opsins

Site-specific LRT's indicate that amino acid sites in Rh2, LWS, and Rh1 have evolved at variable rates. The site-specific LRT's also show that these same opsins have

experienced positive selection, a result that is supported by all model comparisons examined. Further, branch/site-specific LRT's indicate that relative rates of substitution are variable between turbid and clear water species for Rh2 and LWS opsin genes. The failure of all LRT's indicate that SWS2a and SWS2b have evolved solely under purifying selection. The failure of selection and positive selection LRT's indicate that SWS1 has evolved under a regime of neutral evolution.

Variation Between Turbid and Clear lineages

Positive selection was detected among Rh2, LWS, and Rh1 genes using site-specific models. Branch/site-specific models identified Rh2, LWS and Rh1 as having a class of sites with different relative rates of substitution between turbid and clear water species (Table 1.4a). This suggests that longer wavelength-absorbing genes are evolving under selective pressure induced by photic environment. Branch/site-specific differences associated with water clarity are also seen in the SWS1 opsin, despite the failure of site-specific models to detect positive selection. This might indicate that some SWS1 opsins have evolved under neutral evolution while others have been more restrained under purifying selection.

Examination of each turbid lineage separately yields only partially interpretable results (Table 1.4b, c). Since examination of individual turbid lineages required the removal of the other turbid lineage, the data available was also reduced. Reduction in data has been documented to reduce the power of the LRT (Anisimova et al. 2001). This reduction in power was most pronounced upon removal of the Nile lineage, which is among the most basal. The decrease in power caused by lineage removal may have decreased our ability to individually test the Lake Victoria lineage. This explains the

lack of detectable difference between the Lake Victoria lineage and all the clear water lineages (Table 1.4b). Further, Wong et al. (2004) note that in cases of directional selection where mutations rapidly reach fixation, the current methods may have difficulty. Data reduction did not appear to be a problem in the analysis of the Nile lineage (Table 1.4c) as a the results show a similar pattern as when the Lake Victoria and the Nile River lineages are averaged.

Another reason I may not detect differences between Lake Victoria and clear lineages is that these tests average the selective regime over time. The signature of selection can be lost if there are multiple shifts in selective pressure over time. Several studies suggest that ancestors to the Lake Victoria lineage may have colonized other lakes, subsequently returning to the rivers before colonizing Lake Victoria (Nagl et al. 2000; Seehausen et al. 2003; Verheyen et al. 2003). If a previous colonization event or long-term shift in photic environment has occurred, this could potentially eliminate/diminish the difference in relative substitution rates among turbid Lake Victoria and present day clear water lineages because of averaging of selective signature over time.

Evidence of Functional Divergence among Cichlid Opsins

All opsin genes have substitutions that are known tuning sites, except Rh1 (Table 1.5). Only the SWS1, Rh2, and LWS opsin classes have substitutions that are known to tune their respective classes. The trans-specific variation at both possible and known spectral tuning sites demonstrates the potential for functional divergence in all opsin classes. PAML predicts that several known and possible tuning sites listed in Table 1.4 may be under positive selection. Many of the sites predicted by PAML have not been

characterized and therefore are particularly interesting candidates for study by site-directed mutagenesis.

If differences between turbid and clear environments have driven the evolution of East African cichlid opsin genes, one would expect that turbid lineages would have more long wavelength shifting substitutions, since turbid environments are longwave shifted. One would also expect that turbid lineages would have unique sets of possible spectral tuning substitutions, relative to clear lineages. Independently evolved turbid lineages need not use the same spectral tuning substitutions; however, the substitutions should be unique relative to clear lineages. Since many of the substitutions observed are different from those previously studied or are at uncharacterized sites, the magnitude/directionality of spectral shifts caused by some substitutions cannot be predicted. Table 1.6 shows that only in SWS1 do turbid lineages have unique sets of possible spectral tuning substitutions (Nile lineage – 48,114, 118, 197, 204, 208, and 298; Lake Victoria lineage – 114, 160, and 204). All but sites 48 and 160 were predicted by PAML to have been under positive selection. E197Q, a known shortwave shifting substitution, causes a –4 nm spectral shift. This is consistent with our expectations. Also, site 114 is known to be important in SWS1 tuning although in mammals it acts in a synergistic manner in coordination with other sites that are not variable among these data (Shi et al. 2001; Fasick et al. 2002). Among other opsin genes, there may be a lack of functional differentiation with respect to photic environment or alternatively, other sites that have not been considered as possible tuning sites might be responsible for functional differentiation. PAML highlights several uncharacterized possible tuning sites that may be import in spectral tuning (SWS1 – 204 and 208; LWS – 262; Rh1 - 41, 163, 298, and 299). These sites

represent good candidates for site-directed mutagenesis studies, which will be needed to determine the effects and uniform directionality of spectral shifts (directional/positive selection) among lineages.

Conclusions

Given the remarkable ability of cichlids to utilize multiple nonexclusive mechanisms to tune visual sensitivity, the detection of variation in natural selection and the prediction of sites under positive selection are of note. Cichlids show variation in opsin gene expression, with different species expressing different subsets of cone opsins (Carleton and Kocher 2001). Further, variation in the molecular mechanisms of spectral tuning, both across sites and classes, may have an effect on the analysis. For example in bird SWS1, site 86 causes a 75 nm spectral shift (Shi, Radlwimmer, and Yokoyama 2001). Most known tuning sites, however, have a much smaller effect of less than 10 nm (reviewed in Yokoyama 2002; Takahashi and Ebrey 2003). Finally, species specific ecological factors are also likely to play a role (Cummings and Partridge 2001). The present study focuses on gross differences in water clarity, although other factors such as depth are likely to be important in shaping visual sensitivity. A future analysis focusing on depth may therefore prove rewarding.

Unlike other molecules that have been the focus of molecular evolutionary computational studies, the clear link between opsin function and the environment, the availability of robust functional assays (i.e. spectral absorbance and transducin activation), and the rich body of mutagenesis studies provide researchers with a well-characterized system to test molecular evolutionary models and specific ecological hypotheses. In this work, I demonstrated statistical evidence of positive selection in cichlid opsin genes. I then showed that there are differences in selective pressure among lineages that are known to have long-term residence in turbid habitats compared to

lineages that inhabit clear photic environments. Finally, I identified candidate spectral tuning sites in cichlid opsin classes.

Photic environment-driven evolution may have played a significant role in the subsequent evolution of male nuptial hue usage and the diversity of color patterns for which cichlids are so renowned. Already researchers have noted that the color palette used by species living in turbid habitats is generally long wavelength-shifted (Seehausen 1999). This work begins to demonstrate a fundamental mechanism through which changes in hue usage are likely to be modulated.

Table 1.1
Study species.

Species	Location	Photic Environment	Accession Number					
			LWS	Rh2	SWS2b	SWSa	SWS1	Rh1
Oreochromis niloticus	Nile River	Turbid	AF247128	AF247124	AF247120	AF247116	AF191221	AY775108
Opthalmotilapia ventralis	Lake Tanganyika	Clear	AY780512	AY775067	AY775063	AY775060	AY775097	AY775109
Neolamprologus brichardi	Lake Tanganyika	Clear	AY780513	AY775068	AY775062	AY775072	AY775096	AY775110
Tropheus duboisi	Lake Tanganyika	Clear	AY780516	AY775089	AY775082	AY775073	AY775099	AY775111
Pundamilia nyererei	Lake Victoria	Turbid	AY673688	AY673698	AY673708	AY673718	AY673728	AY673738
Pundamilia pundamilia	Lake Victoria	Turbid	AY673689	AY673699	AY673709	AY673719	AY673729	AY673739
Aulonocara heuseri	Lake Malawi	Clear	AY780517	AY775090	AY775082	AY775074	AY775100	AY775112
Labeotropheus fuelleborni	Lake Malawi	Clear	AF247127	AF247123	AF247119	AF247115	AF191223	AY775113
Metriaclima zebra	Lake Malawi	Clear	AF247126	AF247122	AF317674	AF247114	AF191219	AY775114
Melanochromis auratus	Lake Malawi	Clear	AY780518	AY775091	AY775084	AY775076	AY775101	AY775115
Lethrinops parvidens	Lake Malawi	Clear	AY780519	AY775092	AY775087	AY775077	AY775102	AY775116
Tyrannochromis maculatus	Lake Malawi	Clear	AY780520	AY775093	AY775086	AY775078	AY775103	AY775117
Cynotilapia afra	Lake Malawi	Clear	AY780521	AY775094	AY775088	AY775079	AY775104	AY775118
Mylochromis lateristriga	Lake Malawi	Clear	AY780522	AY775095	AY775085	AY775075	AY775105	AY775119
Labiochromis chisumulae	Lake Malawi	Clear	AY780515	AY775069	AY775064	AY775081	AY775098	AY775120
Copadochromis borleyi	Lake Malawi	Clear	AY780514	AY775071	AY775065	AY775061	AY775106	AY775121
Stigmatochromis modestus	Lake Malawi	Clear	AY780523	AY775070	AY775066	AY775080	AY775107	AY775122

Table 1.2
Site variable LRT results. Shading is used to indicate the corresponding gene tree for each opsin gene.

Gene	Tree	Maximum Likelihood Score						Likelihood Ratio Test Statistic			P-value		
		M0	M3	M1	M2	M7	M8	M3-M0	M2-M1	M8-M7	M3-M0	M2b-M1	M8-M7
SWS1	SWS1	-1997.45	-1986.69	-1989.13	-1986.88	-1989.17	-1986.85	21.52	4.49	4.64	2.5E-04	1.1E-01	9.9E-02
	SWS2a	-2086.69	-2050.95	-2067.08	-2052.83	-2074.24	-2052.83	71.47	28.50	42.82	1.1E-14	6.5E-07	5.0E-10
	SWS2b	-2091.41	-2048.35	-2073.52	-2051.63	-2674.81	-2055.12	86.12	43.78	1239.38	8.8E-18	3.1E-10	7.4E-270
	Rh2	-2060.90	-2036.43	-2045.35	-2036.61	-2045.38	-2036.46	48.95	17.47	17.85	6.0E-10	1.6E-04	1.3E-04
	LWS	-2071.16	-2041.62	-2054.50	-2043.27	-2054.69	-2043.38	59.08	22.47	22.61	4.5E-12	1.3E-05	1.2E-05
	Rh1	-2093.04	-2056.67	-2073.84	-2058.70	-2073.96	-2059.85	72.73	30.28	28.23	6.0E-15	2.7E-07	7.4E-07
	Sp	-2070.18	-2041.75	-2052.78	-2042.45	-2052.86	-2042.45	56.86	20.65	20.81	1.3E-11	3.3E-05	3.0E-05
SWS2a	SWS1	-1776.93	-1762.19	-1771.87	-1762.23	-1771.88	-1762.60	29.48	19.29	18.56	6.2E-06	6.5E-05	9.3E-05
	SWS2a	-1744.07	-1739.34	-1742.66	-1739.37	-1742.81	-1739.43	9.48	6.58	6.75	5.0E-02	3.7E-02	3.4E-02
	SWS2b	-1766.13	-1757.74	-1762.99	-1757.76	-1763.00	-1757.86	16.80	10.46	10.29	2.1E-03	5.3E-03	5.8E-03
	Rh2	-1758.69	-1752.08	-1756.27	-1752.08	-1756.34	-1752.08	13.22	8.38	8.51	1.0E-02	1.5E-02	1.4E-02
	LWS	-1766.34	-1748.87	-1760.47	-1748.91	-1760.47	-1749.24	34.94	23.11	22.46	4.8E-07	9.6E-06	1.3E-05
	Rh1	-1780.61	-1763.30	-1774.12	-1763.35	-1774.16	-1763.66	34.62	21.55	21.00	5.6E-07	2.1E-05	2.8E-05
	Sp	-1778.55	-1763.19	-1772.58	-1763.21	-1772.60	-1763.36	30.73	18.74	18.48	3.5E-06	8.5E-05	9.7E-05
SWS2b	SWS1	-1992.33	-1968.13	-1982.05	-1969.41	-1982.28	-1968.14	48.39	25.29	28.27	7.8E-10	3.2E-06	7.3E-07
	SWS2a	-1982.76	-1971.84	-1977.51	-1972.04	-1977.58	-1971.84	21.85	10.94	11.48	2.1E-04	4.2E-03	3.2E-03
	SWS2b	-1923.41	-1921.75	-1922.45	-1921.84	-1921.85	-1921.75	3.32	1.21	0.19	5.1E-01	5.4E-01	9.1E-01
	Rh2	-1934.63	-1931.22	-1932.61	-1931.23	-1932.69	-1931.22	6.81	2.78	2.92	1.5E-01	2.5E-01	2.3E-01
	LWS	-1978.58	-1960.07	-1969.35	-1960.36	-1960.39	-1960.07	37.02	17.98	0.64	1.8E-07	1.2E-04	7.3E-01
	Rh1	-2012.50	-1986.66	-2000.60	-1987.47	-2000.78	-1986.67	51.68	26.26	28.24	1.6E-10	2.0E-06	7.4E-07
	Sp	-2002.82	-1977.88	-1991.35	-1978.49	-1991.60	-1977.88	49.88	25.72	27.43	3.8E-10	2.6E-06	1.1E-06
RH2	SWS1	-2204.48	-2140.95	-2168.81	-2140.95	-2169.12	-2140.95	127.05	55.71	97.47	1.7E-26	8.0E-13	6.9E-22
	SWS2a	-2164.11	-2117.12	-2138.84	-2120.38	-2138.84	-2117.78	93.99	36.92	36.92	1.9E-19	9.6E-09	9.6E-09
	SWS2b	-2160.12	-2114.40	-2135.37	-2115.83	-2135.37	-2115.11	91.45	39.08	39.09	6.5E-19	3.3E-09	3.3E-09
	Rh2	-2053.21	-2037.03	-2042.63	-2037.27	-2042.62	-2037.16	32.36	10.71	10.69	1.6E-06	4.7E-03	4.8E-03
	LWS	-2206.79	-2138.04	-2169.65	-2138.04	-2170.02	-2138.04	137.49	63.21	63.96	9.7E-29	1.9E-14	1.3E-14
	Rh1	-2223.11	-2154.35	-2185.54	-2154.35	-2186.67	-2154.35	137.53	62.39	64.64	9.6E-29	2.8E-14	9.2E-15
	Sp	-2216.11	-2148.87	-2179.90	-2148.87	-2180.20	-2148.87	134.46	62.05	78.49	4.3E-28	3.4E-14	9.1E-18
LWS	SWS1	-2031.30	-1991.32	-2011.29	-1990.77	-2014.82	-1990.94	79.96	41.02	47.76	1.8E-16	1.2E-09	4.3E-11
	SWS2a	-2049.91	-2005.61	-2027.90	-2006.25	-2028.35	-2006.48	88.59	43.30	43.74	2.6E-18	4.0E-10	3.2E-10
	SWS2b	-2054.13	-2009.39	-2031.96	-2008.99	-2033.15	-2010.09	89.46	45.94	46.12	1.7E-18	1.1E-10	9.7E-11
	Rh2	-2032.02	-1989.77	-2011.77	-1989.65	-2012.08	-1989.79	84.49	44.24	44.59	1.9E-17	2.5E-10	2.1E-10
	LWS	-1970.72	-1950.35	-1960.48	-1950.42	-1960.50	-1951.33	40.74	20.12	18.35	3.1E-08	4.3E-05	1.0E-04
	Rh1	-2027.91	-1995.66	-2012.53	-1995.98	-2012.57	-1998.00	64.49	33.09	29.15	3.3E-13	6.5E-08	4.7E-07
	Sp	-2033.96	-1990.41	-2012.90	-1991.02	-2013.22	-1991.22	87.10	43.75	44.01	5.4E-18	3.2E-10	2.8E-10
RH1	SWS1	-2011.59	-1918.58	-1981.39	-1927.21	-1981.74	-1931.22	186.02	108.35	101.04	3.8E-39	3.0E-24	2.8E-19
	SWS2a	-1994.06	-1907.51	-1964.85	-1915.78	-1964.89	-1918.26	173.11	98.14	93.27	2.3E-36	4.9E-22	1.0E-18
	SWS2b	-1985.81	-1900.84	-1956.98	-1910.37	-1957.04	-1911.43	169.94	93.22	91.21	1.1E-35	5.7E-21	1.7E-18
	Rh2	-1990.57	-1901.39	-1960.96	-1910.32	-1961.01	-1913.97	178.37	101.28	94.08	1.7E-37	1.0E-22	4.4E-19
	LWS	-1982.74	-1898.96	-1953.27	-1903.80	-1956.58	-1906.63	167.56	98.93	99.90	3.5E-35	3.3E-22	3.3E-20
	Rh1	-1912.17	-1854.88	-1892.63	-1860.35	-1892.90	-1861.20	114.58	64.57	63.40	7.7E-24	9.5E-15	7.0E-14
	Sp	-2014.57	-1914.67	-1981.71	-1923.41	-1981.71	-1927.38	199.79	116.60	108.65	4.2E-42	4.8E-26	5.3E-21

Table 1.3
Nonsynonymous/synonymous rate ratio (ω).

				Additional class w>1	
Gene	**Model**	**ω**	**% sites**	**ω**	**% sites**
SWS1	M3	2.29	0.155		
SWS2a	M3	7.21	0.057		
	M2	7.16	0.050		
	M8	3.74	0.141		
Rh2	M3	5.57	0.033	1.80	0.236
	M2	2.66	0.229		
	M8	3.03	0.164		
LWS	M3	9.61	0.029	1.91	0.186
	M2	7.00	0.057		
	M8	3.72	0.162		
Rh1	M3	388.72	0.003	11.59	0.080
	M2	17.54	0.055		
	M8	14.07	0.069		

Table 1.4
Branch-site variable LRT results. "MB" refers to Model B. "*" indicates a significant LRT statistic ($p \leq 0.05$; df = 2).

a.

Turbid-Clear Branch-Site Variable LRT Results

Gene	M3	MB	LRT	P-value	
SWS1	-1986.69	-1976.46	20.47	3.6E-05	*
SWS2a	-1739.34	-1738.86	0.96	6.2E-01	
SWS2b	-1921.75	-1918.84	5.84	5.5E-02	
Rh2	-2037.03	-2031.47	11.13	3.8E-03	*
LWS	-1950.35	-1943.59	13.51	1.2E-03	*
Rh1	-1860.42	-1856.51	7.82	2.0E-02	

b.

Lake Victoria-Clear Branch-Site Variable LRT Results

Gene	M3	MB	LRT	P-value	
SWS1	-1780.77	-1779.16	3.22	2.0E-01	
SWS2a	-1655.83	-1655.14	1.37	5.0E-01	
SWS2b	-1754.82	-1753.84	1.95	3.8E-01	
Rh2	-1889.40	-1889.38	0.02	9.9E-01	
LWS	-1813.14	-1813.14	0.00	1.0E+00	
Rh1	-1754.15	-1746.95	14.40	7.5E-04	*

c.

Nile River-Clear Branch-Site Variable LRT Results

Gene	M3	MB	LRT	P-value	
SWS1	-1945.88	-1934.63	22.52	1.3E-05	*
SWS2a	-1700.99	-1700.99	0.00	1.0E+00	
SWS2b	-1880.49	-1877.43	6.12	4.7E-02	*
Rh2	-2011.34	-2003.04	16.58	2.5E-04	*
LWS	-1895.04	-1887.88	14.31	7.8E-04	*
Rh1	-1771.39	-1771.30	2.18	3.4E-01	

Table 1.5
Comparison of PAML predictions and possible tuning sites. Only sites predicted by PAML to have been under positive selection with $p \leq 0.05$, for at least one model, are reported. Sites outside of TM domains are shaded. Possible spectral tuning sites are defined as sites within the chromophore binding pocket that are variable among cichlids. Known tuning sites are sites that have been previously characterized in site-directed mutagenesis studies that are variable among cichlids. "*" indicates that spectral tuning effects were only observed in the presence of substitutions at other specific sites.
[a] Shi et al. 2001
[b] Fasick et al. 2002
[c] Andres et al. 2001
[d] Nathans 1990b
[e] Nakayama and Khorana 1991
[f] Sakmar et al. 1989
[g] Yokoyama et al. 1999
[h] Asenjo et al. 1994

Table 1.5

Gene	PAML Predictions	Possible Tuning Sites	Known Tuning Sites	Spectral Shift	Substitution Studied	Cichlid Substitution
SWS1	21					
	34					
		44				
		46	46[ab]	*[ab]	F-L(SWS1)[ab]	F-T
		48				
		49	49[ab]	*[ab]	F-L(SWS1)[ab]	F-L
	57					
	82					
	114	114	114[ab]	*[ab]	A-G(SWS1)[ab]	A-S
		118				
		125	125[c]	-5[c]	L-N(Rh1)[c]	A-G
		160				
	197	197	197[d]	-4[d]	E-Q(Rh1)[d]	E-Q
	201					
	204	204				
	208	208				
	209					
	214					
		298				
SWS2a	-2					
	88					
	97					
		117	117[e]	-8[e]	A-F(Rh1)[e]	V-A
	147					
	165					
	287					
SWS2b		89				
		265	265[e]	-15[e]	W-Y(Rh1)[e]	W-Y
		269	269[f]	-11[f]	T-A(SWS2)[f]	T-A
		273				
Rh2	**107**					
		122	122[g]	-20[g]	E-Q(Rh1)[g]	E-Q
		207	207[h]	6[h]	L-M(Rh2)[h]	L-M
		212				
	218					
		273				

Gene	PAML Predictions	Possible Tuning Sites	Known Tuning Sites	Spectral Shift	Substitution Studied	Cichlid Substitution
		40				
LWS	**164**	164	164[i]	-7[i]	S-A(LWS)[i]	S-A
		203				
		261	261[i]	-10[i]	Y-F(LWS)[i]	Y-F
	262	262				
Rh1	22					
	41	41				
	42					
	50					
	95	95				
	104					
	133					
	158					
	159					
	162					
	163	163				
	165					
	166					
	169					
	173					
	213					
	217					
	218					
	255					
	256					
	263					
	297					
	298	298				
	299	299				
	304					
	336					

Table 1.6

Possible opsin tuning sites based on previous functional and structural studies. Sites that are known to control spectral tuning are indicated by dark vertical shading. Light horizontal shading indicates species which inhabit turbid habitats. (see Table 1.5 for references)

	Gene	SWS1													SWS2a	SWS2b				Rh2				LWS					Rh1				
	TM Region	TM 1	TM 1	TM 1	TM 1	TM 3	TM 3	TM 3	TM 4		TM 5	TM 5	TM 7		TM 3	TM 2	TM 6	TM 6	TM 6	TM 3	TM 5	TM 5	TM 6	TM 1	TM 4	TM 5	TM 6	TM 6	TM 1	TM 2	TM 4	TM 7	TM 7
	Site	44	46	48	49	114	118	125	160	197	204	208	298		171	198	265	267	273	202	207	212	217	40	46	103	126	132	171	195	163	189	199
Location	Concensus	M	F	F	F	S	S	A	T	Q	T	M	A		V	V	W	A	I	E	M	F	G	A	A	Y	Y	C	G	V	A	A	S
Nile R.	*O niloticus*			L		A	A			E	I	L	S						V						S							S	
L. Tanganyika	*O ventralis*					A		G				I	S		A	T	Y		V	Q	L		V		S							S	
L. Tanganyika	*N brichardi*				L										A	T	Y		V						S			I	A				A
L. Tanganyika	*T duboisi*	K	L			A					I							T															
L. Victoria	*P nyereri*					A			A		I							T												I	G		
L. Victoria	*P pundamilia*					A			A		I							T								F		I		I	G		
L. Malawi	*A heuseri*																																
L. Malawi	*L fuelleborni*																																A
L. Malawi	*M zebra*																																A
L. Malawi	*M auratus*																		F						S								
L. Malawi	*L parvidens*																							S	S							S	
L. Malawi	*T maculatus*																																
L. Malawi	*C afra*																					L					F						A
L. Malawi	*M lateristriga*																		F													S	
L. Malawi	*L chisumulae*																																
L. Malawi	*C borleyi*																		F													S	
L. Malawi	*S modestus*																							S									A
			ab		ab	ab		c		d					e		e	f		g	h				i		i						

Figure 1.1
Cichlid opsin unrooted neighbor-joining tree topologies. Tree topologies were generated based on Tamura-Nei distances and maximum likelihood estimates of gamma shape parameters. Additionally, both the putative species tree and star tree (not shown) were used in the analysis.

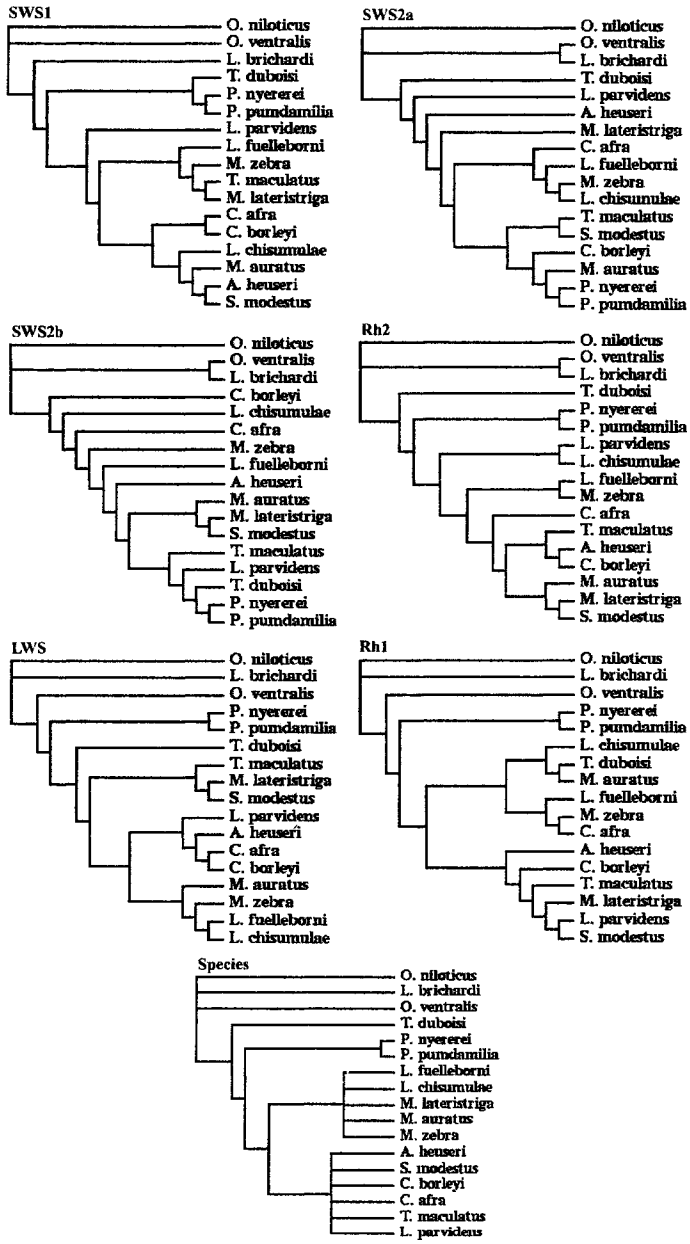
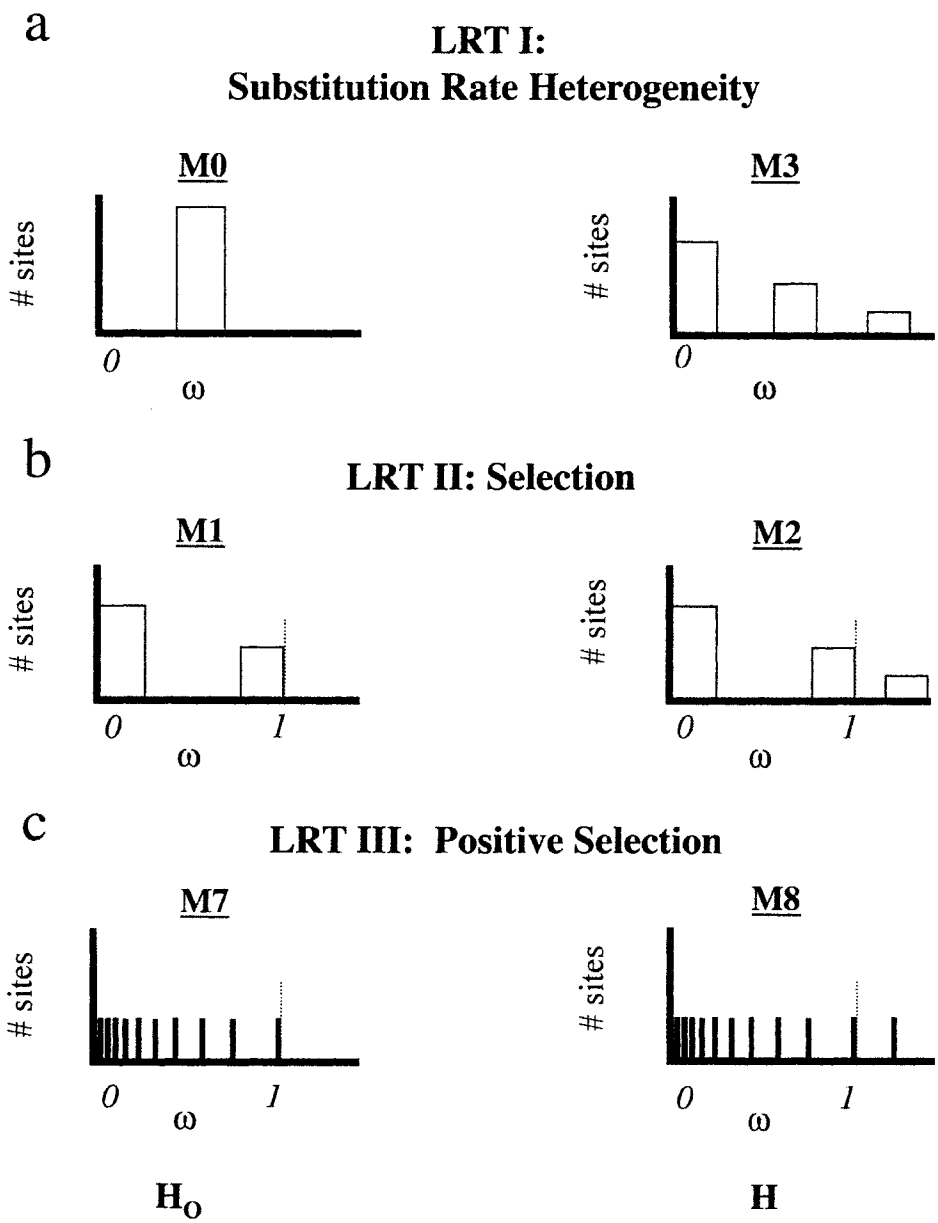


Figure 1.2
PAML model comparisons. PAML calculates a maximum likelihood value (ℓ) for each model (for each gene). Nested models are compared using a LRT (LRT = $2\Delta\ell$). The significance of the LRT is determined using a X^2 distribution, where the degrees of freedom are equal to 4, 2, and 2, respectably, for each test (see below).
a) The comparison of M0 (one rate) and M3 (discrete) was used to test for rate heterogeneity among amino acid sites.
b) The comparison of M1(neutral) and M2 (selection) was used to test for positive selection.
c) The comparison of M7 (beta) and M8 (beta&ω) was used to test for positive selection.



CHAPTER II

EVOLUTION OF THE CICHLID VISUAL PALETTE THROUGH ONTOGENETIC SUBFUNCTIONALIZATION OF THE OPSIN GENE ARRAYS

Abstract

The evolution of cone opsin genes is characterized by a dynamic process of gene birth and death through gene duplication and loss. However, the forces governing the retention and death of opsin genes are poorly understood. African cichlid fishes have a range of ecologies, differing in habitat and foraging style, which make them ideal for examining the selective forces acting on the opsin gene family. In this work, I present data on the riverine cichlid, *Oreochromis niloticus*, which is an ancestral outgroup to the cichlid adaptive radiations in the Great African lakes. I identify seven cone opsin genes with several instances of gene duplication. I also characterize the spectral sensitivities of these genes through reconstitution of visual pigments. Peak absorbances demonstrate that each tilapia cone opsin gene codes for a spectrally distinct visual pigment: SWS1 (360 nm), SWS2b (423 nm), SWS2a (456 nm), RH2b (472 nm), RH2a β (518 nm), RH2a α (528 nm) and LWS (561 nm). Furthermore, quantitative reverse transcription PCR at three ontogenetic time points demonstrates that although only four genes (SWS2a, RH2a α and β, and LWS) are expressed in adults, the other genes are all expressed during ontogeny. Therefore, subfunctionalization through differential ontogenetic expression is a key mechanism for preservation of opsin genes. The distinct peak absorbances of these preserved opsin genes provide a palette from which selection

creates the diverse visual sensitivities found among the cichlid species of the lacustrine adaptive radiations.

Introduction

Gene duplication has been recognized as important in the generation of evolutionary innovation (Ohno 1970; Francino 2005). Opsin genes readily lend themselves to studies of gene duplication and the fate of duplicate gene function. Opsin proteins, in complex with retinal chromophores, form visual pigments which control visual sensitivities. The functional link between opsin gene sequence and visual pigment peak absorption has been well documented through protein expression studies (Nathans et al. 1986; Asenjo et al. 1994; Wilkie et al. 2000; Yokoyama et al. 2000; Cowing et al. 2002; Cowing et al. 2002; Takahashi and Ebrey 2003; Hunt et al. 2004).

Opsin genes have undergone multiple gene duplication events. Early in the radiation of vertebrates, duplications of the ancestral vertebrate retinal opsin gene gave rise to five major evolutionary classes of vertebrate opsins: rod opsin (Rh1) and four cone opsins, long wavelength-sensitive (LWS), rod opsin-like (Rh2), short wavelength-sensitive 2 (SWS2), and short wavelength-sensitive 1 (SWS1) (Hisatomi et al. 1994; Yokoyama 1994; Chang et al. 1995; Collin et al. 2003). Gene duplications within an opsin class have also been found, such as the duplication of the primate LWS opsin, responsible for the independent evolution of trichromatic color vision in both Old and New World primates (Nathans et al. 1986; Jacobs et al. 1996; Dulai et al. 1999).

The duplication of opsin genes has been a common occurrence among teleost fishes. Duplications have been observed in all four cone opsin classes including; LWS (cavefish: (Yokoyama and Yokoyama 1990; Register et al. 1994); zebrafish: (Chinen et al. 2003); killifish: (Fuller and Travis 2004), Rh2 (cichlids: (Carleton and Kocher 2001); zebrafish: (Chinen et al. 2003); goldfish: (Johnson et al. 1993); herring, AF385829,

AF385830; turbot: AF385827, AF385828; smelt: (Minamoto and Shimizu 2005); and pufferfish: (Neafsey and Hartl 2005)), SWS2 (cichlids: (Carleton and Kocher 2001); killifish: (Fuller and Travis 2004)), and SWS1 (smelt: (Minamoto and Shimizu 2005)).

Cichlids had previously been thought to have five spectrally distinct cone opsin gene classes: LWS, Rh2, SWS2a, SWS2b, and SWS1 (Carleton and Kocher 2001). Recent sequencing of BAC clones containing the opsin genes from *Oreochromis niloticus* (Nile tilapia, referred to as tilapia for remainder of the paper) has revealed the presence of two other Rh2 genes (Carleton et al. unpublished data). Opsin genes were detected at three locations within the genome. The Rh2 genes were found in one tandem array, with the SWS2 and LWS genes forming a second array. The single SWS1 gene was isolated in a third location. In combination with recent functional characterization of cone opsin genes of closely related Lake Malawi cichlid species (Parry et al. 2005; Trezise and Collin 2005), these data indicate that tilapia has a total of seven cone opsin genes, not five as had been previously thought.

The revelation that tilapia might have seven cone opsin genes is interesting because opsin gene expression has so far only been detected for a subset of the five genes originally reported (Carleton and Kocher 2001). Why the remaining apparently functional, but unexpressed, cone opsin genes would have been preserved within the tilapia genome is unknown. Studies of gene duplicates show that genes that are not needed are quickly rendered nonfunctional through the accumulation of mutations (Lynch and Conery 2000; Lynch 2002). Nonfunctional genes may eventually be completely excised from the genome or decay to the point of being unrecognizable. Two nonexclusive paths may lead to gene preservation. One or both members of a gene pair

may evolve a new function through functional divergence (neofunctionalization) (Ohno 1970). Alternatively, the duplicate pair may partition the ancestral gene function (subfunctionalization) (Force et al. 1999). Recently, Rastogi and Liberles (2005) have proposed a more integrated view of the two paths. They argue that subfunctionalization is a transitional state in the process of neofunctionalization.

The aim of the current study is to determine why tilapia has maintained such an extensive complement of cone opsin genes when expression has only been detected for a subset. I first expressed each of the tilapia cone opsin genes and determined the peak absorbances of reconstituted visual pigments to establish whether the genes encode for spectrally different products. I then sampled embryonic, juvenile, and adult tilapia to examine the ontogeny of opsin gene expression and determine if temporal subfunctionalization had occurred. Finally, I compared the tilapia visual pigments with those used by the cichlids of the African lacustrine radiations to learn how gene preserveration through subfunctionalization sets the stage for new adult phenotypes. This is the first and most comprehensive in vitro expression and reconstitution study of all the major classes of cone opsin found in a single Acanthopterygian species.

Methods

cDNA Synthesis and Expression Constructs

Expression constructs were made for each of the opsin genes predicted from genomic sequence. Retinal tissues from individuals at different developmental stages were used to extract opsin mRNA. Retinas were homogenized and RNA extracted with Trizol (Invitrogen). Retinal RNA preparations were then reverse transcribed with a poly T primer and Superscript III Reverse Transcriptase (Invitrogen).

Expression primers were based on the sequences of previously reported tilapia opsin sequences (Carleton and Kocher 2001). Expression primers for the new Rh2 genes were designed based on the tilapia BAC sequences. All expression primers contained cloning and expression domains according to established methodologies (Parry et al. 2004). Primer sequences have been reported elsewhere (Parry et al. 2005) for the majority of genes studied. New expression primers were as follows:
GGCGGGAATTCCACCATGGCAGAAGAGTGGGG (LWS-EcoRI),
GGCGGGTCGACCAGGAGCCACAGAGGAGACC (LWS-SalI),
GGCGGGAATTCCACCATGAGGGGTAATCGTGATATGG (SWS2a-EcoRI),
GGCGGGTCGACCAGGCCCAACTTTGG (SWS2a-SalI),
The expression primers and Dynazyme Ext. (MJ Research) were used to amplify full-length cone opsin cDNA's. PCR products were digested with EcoRI (NE Biolabs) and SalI (NE Biolabs), and directionally cloned into pMT3. This mammalian expression vector contains the Rho 1D4 epitope used for the purification of the opsin protein (Franke et al. 1988). Constructs were sequenced through the entire length of the opsin gene insert and compared to previously reported tilapia opsin sequences to ensure fidelity.

Phylogenetic Analysis

Gene trees for each opsin class were generated from the tilapia cone opsin nucleotide coding sequences and a phylogenetically diverse sampling of fish retinal opsin sequences. Chicken (*Gallus gallus*) opsin genes were used as an outgroup in all opsin classes. Sequences were aligned using MEGalign (Lasergene). Gene trees were constructed based on nucleotide sequences from the coding region. Due to the variation in the lengths of both carboxy and amino termini, the regions of variable data were not included in the construction of phylogenies. Bootstrap consensus trees (1000 replicates, 50% majority rule) were calculated using PAUP (Swofford 2002). Bootstrap topologies were then used as a constraint in maximum likelihood estimation of gamma parameters. Maximum likelihood estimates of gamma parameters and Tamura-Nei distances were then used to generate neighbor-joining (NJ) (Saitou and Nei 1987) trees and to calculate bootstrap values.

Expression and Reconstitution of Visual Pigments

HEK 293T cells were transiently transfected with the pMT3 expression constructs using Gene Juice (Merck). Thirty 90 mm plates were used per experiment. Cells were harvested 48 h post-transfection and washed four times with PBS (pH 7.0), and the cell pellets stored at -80°C prior to generation of the pigments. Pigments were generated by suspending cells in PBS (pH 7.0) and incubating them with 40 μM 11-*cis*-retinal in the dark (Oprian et al. 1987). The pigment was solubilized from cell membranes (following (Parry et al. 2004)) and purified by immunoaffinity chromatography using an anti-1D4 antibody coupled to a CNBr-activated Sepharose column following the methods of Molday and MacKenzie (1983). Purified pigment was eluted from the column and stored

on ice. Absorbance spectra were recorded in the dark using a Spectronic Unicam UV500 dual-beam spectrophotometer. The sample was then treated with hydroxylamine to 56 mM or hydrochloric acid to 26 mM to create the retinal oxime, or acid-denatured pigment, respectively, and the spectra were again recorded. The post hydroxylamine or acid treatment spectra were subtracted from the dark absorbance spectra to produce difference spectra which were adjusted to zero by subtraction of a baseline based on points longwave of the absorbance peak. The λ_{max} (peak absorbance) values of expressed pigments were estimated by fitting visual pigment templates (Govardovskii et al. 2000) using the Solver add-in to MS Excel to vary the λ_{max} to obtain the best fit. Difference spectra were fitted with the appropriate Govardovskii template, with the appropriate retinal oxime or acid-denatured product subtracted. Values quoted in the text are taken from the difference spectra, to avoid distortion by the underlying absorbance and scatter of the protein.

Quantitative Real-Time PCR

Tilapia specimens were lab-bred and reared under standard conditions. Two individuals from each of the three age classes, embryo (16 days post fertilization [dpf]), juvenile (62 and 64 dpf), and adult (300 dpf), were sampled. Two replicates were performed for each individual.

Real-time RT-PCR was used to quantify relative cone opsin mRNA levels. Primers and probes were designed to amplify short (60-90 bp) fragments for each gene using Primer Express 1.5 (Applied Biosystems), as previously described by Carleton and Kocher (2001). Because the Rh2a α and β genes were so similar, I first analyzed the sum of these two in comparison to the other five genes (SWS1, SWS2a, SWS2b, Rh2b and

LWS). This utilized our previous set of primers and probes plus a new set for the Rh2b gene (forward: TGCTGCCCCCCCATTG; reverse: AGGTCCACAGGAAACCTGAA; and probe: TGGCTGGTCAAGGTACATTCCTGAGGGA). Then, the ratio between the two RH2a genes was analyzed using forward primers that distinguished them (RH2a α forward: CCATCACCATCACATCAGCTG; RH2a β forward: CACCATCACAATCACGTCTGCTAT). Relative gene expression was determined for the six opsin genes (with RH2a α and β combined) as a fraction of the total cone opsin genes expressed for an individual, following Carleton and Kocher (2001), according to:

$$T_i/T_{all} = (1/(1+E_i)^{Cti})/\sum(1/(1+E_i)^{Cti})$$

where T_i/T_{all} is the relative gene expression ratio for a given gene normalized by the total cone opsin genes expressed, E_i is the PCR efficiency for each gene, and Ct_i is the critical cycle number for each gene. Finally, the Rh2a expression was partitioned between Rh2a α and Rh2a β from the Cts measured using the unique forward primers for the two Rh2a genes to calculate their ratio and this ratio was then used to calculate the relative template amounts.

The extent of cross reactivity amongst the SWS2 and RH2 gene duplicates was quantified using the expression constructs as templates and measuring the critical cycle number for primer/probe combinations from related genes.

The relative PCR efficiency (E_i) of the six primer/probe sets was measured using a novel tool developed for this work. A construct containing amplicons (the amplified region) for each of the six opsin genes (including the fragment of RH2a common to both RH2aα and β) was used to normalize template amounts to a 1:1 ratio for all genes. The concatenated amplicon construct (CAC; Figure 2.1) was generated by first PCR

amplifying separate gene fragments for each of the opsin genes and then, restricting and ligating the fragments. The full length CAC was then sequenced. Rh2a had the highest relative PCR efficiency and was used to normalize the relative PCR efficiencies of the other opsin genes according to:

$(1 + E_{Rh2a})^{Ct\,Rh2a}/(1 + E_i)^{Cti} = 1$

where $C_{t\,Rh2a}$ represents the critical cycle number for the Rh2a gene. The relative efficiencies were averaged between all of the replicates and standard errors were determined.

To determine the absolute efficiency of Rh2a, critical cycle number was measured for a series of nine serial dilutions of cDNA covering a 1000 fold range. Absolute efficiency was then determined from the slope of a plot of ln(concentration) versus critical cycle number such that $E = [(\exp(-\text{slope}))-1]$. The absolute E for other primer/probe sets was calculated based on Rh2a as

absolute E_i=(relative E_i x absolute E_{Rh2a})

Efficiencies for Rh2a α and β were also determined using the slope from a dilution series plot. Tilapia specimens were lab-bred and reared under standard conditions. Two individuals from each of the three age classes, embryo (16 days post fertilization [dpf]), juvenile (62 and 64 dpf), and adult (300 dpf), were sampled. Two replicates were performed for each individual.

Results

Tilapia Opsin Gene Sequences

Complete opsin coding sequences were obtained for all seven tilapia cone opsin genes (LWS, Rh2a α, Rh2a β, Rh2b, SWS2a, SWS2b, SWS1). Individuals used in the generation of the opsin expression constructs were not the same as those used previously (Carleton et al. 2000; Carleton and Kocher 2001). There were a small number of nucleotide differences among the expression constructs when compared to the previous tilapia individual. All except two of these substitutions were synonymous. The two exceptions were both in the SWS1 coding sequence, although only one encoded an amino acid change (F214I; bovine rhodopsin numbering) within a transmembrane region (IV). However, structural studies (Palczewski et al. 2000) indicate that site 214 does not face into the chromophore binding pocket. This site varies among other African cichlid species, which suggests that this nonsynonymous difference is part of natural allelic variation and not a cloning artifact. Opsin gene sequences obtained have been deposited in the GenBank database (DQ235678-DQ235684).

Phylogenetic Relationships of Fish Opsin Genes

Figure 2.2 shows phylogenies of the fish cone opsin genes found in the major superorders of euteleost fish. Within each opsin class, gene relationships were generally consistent with the previously published evolutionary relationships of fishes, except where gene duplications had occurred (Nelson 1994; Kumazawa et al. 1999; Miya et al. 2003; Saitoh et al. 2003; Chen et al. 2004).

LWS genes from the two cichlid species cluster together with 100% bootstrap support (Figure 2.2A). Acanthopterygian LWS genes form a clade supported by a 99%

bootstrap score. The remainder of the tree is in agreement with previous studies of zebrafish and killifish LWS duplications and other vertebrate LWS genes (Chinen et al. 2003; Fuller and Travis 2004).

The Rh2 clade shows the greatest number of gene duplications. The tilapia Rh2a α and Rh2a β cluster has 100% bootstrap support, to the exclusion of killifish and medaka Rh2 genes (Figure 2.2B). This suggests that the divergence of tilapia Rh2a α and Rh2a β occurred after the cichlid/killifish-medaka split. Furthermore, Lake Malawi cichlids also have orthologs to all of the tilapia Rh2 genes (Parry et al. 2005), demonstrating that the duplication event that generated Rh2a paralogs occurred before the divergence of tilapia from the rapidly speciating lacustrine cichlids. In contrast, the tilapia Rh2a/Rh2b split is far older. Tilapia Rh2b clusters with medaka Rh2b with 100% bootstrap support, to the exclusion of Rh2a genes. Paracanthopterygii (e.g. cod) and Acanthopterygii (e.g. cichlids) Rh2a genes form a clade with 99% bootstrap support. This suggests that the divergence of the ancestral tilapia Rh2a and Rh2b predates the Acanthopterygii/Paracanthopterygii split, but occurred after the Paracanthopterygii/Protocanthopterygii (e.g. trout) split. These data support the findings of Neafsey and Hartl (2005), which suggest that other Paracanthopterygii and Acanthopterygii may have an ortholog to tilapia Rh2b, giving them at least two Rh2 genes. The remainder of the tree is in agreement with previous studies of ostariophysian Rh2 duplications (Chinen et al. 2003; Minamoto and Shimizu 2005).

Both tilapia and Malawi cichlid SWS2a and SWS2b opsins cluster independently with 100% bootstrap scores (Figure 2.2C). Cichlid SWS2a and SWS2b opsin genes cluster with killifish SWS2a and SWS2b with generally high bootstrap support, 79% and

98% respectively. Medaka SWS2 clusters with killifish and cichlid SWS2b genes (98% bootstrap support), suggesting that medaka might have or have had an additional SWS2 gene, orthologous to killifish and cichlid SWS2a. The SWS2 tree, as reported, parallels the Rh2 tree, with gene duplication events occurring near the base of the Paracanthopterygian/Acanthopterygian radiation. However, the topology of the SWS2 tree suggests that the SWS2a/SWS2b split occurred after the divergence from cod, in contrast to the Rh2a/Rh2 split, although the low bootstrap value (53%) cannot rule out the possibility that the duplication that led to SWS2a and SWS2b predates the Paracanthopterygii/Acanthopterygii divergence. The SWS2 tree is consistent with previous studies of SWS2 opsin genes (Carleton and Kocher 2001; Neafsey and Hartl 2005).

No new duplication events were observed or inferred among SWS1 opsins. Further, SWS1 gene relationships are in agreement with those of previous studies of SWS1 opsin duplications (Minamoto and Shimizu 2005).

Spectral Characteristics of Tilapia Visual Pigments

Expression and in vitro reconstitution of the seven tilapia cone opsin genes gave seven photosensitive pigments, confirming that all genes are indeed functional. Each pigment showed a spectrally distinct λ_{max}, spread across the entire visible spectrum: LWS 561 nm; Rh2Aα 528 nm; Rh2Aβ 518 nm; Rh2B 472 nm; SWS2A 456 nm; SWS2B 425 nm; SWS1 360 nm (Figure 2.3). The three Rh2 genes cover a large range from 472 to 528 nm, making this class spectrally very broad. The λ_{max} values obtained for these pigments agree well with those observed for closely related species (Parry et al. 2005; Jordan et al. in press).

Relative Opsin mRNA Expression by Quantitative Real-Time RT-PCR

The absolute PCR efficiencies determined from the relative PCR efficiencies in the CAC data were 0.84 (LWS), 0.93 (Rh2a), 0.78 (Rh2b), 0.85 (SWS2a), 0.84 (SWS2b), and 0.84 (SWS1). The absolute efficiencies for the Rh2a α and β genes were 0.75 and 0.8, respectively. Relative PCR efficiencies were used to calculate an average relative opsin expression for each of the three age classes.

Cross reactivities were minimal for the SWS2 gene duplicates with cross amplifications of 10^{-4} and 10^{-8} with the SWS2a and SWS2b primer sets respectively. Cross amplification was also small for the Rh2a and Rh2b primer sets at 10^{-7} and 10^{-5} respectively. There was some cross reactivity for the Rh2a α and β primer sets as these two genes are so similar in sequence. Cross amplification was 0.06 and 0.007 for Rh2 α and β respectively. However, this level of cross amplification is sufficiently low that these genes can be distinguished.

Gene expression changed considerably through the three ontogenetic stages examined (Figure 2.4). Net increases in relative gene expression were observed for LWS and SWS2a. Net decreases were observed for Rh2a α, Rh2b, SWS2b, and SWS1. The expression of Rh2a β was relatively constant through time. LWS was the most highly expressed of all opsins, making up nearly 70% or more of the total cone opsin gene expression for juvenile and adult age classes (Table 2.1). In the embryonic class, all opsins are expressed except SWS2a. By the juvenile age class, SWS2a is expressed while Rh2b and SWS1 expression fall to essentially zero. By the juvenile age class, Rh2a α expression is also dramatically reduced, dropping from 16% (embryonic) to 6.5%. By the adult age class, LWS opsin expression makes up 82.6% of the total cone

opsin expression. SWS2b expression falls from 5.2% (embryonic) to less than 1% of the total cone opsin expression. In contrast, SWS2a expression increases from undetected (embryonic) to 8.2%.

These results demonstrate that each of the opsin genes is functional and is expressed within the retina at some developmental stage. Visual system sensitivities change considerably from larva to adult. Two of the genes, SWS1 (360 nm) and RH2b (472 nm), are primarily larval genes. These have shorter wavelength-sensitivities relative to the adult genes, suggesting that larvae benefit from a shorter wavelength-sensitivity.

Discussion

Phylogenetic analysis of fish retinal opsin gene sequences in both current and previous studies provides overwhelming evidence of widespread gene duplication (Yokoyama and Yokoyama 1990; Johnson et al. 1993; Register et al. 1994; Carleton and Kocher 2001; Chinen et al. 2003; Fuller and Travis 2004; Minamoto and Shimizu 2005; Neafsey and Hartl 2005). However, despite strong phylogenetic evidence for their existence, many genes have yet to be isolated in the fishes studied to date. For example, phylogenetic inference would predict that acanthopterygian fishes may all have at least two SWS2 and two Rh2 genes. The divergence of SWS2a and SWS2b occurred after the divergence of Paracantopterygii (cod) and Acanthopterygii (cichlid) approximately 260 MYA: (Kumazawa et al. 1999), but in the early stages of the radiation of Acanthopterygii. The more ancient divergence of Rh2a and Rh2b predates the divergence of cod and Acanthopterygii, again, approximately 260 MYA (Kumazawa et al. 1999). Yet, only in pufferfish and cichlids have these genes been sequenced and only in cichlids have their expression been confirmed (Neafsey and Hartl 2005; Parry et al. 2005). It seems likely that orthologous genes will be found in other fish species.

All tilapia opsin genes code for spectrally distinct photopigments. Even the products of the most recent duplication event (Rh2a), which occurred over 10 MYA (Kocher et al. 1995), have diverged in λ_{max} by 11nm. The recent nature of this duplication would suggest that it is likely to be limited to the East African cichlids, which includes the adaptive radiations of Lakes Malawi, Tanganyika, and Victoria.

Among most other species sampled by either MSP or retinal mRNA extraction, there is no evidence that the full complement of cone opsin genes is expressed (Levine

and MacNichol 1979; Carleton and Kocher 2001). Our data for tilapia now demonstrate that these genes are expressed at different lifestages, and this may be true for other species. Alternatively, the possibility remains that extra cone opsin genes may be expressed outside of the retina, for example in the brain (Forsell et al. 2001; Forsell et al. 2002) and skin (Ban et al. 2005), although none have been shown to be expressed exclusively outside of the retina.

Genes that are not expressed are expected to evolve free of the constraints of selection. In the absence of selection, random substitutions rapidly accumulate, many of which degrade gene function or result in complete nonfunctionalization (i.e., Lynch and Conery 2003). Sampling tilapia opsin expression across ontogeny revealed that all tilapia opsin genes are expressed within the retina at some point in development. This would explain the retention in the tilapia genome of functional cone opsin genes, which are not expressed in adults. The current study reveals that differential expression across ontogeny may allow the functionality of all of the genes to be maintained by selection.

Ontogenetic changes in cone opsin gene expression have been reported across a diverse assemblage of fishes that include salmon (Cheng and Novales Flamarique 2004), zebrafish (Takechi and Kawamura 2005), and flounder (Mader and Cameron 2004). The phylogenetic diversity of these fishes suggests that ontogenetic changes in opsin gene expression are likely to be a common occurrence among fishes. Such changes in gene expression could have occurred in the ancestors of the East African lacustrine cichlid species, and may account therefore for the maintenance and retention of opsin genes in those species. Taken together, these data suggest that tilapia cone opsin genes have been retained through a process of both neofunctionalization, by accumulation of spectrally

modifying amino acid substitutions, and subfunctionalization, by differential expression over ontogeny.

A comparison of the peak absorbances of reconstituted pigments from tilapia and both in situ (determined by MSP) and reconstituted pigments from the Lake Malawi cichlid, *Metriaclima zebra*, shows that peak absorbances are largely similar among these species (Table 2.2), as has been predicted from sequence comparisons (Carleton and Kocher 2001). This is in spite of the 10 MY divergence time between these species (Kocher et al. 1995), as well as the differences in habitats where these species are found (Spady et al. 2005; Carleton et al. in press). In spite of the 12 nm difference in peak absorbance of Rh2b and the 8 nm difference in peak absorbance of SWS1, the tilapia cone pigments can be used to roughly predict the peak absorbances of the corresponding opsin genes of other East African cichlids, and therefore the visual sensitivities of those same species.

MSP recordings of single photoreceptor sensitivities have identified variable expressed major-photopigment complements among East African lacustrine cichlids. In the only Lake Tanganyika species sampled to date, *Astatotilapia burtoni*, photopigments with peak absorbances of 562 nm, 523 nm, and 455 nm have been identified (Fernald and Liebman 1980). In addition, the opsin genes sequenced from retinal cDNA for this species include SWS2a, Rh2a β and LWS (Halstenberg et al. 2005), although the presence of Rh2a α cDNA was not assayed, and therefore cannot be ruled out. Among Lake Malawi species three different cone pigment combinations have been observed. The most long-wave-sensitive complement has pigments with peak absorbances at 569 nm, 532 nm, and 455 nm (e.g. *Tramitochromis intermedius*, (Parry et al. 2005). The

other two pigment complements differ from each other only in the peak absorbance of the shortest wavelength pigment. In one complement, this pigment peaks in the violet (e.g. *Melanochromis vermivorous* with a 418 nm pigment (Parry et al. 2005)) whereas in the other, it peaks in the ultraviolet (e.g. *Metriaclima zebra* with a 368 nm pigment (Carleton et al. 2000)). These two pigment sets both include double cone pigments around 530 nm and 485 nm, (Levine and MacNichol 1979; Parry et al. 2005). Like the Lake Tanganyika species, *A. burtoni*, and the Lake Malawi species, *T. intermedius*, the Lake Victoria cichlid species, *Pundamilia nyererei* has pigments peaked at 568 nm, 535 nm, and 451 nm (Carleton et al. in press).

In vitro expression of visual pigments from tilapia and *M. zebra* (Parry et al. 2005) provide a link between cichlid photoreceptor sensitivities and the underlying opsin genes. Comparisons between MSP and opsin sequence data from the lacustrine species demonstrate that the visual pigments that are differentially expressed across cichlid species of the East African adaptive radiations of Lakes Tanganyika, Malawi and Victoria correspond to the full set of tilapia cone opsin genes (Figure 2.5).

With regard to Rh2a α and Rh2a β, the spectral similarity and the differences between the peak absorbances determined by MSP and in vitro expression (Parry et al. 2005) makes the designation of a cone class difficult, particularly when both cone types have not been identified, as is the case for the Lake Tanganyika species, *A. burtoni.* In several species from both Lakes Malawi and Victoria, both Rh2a α and Rh2a β cone classes have been observed, although Rh2a β cones are always very rare (Parry et al. 2005; Carleton et al. in press). The full set of cichlid opsin genes have therefore been used across the species to generate at least three different photopigment combinations.

The riverine tilapia is an outgroup to the lacustrine cichlid species and is in many ways representative of the ancestral state. This suggests that all genes are/have been available for expression in the lake species. Many of these species differentially utilize a subset of available genes to tune their visual sensitivities. The genes that I have characterized in tilapia represent the visual pigment palette from which the species of the East African adaptive radiations mix and match to generate diverse complements of photoreceptor sensitivities.

Table 2.1
Percentage of total opsin expression.

Gene	Peak Absorbance (nm)	Age Class		
		Embryo	Juvenile	Adult
LWS	560	38.6 ±15.9	69.4 ±4.8	82.6 ±2.9
Rh2a α	528	16.0 ±3.7	4.8 ±2.3	6.5 ±2.6
Rh2a β	517	1.5 ±1.1	6.9 ±1.6	2.2 ±1.3
Rh2b	472	19.1 ±6.1	0.3 ±0.3	0.1 ±0.1
SWS2a	456	0.0 ±0.0	5.9 ±2.1	8.2 ±2.1
SWS2b	425	5.2 ±1.6	11.9 ±2.2	0.3 ±0.2
SWS1	360	19.7 ±10.7	0.9 ±0.4	0.1 ±0.1

Table 2.2
Comparison of tilapia and Lake Malawi cichlid visual pigments. Peak absorbances marked with a "*" are from MSP recordings of M. vermivorus (556 nm) and T. intermedius (455 nm). All other peak absorbances are from in vitro expression and reconstitution experiments.

Species	LWS	Rh2a α	Rh2a β	Rh2b	SWS2a	SWS2b	SWS1
Tilapia	560	528	517	472	456	425	360
Metriaclima zebra[a]	556*	528	519	484	455*	423	368

[a] Parry et al. 2005

Figure 2.1
Concatonated amplicon construct (CAC). The CAC is a novel tool developed to obtain relative PCR efficiencies for real-time RT-PCR comparisons. Cichlid opsin cDNA fragments were directionally ligated using the indicated restriction sites. The fragments correspond to cDNA regions of the primers/probe used in quantitative RT PCR experiments. Arrows have been used to indicate fragment directionality.

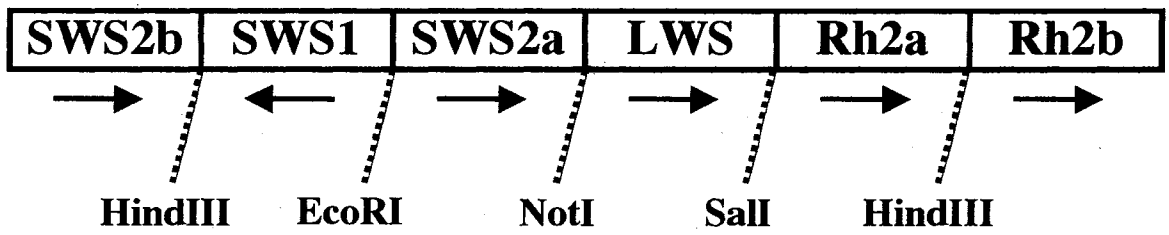


Figure 2.2
Fish opsin phylogenies. NJ trees were constructed for fish LWS (A), Rh2 (B), SWS2 (C) SWS1 (D), using gamma corrected Tamura-Nei distances. Bootstrap values are indicated when greater than 50%. Scale bars indicate the number of substitutions per 100 sites. The following sequences were included: cavefish (LWS g103, U12025; LWS g101, U12024; LWS R007, M90075), zebrafish (LWS1, AB087803; LWS2, ĀB087804; Rh21, AB087805; Rh2 2, AB087806; Rh2 3, AB087807; Rh2 4, AB087808; SWS2, BC062277; SWS1, AB087810), goldfish (LWS, L11867; Rh2 1, L11865; Rh2 2, L11866; SWS2, L11864; SWS1, D85863), smelt (LWS, AB098702; Rh2 1, AB098703; Rh2 2, AB098704; SWS1 1, AB098705; SWS1 2, AB098706), trout (LWS, AF425073; Rh2, AF425076; SWS2, AF425075; SWS1, AF425074), halibut (LWS, AF316498; Rh2, AF156263; SWS2, AF316497; SWS1, AF156264), flounder (LWS, AY631039; SWS2, AY631038), turbot (LWS, AF385826), pufferfish (LWS, AY598942; Rh2a, AF226989;SWS2, AY598947), medaka (LWS, AB001604; Rh2a, BJ495952, BJ491781; Rh2b, AB001603; SWS2, AB001602; SWS1, AB001605), killifish (LWSa, AY296740; LWSb, AY296741; Rh2, AY296739; SWS2a, AY296737; SWS2b, AY296736; SWS1, AY296735), Lake Malawi cichlid (LWS, AF247126; Rh2a α, DQ088651; Rh2a β , DQ088650; Rh2b, DQ088652; SWS2a, AF247114; SWS2b, AF317674; SWS1, AF191222), cod (Rh2, AF385824; SWS2, AF385822), bullhead (SWS2, CGO430489), and tilapia (LWS, AF247128; Rh2a α, DQ235683; Rh2a β, DQ235682; Rh2b, DQ235681; SWS2a, AF247116; SWS2b, AF247120; SWS1, AF191221). The outgroups were chicken (LWS, M62903; Rh2, M92038; SWS2, M92037; SWS1, M92039) and coelacanth (Rh2, AH007713).

Figure 2.2

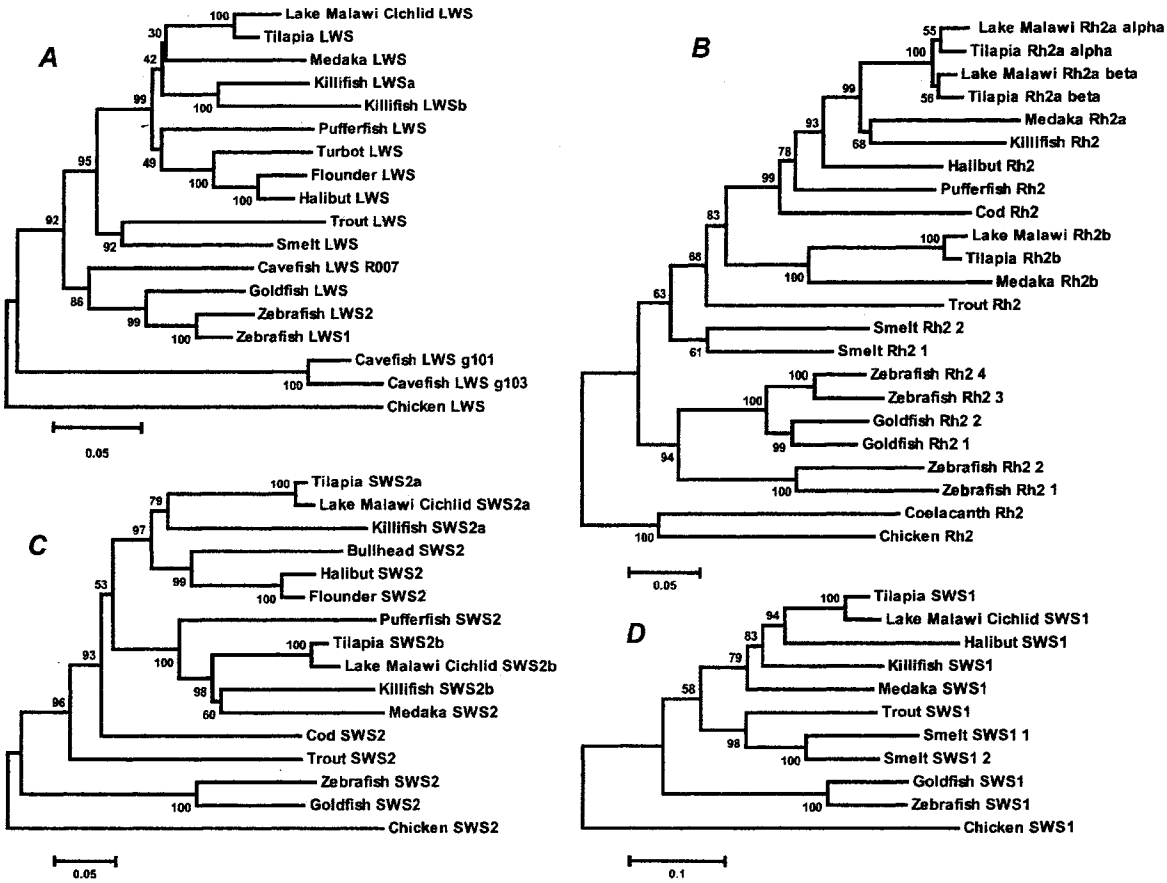


Figure 2.3
Difference spectra of reconstituted tilapia visual pigments. Absorbance spectra were measured before the pigment was denatured with acid (A) or hydroxylamine-treated (B-G). The latter spectra were subtracted from the former and resulting difference spectra were adjusted to zero by subtraction of a baseline based on points longwave to the absorbance peak, before fitting with visual pigment templates (Govardovskii et al. 2000). Visual pigments were peaked as follows: (A) SWS1, 360 nm; (B) SWS2b, 425 nm; (C) SWS2a, 456 nm; (D) Rh2b, 472; (E) Rh2a β, 518 nm; (F) Rh2a α, 528 nm; (G) LWS, 561 nm; and (H) LWS (minus chromophore peak), 561 nm. Due to the instability of the reconstituted LWS pigment, there is a large peak at 360nm (G), which is due to dissociated chromophore. A 360 nm template curve was used to subtract the chromophore peak from the visual pigment spectra (H).

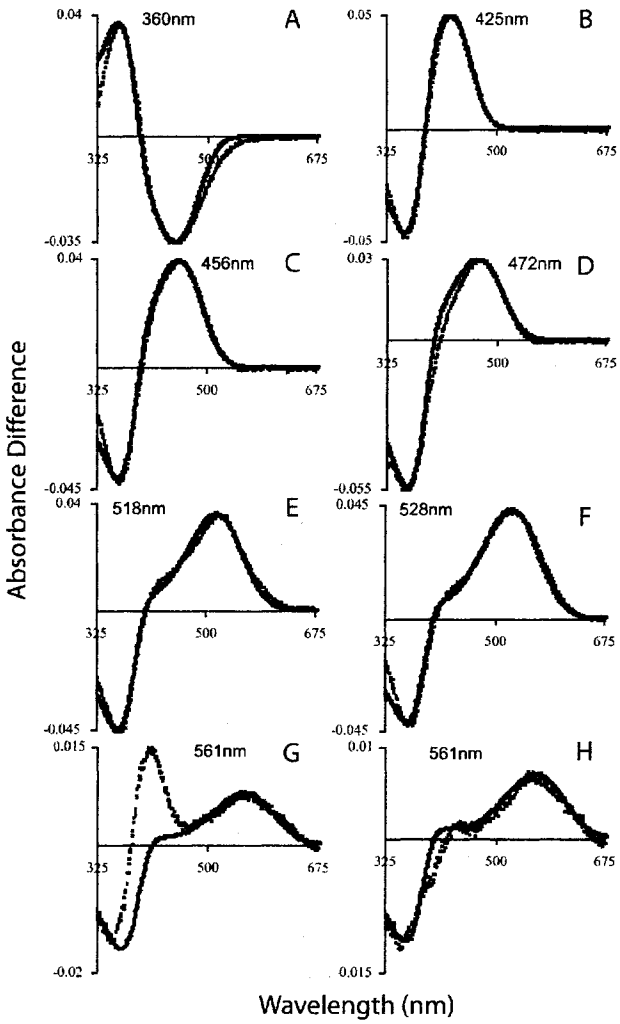


Figure 2.4
Relative cone opsin expression profiles for embryonic, juvenile, and adult age classes as determined by real-time RT-PCR. Expression levels are given as percentages of the total cone opsin genes expressed for a given age class. Error bars are ±1 SD.

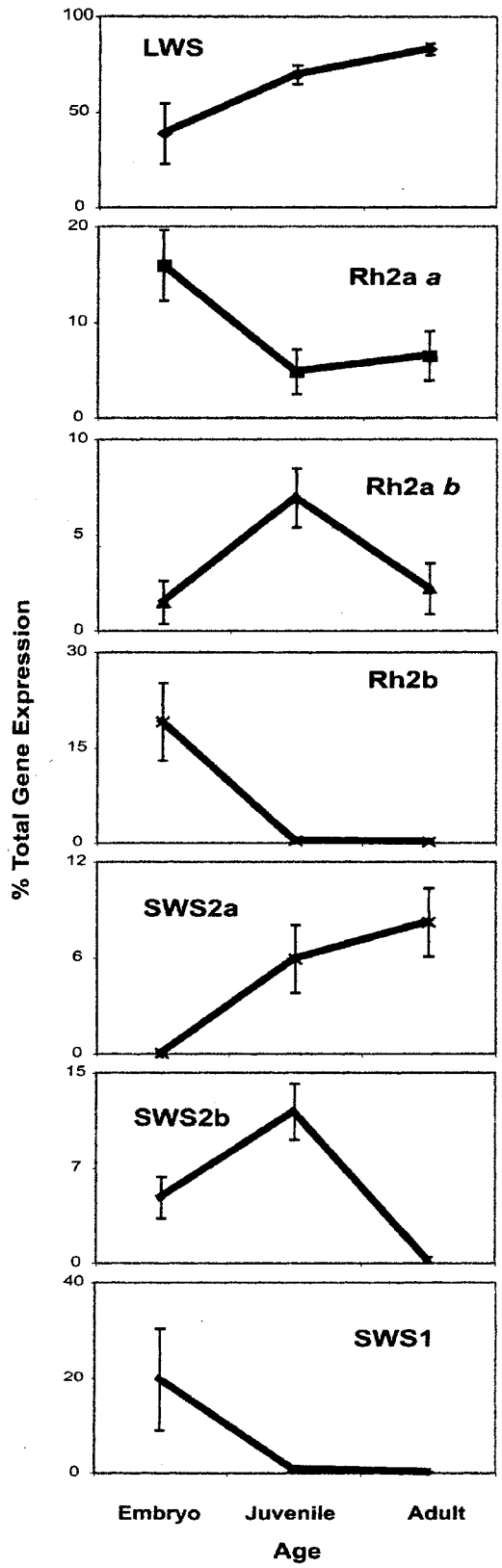
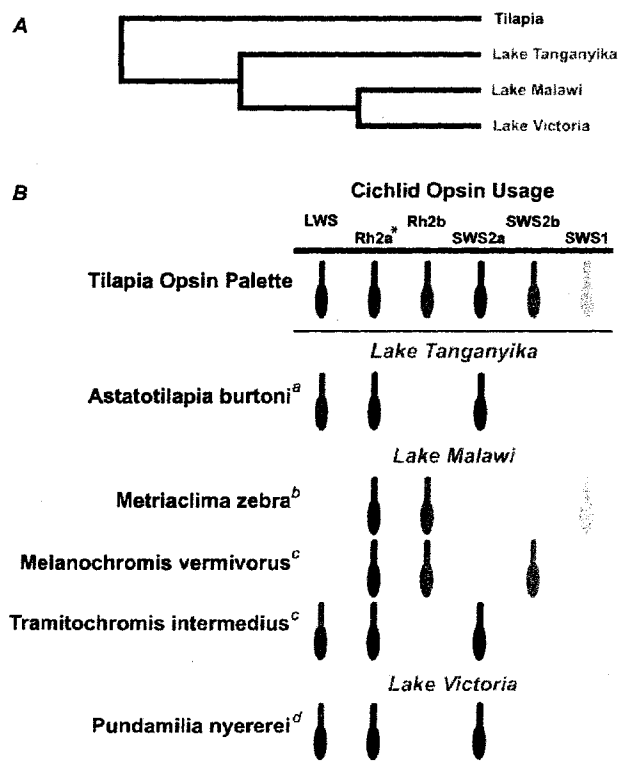


Figure 2.5
Tilapia opsin genes correlated to lacustrine cichlid photopigment usage. (A) The basal phylogenetic relationship of tilapia relative to the lacustrine cichlids is shown (Kocher et al. 1995). (B) Photoreceptor markers are used to indicate opsin gene usage. Opsin gene usage was inferred based on the comparison of the peak absorbances of the reconstituted tilapia cone photopigments and the MSP-derived spectral sensitivities of the lacustrine cichlid cone photoreceptors. Photoreceptor markers do not indicate cone morphology. *The spectral similarity and the differences between the peak absorbances determined by MSP and in vitro expression between Rh2a α and Rh2a β makes the designation of a cone class difficult when both cone types are not assayed for a given species. Therefore, Rh2a α and Rh2a β have been grouped together as Rh2a. Both Rh2a α and Rh2a β are however, thought to be expressed in cone photoreceptors among Lake Malawi and Lake Victoria cichlid species (Parry et al. 2005; Carleton et al. in press).

[a] (Fernald and Liebman 1980)
[b] (Levine and MacNichol 1979; Carleton et al. 2000)
[c] (Parry et al. 2005)
[d] (Carleton et al. in press)



CHAPTER III

DIFFERERENCES IN VISUAL SENSITIVITY AMONG THE *LABIDOCHROMIS* GENUS OF RAPIDLY SPECIATING LAKE MALAWI CICHLIDS

Abstract

The cichlids of East Africa are among the most rapidly speciating animals of recent evolutionary history. Sexual selection on male coloration is thought to be an important ingredient driving the cichlid radiations. Microspectrophotometry (MSP) and real-time RT-PCR studies have demonstrated that, among more divergent taxa, cichlids have diverse color visual systems. However, it remains unclear at what phylogenetic scale major differences in cichlid visual systems occur. Of the Lake Malawi cichlids, the genus *Labidochromis* is among the most species-rich groups. In the current study, I examined the visual systems of an ecologically diverse cross section of seven species of *Labidochromis* to determine if cichlid visual systems vary within a genus. Using real-time RT-PCR, relative cone opsin gene expression was assayed for five species. Three visual system types were identified, roughly corresponding to long, long + short, and short wavelength-sensitive systems. MSP was used to examine individual photoreceptor sensitivities of four *Labidochromis* species, two of which were also examined by real-time RT-PCR. MSP was used to confirm gene expression and the functionality of all cone visual pigment classes within the genus. Of the seven known East African cichlid visual pigment classes, photoreceptor classes corresponding to six were confirmed by MSP. Peak sensitivities matched those previously determined from in vitro expressed

pigments. The *Labidochromis* Rh2a β photoreceptor class had a peak sensitivity (518 nm), which was much closer to the in vitro expressed Rh2a β pigments than previously studied Lake Malawi cichlids. Despite having the ancestral capacity to express all cone opsin gene classes, those gene classes are differentially expressed among species of the *Labidochromis* genus. Differential cone opsin gene expression did not correlate to foraging strategy or depth, but was suggestive of major differences in color perception. This work is the first demonstration of major intergeneric variation in visual sensitivity among cichlid fishes. This suggests that differential gene expression is an important mechanism of modifying, among closely related species, the colors to which cichlid females are maximally sensitive and may therefore be important in driving cichlid speciation.

Introduction

East African cichlids are famous for their extraordinary rates of speciation (Kocher 2004, Genner and Turner 2005). Specifically among the Lake Malawi cichlids, researchers have proposed that more than 23,000 species may have evolved within the lake's one million year history (Won et al. 2005). Current estimates place the number of extant cichlid species within Lake Malawi at about 500 (Genner and Turner 2005). Rapid cichlid speciation and phenotypic diversification are thought to be the products of sexual selection via the polygamous mouth-brooding mating system utilized by cichlids (Dominey 1984; Danley and Kocher 2001). The mating system is characterized by strongly sexually dimorphic males soliciting mates through highly conserved courtship rituals (Fryer and Iles 1972; McElroy and Kornfield 1990). If a male is worthy, these courtship rituals may culminate in mating. Males play no part in rearing/guarding of young but females mouthbrood the developing young for several weeks. This highly asymmetric parental investment creates an opportunity for sexual selection which has been suggested to be an important factor in explaining cichlid diversity (Dominey 1984; Danley and Kocher 2001).

Sexual selection on visual cues, specifically male color pattern is thought to have been important (Dominey 1984, Carleton et al. 2005). Although other types of cues may influence mating success, color cues are clearly key (Seehausen 1997). The color pattern diversity of Lake Malawi cichlids is surpassed only by that of the fishes of the coral reef. Cichlids are overwhelmingly sexually dimorphic for color pattern (Fryer and Iles 1972). Color pattern alone has been shown to be sufficient in conspecific identification (Seehausen 1997; Knight and Turner 1999; Kidd et al. in press) and important in mate

assessment in cichlids (Pauers et al. 2004). In fact in the absence of color information, cichlids do not mate assortively (Seehausen 1997).

Given the importance of color visual cues, understanding the perceptual abilities of cichlids is crucial to understanding cichlid speciation. Color vision begins with excitation of cone photoreceptors, within the retina. Within cones are various spectrally distinct, light absorbing molecules known as visual pigments. Each visual pigment is composed of a light absorbing chromophore covalently bound to an opsin protein. Interactions between the chromophore and specific amino acids within the opsin determine the unique spectral absorbance of a visual pigment (reviewed in Yokoyama and Tada 2002; Takahashi and Ebrey 2003). Cichlids have representatives of all four major vertebrate cone opsin classes (Carleton et al. 2000; Carleton and Kocher 2001; Carleton et al. 2005a, b; Spady et al. 2005, Chapter 1; Parry et al. 2005). In total, cichlids have at least seven cone opsin genes (Parry et al. 2005). In vitro expression studies by Spady and coworkers have demonstrated that all seven genes code for spectrally distinct visual pigments (in review, Chapter 2).

Several previous studies of the cichlid cone opsin genes have focused on sequence variation among species (Terai et al. 2002; Spady et al. 2005, Chapter 1). Although these sequence studies found evidence of limited variation, studies examining the expression of the opsin mRNAs or the spectral sensitivities of individual cone photoreceptors have found far greater variation in visual spectral sensitivity (Levine and MacNichol 1979; van der Meer and Bowmaker 1995; Carleton et al. 2000; Carleton and Kocher 2001; Halstenberg et al. 2005; Parry et al. 2005). Although cichlids possess at least seven cone opsin genes, only a subset of these have been shown to be significantly

expressed in adults (Carleton and Kocher 2001, Parry et al. 2005). This highlights that cichlids have variable visual sensitivities. If vision were important in speciation, one would predict that closely related species would show variation in visual sensitivity, either in terms of differences in the spectral sensitivities of cone photoreceptors or differences in cone opsin mRNA expression.

Carleton and Kocher (2001) and Parry et al. (2005) have shown that within Lake Malawi, there are major differences in visual sensitivity among the most divergent taxa. However, it remains unclear at what phylogenetic scale major differences in cichlid visual systems occur. The genus *Labidochromis* represents one of Lake Malawi's most species-rich groups of rock-dwelling cichlids with an estimated 38 species (reviewed in Genner and Turner 2005). In the current study, I examine the visual sensitivity of several species of *Labidochromis* to determine if major variation in visual sensitivity can occur within a genus.

Methods

Study Species

Cichlid specimens were obtained from Old World Aquatics, an ornamental fish dealer specializing in East African rift lake cichlids. Individuals were housed in our fish facility at the University if New Hampshire for at least one month. The visual sensitivities of seven *Labidochromis* species were sampled by real time RT-PCR for cone opsin gene expression and/or microspectrophotometry (MSP) for cone photoreceptor sensitivity (Table 3.1). The species sampled represent an ecological cross section that includes both shallow and deep-water species, as well as algivores and invertebrate predators.

cDNA Synthesis

Retinas were homogenized and the total retinal mRNA was extracted using Trizol (Invitrogen). Retinal RNA preparations were then reverse transcribed with a poly T primer and Superscript II Reverse Transcriptase (Invitrogen). cDNA's were extracted from three individuals of *L. caeruleus*, two *L. sp. "zebra Lundo"*, one *L. strigatus*, two *L. freibergi*, and one *L. sp. kimpuma.*

Quantitative Real-Time RT-PCR

Real-time RT-PCR was used to quantify relative cone opsin mRNA levels (Carleton and Kocher 2001). Total retinal RNA (1 μg) was reverse transcribed using a poly T primer and Superscript III (Invitrogen) at 42° C to create a retinal RT cDNA mixture. Parallel real-time PCR reactions were set up for each of the opsin genes using the same master mix, such that each 30-μl real-time PCR reaction contained equal

amounts of the retinal cDNA mixture. Gene-specific Taqman primers and probes were then added.

The gene-specific cichlid opsin primers and probes used for this work were designed to amplify across a wide variety of species (Carleton et al. 2001; Spady et al. in review, Chapter 2). Real-time RT-PCR primers amplify short (60-90 bp) fragments for each gene. Because the Rh2a α and β genes were so similar, I analyzed the sum of these two.

Briefly, fluorescence was monitored during 40 cycles of PCR on a Gene Amp 5700 Sequence Detection system (Applied Biosystems; 95° C for 15 s, 55° C for 30 s, 65° C for 1min). Critical cycle number was determined when the fluorescence exceeded a threshold set close to the background fluorescence. Relative gene expression was determined for the six opsin genes (with RH2a α and β combined) as a fraction of the total cone opsin genes expressed for an individual according to:

$$T_i/T_{all} = (1/(1+E_i)^{Cti})/\sum(1/(1+E_i)^{Cti})$$

where T_i/T_{all} is the relative gene expression ratio for a given gene normalized by the total cone opsin genes expressed, E_i is the PCR efficiency for each gene, and C_{ti} is the critical cycle number for each gene. Three replicates were performed for each individual. All replicates for a species were averaged unless otherwise indicated. Efficiencies were determined from a concatenated amplicon construct (CAC) as described in Spady et al. in review, Chapter 2.

MSP

Photoreceptor spectral sensitivities were measured using a single-beam, computer controlled microspectrophotometer (MSP) (Loew 1994; Britt et al. 2001). Fish were first

dark adapted for at least 1.5 hrs, then anesthetized with MS-222. Single individuals of all species, except *L. caeruleus* (two individuals), were sampled for MSP. Eyecups were removed under infrared and/or dim red illumination. Retinas were dissected out of the eyecups and placed in PBS with 7.5% sucrose. Using scalpel blades, retinas were teased apart. Portions of processed retina were covered with a second cover slip and sealed with silicone grease. Spectral regions scanned ranged from 350 nm to 750 nm, although it often started at 400 nm because of excessive noise in the region from 350 nm to 400 nm. To establish the baseline, blank recordings were taken in regions adjacent to photoreceptor outer segments. The logarithm of the ratio of the baseline to actual photoreceptor recordings was used to determine absorbance.

The resulting absorbance curves were fitted to determine the peak absorbance. Absorbance curves were digitally filtered using Smooft (Press et al. 1987) and then fitted to visual pigment templates (Loew 1994). Absorbance curves were subjected to six fitting methods based on the following: the long wavelength limb, short wavelength limb, or both, with either the A_1 or A_2 derived chromophore. Data from cone classes were first partitioned by cell type/general spectral region. Double cones, single cones, and rods could readily be identified and differentiated based on cell morphology. All cones have roughly conical-shaped outer segments (photoreceptor organelle containing the visual pigment). Cones also possess a much larger inner segment relative to rods. Cones occur either as single cells or in pairs and are referred to as single and double cones, respectively. Rods usually have very long rod-like outer segments. For each cell type/spectral class, each type of limb fit was averaged and the limb fit with the lowest SD was used as the sensitivity of that photoreceptor class.

Results

Relative Opsin mRNA Expression by Quantitative Real-Time RT-PCR

Figure 3.1 shows relative opsin mRNA expression as a percentage of the total opsin expression (for discussion of PCR efficiencies and cross reactivities, see Spady et al. in review, Chapter 2). There were both quantitative and qualitative differences in relative opsin gene expression among species assayed (Figure 3.1, Table 3.2). There are at least three distinct types of expressed gene sets observed among *Labidochromis*. Each set includes four or five major genes that comprise roughly 99% of cone opsin gene expression. In the type 1 set, expressed by *L. caeruleus*, *L. strigatus*, and *L. sp. "zebra Lundo"*, all genes are highly expressed except Rh2b and SWS1. For *L. strigatus*, real-time PCR replicates were not uniform for Rh2a, SWS2a, and SWS1, indicating amplification failure for specific genes for some runs. These replicates (for specific genes) were not included. Although this has an effect on the apparent relative expression, the data removal does not affect the overall qualitative pattern of the *L. strigatus* data. The type 2 set, which is only observed in *L. freibergi*, is similar to the first set except that instead of expressing SWS2a, SWS2b is expressed. In the type 3 set, observed in *L. sp. kimpuma*, Rh2a, Rh2b, SWS2b, and SWS1 are expressed.

MSP

Six spectral classes of photoreceptor were observed among the species sampled (Table 3.3). The classes correspond to the peak absorbances of the genes that are known to make up the cone opsin array of the Nile tilapia, a closely related species (Spady et al. in review, Chapter 2). The average peak absorbances for each class were 558 nm, 533 nm, 517 nm, 494 nm, 450 nm, and 430 nm (Figure 3.2 – representative spectra).

Different subsets of the six classes were found among species. The LWS class was only observed among *L. caeruleus*. The Rh2a α class, which encompasses pigments with peak absorbances near 533 nm, was observed in all species. The Rh2a β class was also observed in all species. The close spectral proximity and the small number of recordings make it difficult to differentiate the Rh2a classes with absolute certainty. The Rh2b class was observed in all species, except *L. chisumulae*. Only *L. caeruleus* and *L. chisumulae* had SWS2a pigments. *L. caeruleus* and *L. sp. Hongi* were the only species to have a 430 nm SWS2b pigment. No SWS1 pigments were detected among any of the species. The number of MSP recordings does not always correspond to the number of photoreceptors measured as MSP does not sample a large number of photoreceptors and examines only certain areas of the retina.

Discussion

The photoreceptor classes found among *Labidochromis* are largely the same as those found among other Lake Malawi cichlids (Table 3.4). Lake Malawi cichlids have previously been shown to possess cone classes that range in peak spectral sensitivity from the yellow (560 nm) to the ultraviolet (370 nm) region. In total, seven cone classes have been identified among Lake Malawi species (Levine and MacNichol 1979; Carleton and Kocher 2001; Parry et al. 2005, Jordan et al.. in press). In vitro expression and reconstitution experiments have linked each cone class to the corresponding opsin gene (Parry et al. 2005, Spady et al. in review, Chapter 2). These seven cone classes correspond to the full suite of known East African cichlid cone opsin genes. In the current study, all East African cichlid cone classes have been identified among *Labidochromis* species, except the ultraviolet-sensitive class. Because of the high relative noise in the ultraviolet, I was usually unable to sample this spectral region. Rarity and restricted distributions could also explain why ultraviolet cones were not observed.

One notable difference between the *Labidochromis* and other Lake Malawi species previously examined is in the peak sensitivity of the cone class corresponding to the Rh2a β opsin gene. Parry et al. (2005) have reported a peak sensitivity of 505 nm for the cone class corresponding to the Rh2a β gene. However, in vitro expression and reconstitution experiments in a Lake Malawi cichlid and tilapia placed the peak absorbance of Rh2a β closer to 520 nm. Parry and coworkers argue that small inconsistencies between MSP and in vitro expression are common. They also suggest that the 505 nm photopigment might represent an undescribed gene or possible

coexpression of Rh2a β and Rh2b. I did not observe the 505 nm cone photopigment, and instead observed the 520 nm photopigment. Ultimately, in situ hybridation and Southern blot experiments are needed to examine coexpression and "extra gene" explanations, respectively.

L. caeruleus and *L. sp. kimpuma* were the only species assayed by both MSP and real-time RT-PCR. As a nonrandom regional sampling technique, MSP can miss rare cones or cones types that have a more restricted distribution. Differences in the spatial distribution of photopigment expression have been described in fish, with some cone types being restricted to relatively small regions of the periphery of the retina (Takechi and Kawamura 2005; Levine et al. 1979). In the current study, MSP recordings, instead, are used to confirm photopigment expression. For *L. caeruleus*, all photopigment classes that made up more than 1 % of relative cone opsin gene expression were also detected by MSP. For *L. sp. kimpuma*, SWS2b and SWS1 photopigments were not observed by MSP although expression was detected by real-time RT-PCR. However, the SWS2b photopigment is observed in *L. sp. Hongi*, which is thought to be conspecific to *L. sp. kimpuma*. *Labidochromis* MSP results confirm the expression of all the cichlid cone classes within this genus, except that of the SWS1. Although no SWS1 cones were recorded, real-time quantitative PCR results indicate that this gene is highly expressed among some species (*L. freibergi* and *L. sp. kimpuma*). This indicates that the ancestor of the *Labidochromis* genus had functional copies of all the cone opsin genes surveyed.

Despite having the ancestral capacity to express all six cone opsin gene classes, those gene classes are differentially expressed among species of the *Labidochromis* genus. Although several studies have shown that visual sensitivity is variable among the

Lake Malawi cichlids (Levine and MacNichol 1979; Carleton and Kocher 2001; Parry et al. 2005, Jordan et al.. in press), this is the first to establish that differential opsin gene expression can occur within a genus.

Labidochromis can be grouped into two trophic guilds, the algivores and the insectivores (Table 3.1)(Lewis 1981; Koning 95). These species are found over a range of depths. Among those species sampled by real-time PCR, *L. caeruleus* is the only insectivore and is primarily found at depth (10-25 m). However, *L. caeruleus* shares the type 1 expressed gene set along with *L. strigatus*, an algivore that inhabits the shallows. This suggests that among this species pair, differences in feeding and depth distributions have not resulted in divergence in visual sensitivity. Alternatively, among algivores *L. strigatus*, *L. freibergi*, and *L. sp. kimpuma*, there are marked differences in their respective profiles of gene expression despite their trophic similarity. *L. strigatus* and *L. freibergi* have the types 1 and 2 expressed gene sets, respectively, yet they share similarities in both feeding and depth distribution. (Since depth distribution information has not been gathered for *L. sp. kimpuma*, it cannot be compared.) Differential opsin gene expression does not seem to be correlated with feeding strategy or habitat depth.

Understanding the relationship between color vision and ecology is complicated because of limited information for these species. The phylogenetic relationships among *Labidochromis* are unknown, which further hinders attempts to examine potential adaptive relationships between ecology and vision. Thus, determining the causative factors driving the evolution of cone opsin gene expression will have to wait for further ecological characterization and phylogenetic analysis of these species.

Although the ecological factors that have been considered in the current study do not explain the variation in cone opsin gene expression observed among *Labidochromis* species, adaptive explanations are not a prerequisite for such differences to be important in speciation. For differential cone opsin gene expression to be important in speciation, one would expect sister taxa to be divergent in gene expression. A less stringent standard would simply be the presence high levels of variation within a closely related group. When comparisons of gene expression are limited to qualitative/presence or absence differences, there are three distinct expression profile types. If quantitative variation is considered as well, then each of the five species can be considered to have a distinct expression profile. Thus, the interspecific variation in cone opsin expression may be high enough to suggest that it is important in speciation among *Labidochromis*.

In the most simplistic framework, assuming a uniform distribution across the retina, the three *Labidochromis* visual system types correspond to a long wavelength-sensitive type (type 1), a long + short wavelength-sensitive type (type 2), and a short wavelength type (type 3). Based on these differences, one would predict that each type of visual system would be maximally sensitive to different regions of color space. Type 1, the long wavelength-sensitive type, should be relatively more sensitive to longer wavelength colors (red and orange [color appraisals based on human perception]). Type 3, the short wavelength-sensitive type, should be relatively more sensitive to shorter wavelength colors (ultraviolet). Type 2, the long + short wavelength-sensitive type, should be broadly sensitive (from red to ultraviolet). If male nuptial color has evolved to maximally stimulate female spectral sensitivity, then across species of a given visual system type, male nuptial color usage should be enriched for the spectral region of

maximum sensitivity. Thus, species with type 1 visual sensitivities should more often use reds and oranges, whereas species with type 3 visual sensitivities should more often use ultraviolets. Species with type 2 visual sensitivities should have the broadest color usage, encompassing type 1 and 3 ranges. There are however, physiological and ecological constraints that may limit color usage.

In the current study, five species were examined (real-time RT-PCR) and two of the three visual system types identified have only a single representative, which makes the detection of trends difficult. Among the type 1 species, *L. caeruleus* males are yellow and black (a white variant also exists), *L. strigatus* males are blue, and *L. sp. "zebra Lundo"* males white and black. Males of the type 2 species, *L. freibergi*, are blue. Males of the type 3 species, *L. sp. kimpuma*, are blue and yellow. To test these predictions of nuptial color usage in relation to visual sensitivity, a comprehensive spectral characterization of *Labidochromis* color usage and characterization of the visual sensitivities of more *Labidochromis* species will be required.

The current study demonstrates that *Labidochromis* has seven cone opsin genes. These genes are differentially expressed and likely result in major differences in color perception within the genus. This is the first demonstration of major variation in cone opsin expression within a genus. The differential perception of male nuptial coloration may, in turn, have important consequences to female mate choice and cichlid speciation.

Table 3.1
Study species. "*"*L. sp. Hongi* may be conspecific to *L. sp. "red top kimpuma"*. Ecological information for undescribed species is from Konings (1995).

Species	Reference	Location	Depth	Food	Assay
L. caeruleus	Fryer, 1956	Charo/Chizu Pt	10-25 m	invertebrates	Real Time/MSP
L. chisumulae	Lewis, 1982	Chizumulu	3-20 m	invertebrates	MSP
L freibergi	Johnson, 1974	Likoma	2-10 m	algae	Real Time
L. strigatus	Lewis, 1982	Chizumulu/Likoma	shallow	algae	Real Time
L. sp. "red top kimpuma"	undescribed	Hongi Island	unknown	algae	Real Time/MSP
*L. sp. Hongi**	undescribed	Hongi Island	unknown	algae	MSP
L. sp. "zebra Lundo"	undescribed	unknown	unknown	unknown	Real Time

Table 3.2
Labidochromis relative cone opsin gene expression as determined by real-time RT-PCR. The peak absorbances of in vitro expressed and reconstituted Nile tilapia orthologs are shown for each opsin class (Spady et al.. in review, Chapter 2).

	Opsin Gene					
Species	**LWS**	**Rh2a**	**Rh2b**	**SWS2a**	**SWS2b**	**SWS1**
L. caeruleus	58.9	7.5	0.5	3.7	29.4	0.1
L. "zebra Lundo"	80.1	2.3	0.0	5.2	12.3	0.1
L. strigatus	48.1	18.6	0.0	16.9	14.8	1.6
L. friebergi	82.6	3.8	1.5	0.0	5.0	7.0
L. "red top kimpuma"	0.2	20.2	42.5	0.1	26.5	10.6
Tilapia Cone Pigment Peak Absorbance (nm)	*560*	*528/517*	*472*	*456*	*425*	*360*

Table 3.3
Labidochromis cone visual pigment sensitivities determined by MSP, ± 1 SD. Six spectral classes of visual pigment were found among *Labidochromis*. The numbers of cones measured for each cone class are shown in parentheses.

Cone Type	Inferred Visual Pigment Class	*L. cauruleus*	*L. sp. "redtop kimpuma"*	*L. sp. hongi*	*L. chisumulae*
Double	LWS	558 ± 3 (7)			
	Rh2a α	527 ± 5 (8)	539 (1)	535 ± 2 (3)	532 ± 3 (2)
	Rh2a β	519 ± 2 (4)	519 ± 1 (3)	515 ± 1 (3)	518 (1)
	Rh2b	498 ± 6 (5)	490 ± 5 (25)	493 ± 3 (3)	
Single	SWS2a	456 ± 5 (5)			444 ± 7 (3)
	SWS2b	425 ± 3 (3)		435 ± 1 (3)	

Table 3.4
Comparison of the visual pigment classes found among cichlids. MSP determined visual pigment classes are shown for *Labidochromis* and other Lake Malawi cichlids. The peak absorbances of in vitro expressed and reconstituted Nile tilapia and *Metriaclima zebra* orthologs are shown for each opsin class. The peak absorbances of LWS and SWS2a visual pigments were not determined for *Metriaclima zebra.*

[a] Levine and MacNichols 1979; Carleton et al. 2000

[b] Parry et al. 2005

[c] Spady et al. in review, Chapter 2

Species	LWS	Rh2a α	Rh2a β	Rh2b	SWS2a	SWS2b	SWS1
MSP							
Labidochromis cauruleus	558	527	519	499	456	425	
Labidochromis sp. kimpuma		539	519	490			
Labidochromis sp. hongi		535	515	493		435	
Labidochromis chisumulae		532	518		444		
Metriaclima zebra [a]		533		488			368
Pseudotropheus acei [b]	566	534	505	482	452	415	378
Melanochromis vermivorus [b]	556	534	504	485		418	
Tramitichromis intermedius [b]	569	532			455		
In vitro Expression							
Metriaclima zebra [b]	*	528	519	484	*	423	368
Tilapia [c]	560	528	517	472	456	425	360

Figure 3.1
Relative retinal opsin mRNA expression for five *Labidochromis* species as determined by real-time RT-PCR. Relative expression is given as a percentage of the total cone opsin gene expression. Error bars are ±1 SD. *L. caeruleus* and *L. sp. "red top kimpuma"* were assayed by both real-time RT-PCR and MSP. "*" indicates that the corresponding cone class was found by MSP.

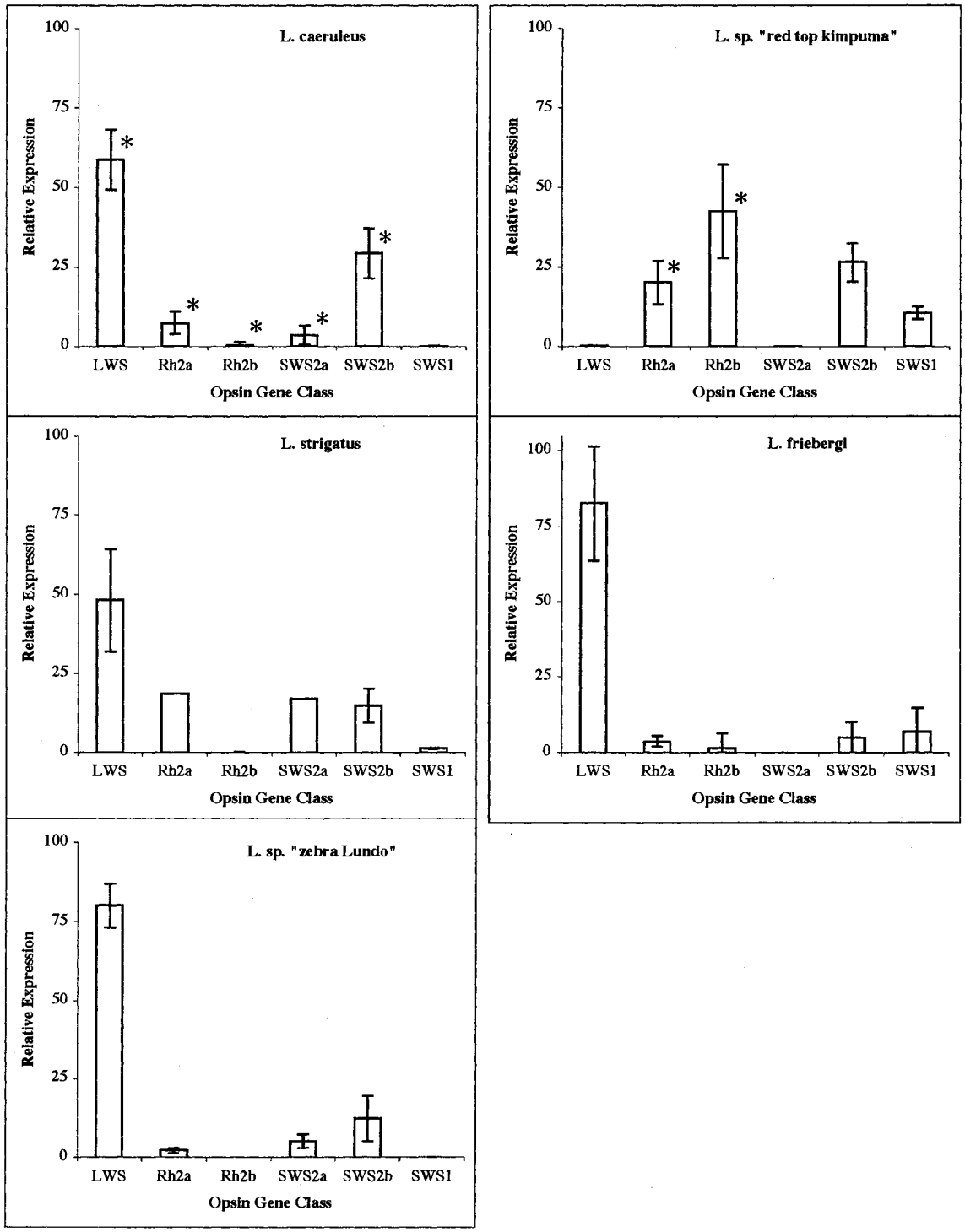
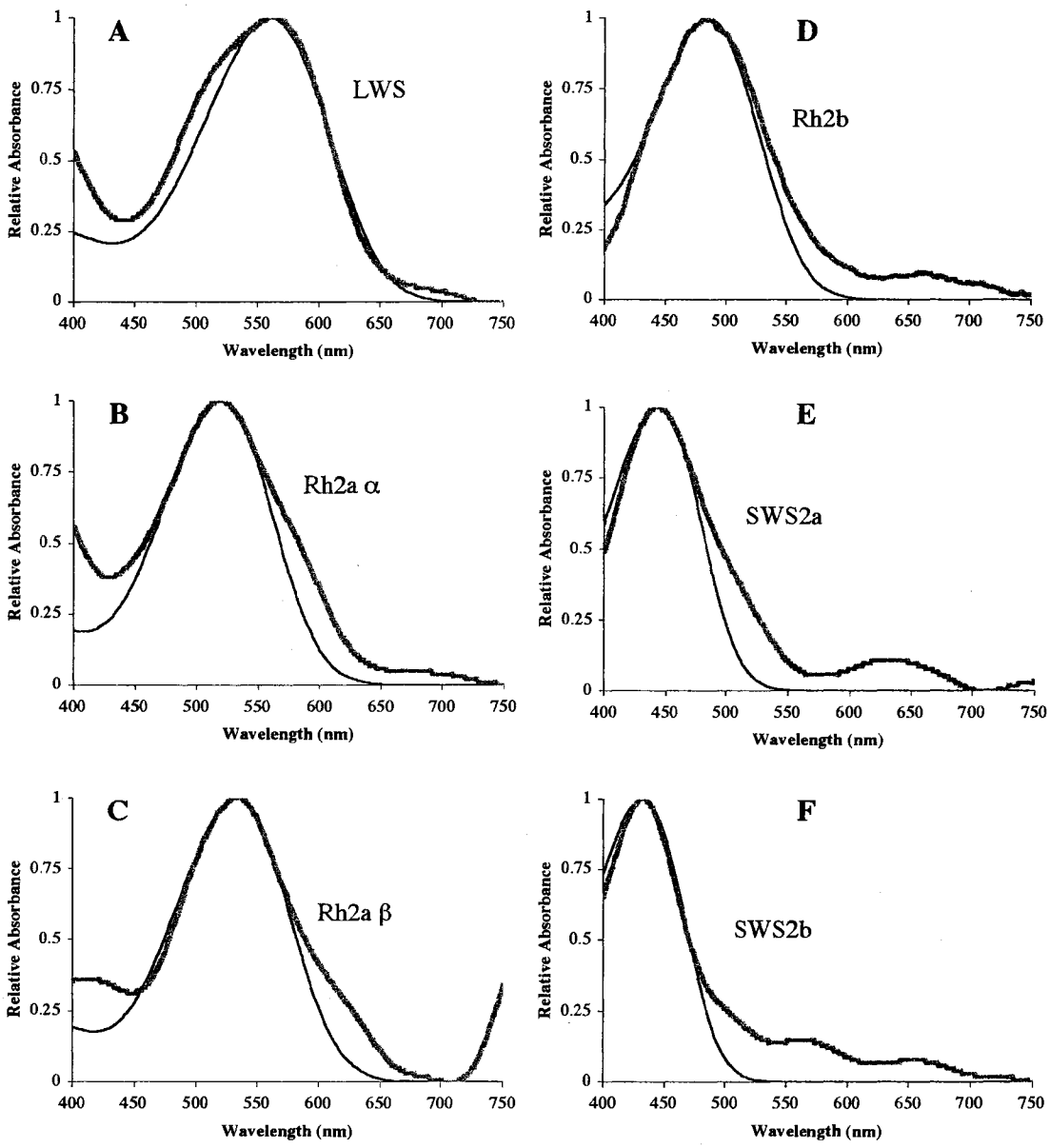


Figure 3.2
Representive MSP photopigment absorbance curves (thick grey) along with the best-fit template curves (Govardovskii et al. 2000) for each opsin class. (A) P560; (B) P533; (C) P520; (D) P485; (E) P444; (F) P432. All absorbance curves are from *L. caeruleus*, except (D) which was from *L. sp. Hongi.*



CHAPTER IV

EXTENSIVE GENE DUPLICATION OF FISH CONE OPSIN GENES

Abstract

Gene duplication provides a genetic substrate for evolutionary innovation. Ancient duplications of the opsin genes in early vertebrates were responsible for generating four functionally divergent classes of photopigment. Among fish cone opsin genes in particular, several more recent instances of gene duplication have been reported. In the current study, phylogenetic relationships are reconstructed from published gene sequences from a diverse assemblage of fishes, to examine the rates and patterns of gene duplication across fishes. The phylogenetic relationships of fish opsin genes are in agreement with the organismal relationships, except where gene duplications have occurred. I report extensive gene duplication among fish cone opsin genes. The rod opsin-like class shows the highest number of duplications, seven, followed by four among the long wavelength-sensitive class. Short wavelength-sensitive 1 and 2 classes both show a single duplication. These results suggest that, across fishes, the rates of retention of cone opsin gene duplicates vary. Standard calibrations of the rates of divergence (molecular clock) of gene duplicates based on several species divergence events failed to consistently reproduce established divergence estimates. We, therefore, employed a multiple rate calibration to estimate the divergence times of opsin gene duplicates. Notably, almost all opsin gene duplicates that have been retained began to

accumulate around the time of the radiation of higher teleosts, suggesting that the expansion and elaboration of the cone opsins might be associated with adaptive radiation. This is the first comprehensive study of gene duplication among fish cone opsin genes.

Introduction

The duplication of genes is thought to facilitate the process of adaptive evolution (Ohno 1970, Francino 2005). Upon duplication, the resulting gene pair undergoes functional divergence. Functional divergence may consist of neofunctionalization, the evolution a novel function among a member of a duplicate pair (Ohno 1970), and/or subfunctionalization, the partitioning of the ancestral gene functions (Force et al.1999). However, the most common fate for a duplicated gene is nonfunctionalization, the loss of function through the accumulation of deleterious mutations or excision from the genome (Lynch and Conery 2000).

Despite the abundance of phylogenetic evidence of gene duplication and functional divergence, only a handful of studies have provided empirical evidence of adaptation via gene duplication (Francino 2005). For example, Henderickson et al. (2002) showed that in *E. coli* mutants (lacZ-), duplication of the lacZ locus allowed for adaptation to lactose-minimal media growth conditions. Here, gene duplication resulted in a large increase in the expression of the defective lacZ- gene product allowing the bacteria to survive. Gene duplication also increased the probability of the occurrence of a mutation of adaptive value. Similar phenomena have been observed with regard to antibody production in mammalian cell lines (Kim and Lee 1999), insecticide resistance in mosquitos (Gillemaud et al. 1999), as well as other cases of adaptation among prokaryotes (referenced in Francino 2005).

The duplication of opsin genes has been described across a diverse array of species ranging from insects (Spaethe and Briscoe 2004) to mammals (Nathans et al.

1986). Opsin genes code for the protein component of visual pigments and are therefore key determinants of visual sensitivity. Nowhere is the duplication of opsin genes more pronounced than among fishes. Studies of fish opsin genes have identified occurrences of gene duplication among all major classes of vertebrate visual pigments, which themselves are the products of ancient duplication events that predate the divergence of jawed and jawless vertebrates. The major cone photoreceptor opsin classes are characterized as long wavelength-sensitive (LWS), rod opsin-like (Rh2), short wavelength-sensitive 2 (SWS2), and short wavelength-sensitive 1 (SWS1) (Hisatomi et al. 1994; Yokoyama 1994; Chang et al.1995; Collin et al. 2003).

The aim of the current study is to examine the relationship between opsin gene duplication and the adaptive evolution of fishes. I reconstruct the phylogenetic relationships of fish opsin genes and employ a multiple calibration approach to estimate the divergence times of fish cone opsin gene duplications. I also look for evidence of pattern in both the phylogenetic and chronological distribution of opsin duplication events.

Methods

Phylogenetic Analysis

Gene trees were generated from newly reported opsin coding sequences and a phylogenetically diverse sample of fish and chicken (outgroup) retinal opsin sequences. Within each opsin class, amino and carboxy termini were variable in length. To eliminate alignment gaps in the termini, all termini were trimmed of sequence regions not represented in all other species. This did not affect the alignment of the more conserved seven transmembrane domains. Bootstrap consensus trees (1000 replicates, 50% majority rule) were calculated using PAUP (Swofford 2002). Each opsin class was analyzed separately. Bootstrap topologies were then used as a constraint in maximum likelihood estimation of gamma parameters. Maximum likelihood estimates of gamma parameters and Tamura-Nei distances were then used to generate neighbor-joining (NJ) (Saitou and Nei 1987) trees and to calculate bootstrap values. Figure 4.1 shows the evolutionary relationships of species included in the study (Nelson 1994; Kumazawa et al. 1999; Miya et al. 2003; Saitoh et al. 2003; Chen et al. 2004). Sequences were aligned using MEGalign.

Estimation of Divergence Times

Using gamma corrected Tamura-Nei genetic distances, the timing of all fish duplication events among the opsin genes were estimated using the following relationship:

Rate = Distance/(2*Time)

Several molecular clocks were tested (3). Molecular clocks were calibrated to the average gamma corrected pairwise distance and the 10 MY divergence time of tilapia vs.

haplochromine cichlids (TH) (Kocher et al. 1995), the 296 MY divergence time of Acanthopterygii/Paracanthopterygii/Protacathopterygii vs. Ostariophysii (AO), and the 455 MY divergence time of Sarcopterygii vs. Actinopterygii (SA) (Kumar and Hedges 1998; Kumazawa et al.1999). Divergence estimates were calculated from the above rate calibration and the average pairwise distance.

Although the robust use of a molecular clock is contingent upon rate constancy among genes, rate acceleration following gene duplication is known to be a common phenomenon (Graur and Li 2000 and references therein). This is confirmed by relative rate tests within each opsin class. The relative rate test also indicated interspecific differences (see also Spady et al.2005, Chapter 1). I therefore employ a correction tool to compensate for the violation of rate constancy. To determine the extent to which the rate calibrations are inaccurate, I calculated species divergence times and compared these to previously published, mitochondrial based divergence times of Kocher et al.(1995) and Kumazawa and coworkers (1999). To correct for variation in intra-gene class divergence rates due to differences in selection pressure across the tree, a calibration correction factor was used to fit raw divergence estimates to expected divergence times.

Results

Phylogenetic Relationships of Fish Opsin Genes

Figure 4.2 shows phylogenies of all fish cone opsin genes from representatives of all major superorders of euteleost fish. Phylogenetic relationships are based on nucleotide sequences from the coding region. Due to the variation in the lengths of both carboxy and amino termini, the regions of variable data were not included in the construction of phylogenies. Chicken (*Gallus gallus*) opsin genes were used as an outgroup in all opsin classes. Within each opsin class, gene relationships were generally congruent with the previously published evolutionary relationships of fishes (Figure 4.1), except where gene duplications had occurred (Nelson 1994; Kumazawa, Yamaguchi, and Nishida 1999; Miya et al. 2003; Saitoh et al.2003; Chen et al.2004).

Cichlid LWS genes cluster with 100% bootstrap support (Figure 4.2a). Acanthopterygii LWS genes form a clade supported by a 99% bootstrap score. The remainder of the tree is in agreement with previous studies of cyprinodontiform and Ostariophysan LWS duplications (duplications A, E, and D) as well as other vertebrate LWS genes (Fuller et al.2004, Chinen et al.2003).

The Rh2 clade shows the greatest number of gene duplications. The tilapia Rh2a α and Rh2a β (duplication G) cluster has 100% bootstrap support, to the exclusion of pleuronectiformes Rh2 gene (Figure 4.2b). This suggests that the divergence of tilapia Rh2a α and Rh2a β occurred after the cichlid /flatfish split. Furthermore, Lake Malawi cichlids also have orthologs to all the of the tilapia Rh2 genes (Parry et al.2005), suggesting that the duplication event that generated Rh2a paralogs occurred before the divergence of tilapia and the haplochromines. In contrast, tilapia Rh2b clusters with

killifish Rh2, with 100% bootstrap support. Paracanthopterygii and Acanthopterygii Rh2 genes form a clade with 86% bootstrap support. This would suggest that the divergence of the ancestral tilapia Rh2a and Rh2b (duplication A) predates the cichlid/cod split, but occurred after the cod /trout split. These data also suggest that other Paracanthopterygii and Acanthopterygii may have at least two Rh2 genes. This has recently been confirmed in pufferfish and medaka (Neafsey and Hartl 2005). Paracanthopterygii and Acanthopterygii may have an ortholog to tilapia Rh2b. Similar to Minamoto and Shimizu (2005), I found that smelt Rh2 genes cluster, although with only 60% bootstrap support. This provides weak corroboration for the assertion that the duplication event that lead to the two contemporary smelt Rh2 genes was unique to Osmeroidei. The remainder of the tree is in agreement with previous studies of Ostariophysii Rh2 duplications (duplications B, D, E, and F) and other vertebrate Rh2 genes (Chinen et al.2003).

Cichlid SWS2a and SWS2b opsins cluster with 100% bootstrap scores, in both cases (Figure 4.2c). Cichlid SWS2a and SWS2b opsin genes cluster with killifish SWS2a and SWS2b with generally high bootstrap support, 77% and 97% respectively (duplication A). Medaka SWS2 clusters with killifish and cichlid SWS2b genes (97% bootstrap support), suggesting that medaka might have or have had an additional SWS2 gene, orthologous to killifish and cichlid SWS2a. Although the SWS2 tree as it is reported suggests that duplication A occurred after the divergence of Paracanthopterygii and Protacanthopterygii, the low bootstrap value (52%) can not rule out the possibility that duplication A predates the divergence of Paracanthopterygii and Protacanthopterygii.

The SWS2 tree is consistent with previous studies of SWS2 opsin genes (Carleton and Kocher 2001; Neafsey and Hartl 2005).

No new duplication events were observed or inferred among SWS1 opsins (Figure 4.2d). Further, SWS1 gene relationships are in agreement with those of previous studies of SWS1 opsin duplications (Minamoto and Shimizu 2005).

Estimation of Divergence Times

Species divergence times estimated using the three rate calibrations (SA, OA, and TH) are plotted alongside previously published mitochondrial-based divergence times (Figure 4.3)(Kocher et al. 1995; Kumazawa et al. 1999). Species divergence times calculated using the SA rate calibration overestimated divergence times for all opsin genes except SWS1. For SWS1, the SA rate calibration overestimated recent speciation events while underestimating more ancient events. The OA rate calibration showed the same pattern of overestimation of recent events and underestimation of more distant events for all opsin classes. Despite this pattern, the OA rate calibration generally gave estimates closest to Kumazawa and coworker's (1995) species divergence times. The TH rate calibration yielded species divergence times that were drastic underestimates, except for the earliest time point. Since it gave estimates closest to previously published divergence times, I focus on the OA-based divergence estimates.

Although the OA-based divergence estimates were closest to the previously published divergence times, they were still significantly different. A correction factor was used to fit the OA-based divergence estimates to previously published species divergence times (Kocher et al. 1995; Kumazawa et al. 1999). The correction of raw divergence estimates (Table 4.1) yielded times that were much more in agreement with

both the fossil and independently derived molecular data (Kocher et al. 1995; Kumazawa et al. 1999). Corrected divergence estimates indicate that most duplications have occurred within the past 250 MY (Figure 4.4).

Discussion

Extensive Opsin Gene Duplication Among Fishes

Phylogenetic analysis of fish retinal opsin gene sequences establishes that gene duplication and the subsequent differential retention (fixation) and loss of those products has had a major impact on the evolution color vision in fishes (Yokoyama and Yokoyama 1990; Johnson et al.1993; Register et al.1994; Carleton and Kocher 2001; Chinen et al. 2003; Fuller et al.2004; Minamoto and Shimizu 2005; Neafsey and Hartl 2005; Fuller et al.2004, pers com; Drivenes and Helvik personal communication). The cichlid Rh2 genes specifically indicate at least one gene duplication event in the ancestor to acanthopterygian fishes and one duplication event that may be unique to African cichlids. The more ancient Rh2 duplication is supported by the studies of pufferfish and medaka Rh2 genes of Neafsey and Hartl (2005) and Kawamura et al. (personal communication).

Since the opsin gene arrays of most fishes have not been exhaustively examined by genomic methods, the opsin gene counts reported for some species may be underestimates. Relying on cDNA based methods may not detect genes expressed at very low levels, expressed differentially across ontogeny, or duplicates which have very similar sequences either due to their recent occurrence and/or gene conversion. However, those taxa that have been examined, cichlids (Carleton et al. unpublished data), pufferfish (Neafsey and Hartl 2005), smelt (Minamoto and Shimizu 2005), and zebrafish (Chinen et al. 2003), have been useful in predicting the existence of duplicates in other species.

Differences in Rates of Fixation of Duplicates Among Fish Opsin Genes

The high number of observed opsin gene duplications is consistent with the work of Lynch and Conery (2003). They argue that rates of eukaryotic gene duplication are

similar to rates of single nucleotide substitutions. In their survey of genomic databases, Lynch and Conery (2000) found that gene duplication occurs at rate of about 0.01 per gene per MY. Based on this rate of duplication, one would expect to see about 350 gene duplications per MY (for a genome with 35000 genes). That would be equivalent to one duplication per gene per 100 MY or 3.5 primary duplications per ancestral gene over the 350 MY of teleost evolution. Among the teleost opsin genes, I find evidence of three primary duplications of the ancestral teleost opsins among Rh2 genes. LWS, SWS2 and SWS1 only have one duplication of their respective ancestral opsin gene. Further, LWS and Rh2 have all had secondary duplication events (duplication of a duplicate).

With eight duplications (including duplicates indicated from partial gene sequences from turbot (Drivenes and Helvik personal communication)), the Rh2 gene has experienced far more duplication events then any other retinal opsin class. This brings several important questions to light. First, why are teleost Rh2 genes duplicating (and becoming fixed) so rapidly, relative to other opsins? The other opsin classes do not show nearly as many gene duplications. The rates of substitution and gene duplication are proportional across genomes (Lynch and Conery 2003) and one might expect local rates within a genome to show a similar relationship. In our comparative study of the rates of evolution of cichlid opsin genes, Spady et al. (2005) found that different opsin classes evolved at different rates. Although, the Rh2 class had a higher rate of evolution, its rate was still lower than SWS1, which had the highest rate. In contrast, only one gene duplication has been found in the SWS1 opsin class (smelt)(Minamoto and Shimizu 2005). Although the current study employs a much greater phylogenetic breadth than Spady et al. (2005), the disparity between local rates of substitution and gene duplication

suggests that differences in local rates of substitution may not be good indicators of rates of gene duplication. This hints at a possible functional/adaptive explanation for the preponderance of teleost Rh2 duplications.

Although the rapidity of gene duplication among the Rh2 opsin genes is of note, the persistence of the duplicates as functional copies within the genome is equally remarkable, especially since the loss of Rh2, SWS2, and SWS1 genes has been documented in fugu (Neafsey and Hartl 2005), smelt (Minamoto and Shimizu 2005), and some cichlid species (Spady et al.2005, Chapter 1), respectively. One member of a duplicate pair is typically doomed due to the accumulation of deleterious mutations. Thus, the gene duplications observed here likely represent a small portion of the duplications that have actually occurred among fish opsin genes. In animals, the process of nonfunctionalization takes about 4 MY, on average (Lynch and Conery 2000; Lynch 2002 and references therein). Genes that have been retained for longer periods are less likely to be lost. All duplications observed in teleost Rh2 genes (and other cone opsin genes) are older than 4 MY and in most cases more than an order of magnitude older (Figure 4.4). This would suggest that these genes are being retained in their respective genomes, but the reason is not clear.

Distribution of Duplication Events

It is difficult to draw inferences from the phylogenetic patterns of cone opsin duplication (and retention) because few species have had their genomes screened exhaustively (Chinen et al. 2003; Minamoto and Shimizu 2005; Neafsey and Hartl 2005; Kawamura et al. pers. com.; Carleton et al. unpublished data). Of these species, zebrafish belongs to the superorder Ostariophysii, tilapia, fugu, medaka belong to the

Acanthopterygii, and smelt belongs to the Protacanthoptergii. These do, however, represent the most prominent superorders of fishes. Further, the conserved genomic organization across these taxa could simplify future genomic screens in the other fish species (Carleton et al. unpublished data).

Despite our inability to draw conclusions from the phylogenetic distributions of opsin duplication events, a clear pattern does exist in the chronological distribution. With the exception of LWS A, all observed duplication events occurred after about 260 MYA. This roughly corresponds to the early period of higher teleost adaptive radiation.

Gene Duplication and Adaptive Radiation

Adaptive radiations are characterized by the evolution of divergent, ecologically specialized phenotypes. As ancestral teleosts evolved to exploit a range of light environments and foraging strategies, their visual systems are likely to have adapted accordingly (Levine and MacNichol 1979). Specifically with respect to the cone opsin genes, the overwhelming majority of observed gene duplications do not start to become evolutionarily fixed until the higher teleosts begin to diversify. Although it is speculative at this point to conclude that expansion and elaboration of the cone opsins is associated with adaptive radiation, this might suggest an interesting link. The highly vision-dependent species of the East African cichlid adaptive radiations would be ideal to examine this association. Younger adaptive radiations, such as those of Lakes Malawi and Victoria, might be more useful in detecting short-lived gene duplicates as they emerge. In contrast, an older radiation, such as that of Lake Tanganyika, might be more useful for examining gene retention.

Table 4.1
Divergence estimates for each opsin duplication. Each duplication has a letter designation that corresponds to the duplication markers used in Figure 4.2. Divergence times are in MYA.

Gene	Duplication	Correction Factor	Raw Divergence Estimate	Final Divergence Estimate
LWS	A	1.221	369±77	451±94
	B	0.524	138±29	72±15
	C	0.299	64±13	19±4
	D	0.267	53±11	14±3
Rh2	A	0.906	253±32	229±29
	B	0.870	237±32	206±28
	C	0.694	158±20	110±14
	D	0.661	143±18	94±12
	E	0.510	75±10	38±5
	F	0.477	60±8	29±4
	G	0.413	31±4	13±2
SWS2	A	0.889	222±10	198±8
SWS1	A	0.496	117 ±11	58 ±5

Figure 4.1
Phylogenetic relationships of all species (orders) sampled according to (Nelson 1994; Kumazawa et al. 1999, Miya et al. 2003; Saitoh et al. 2003; Chen et al. 2004).

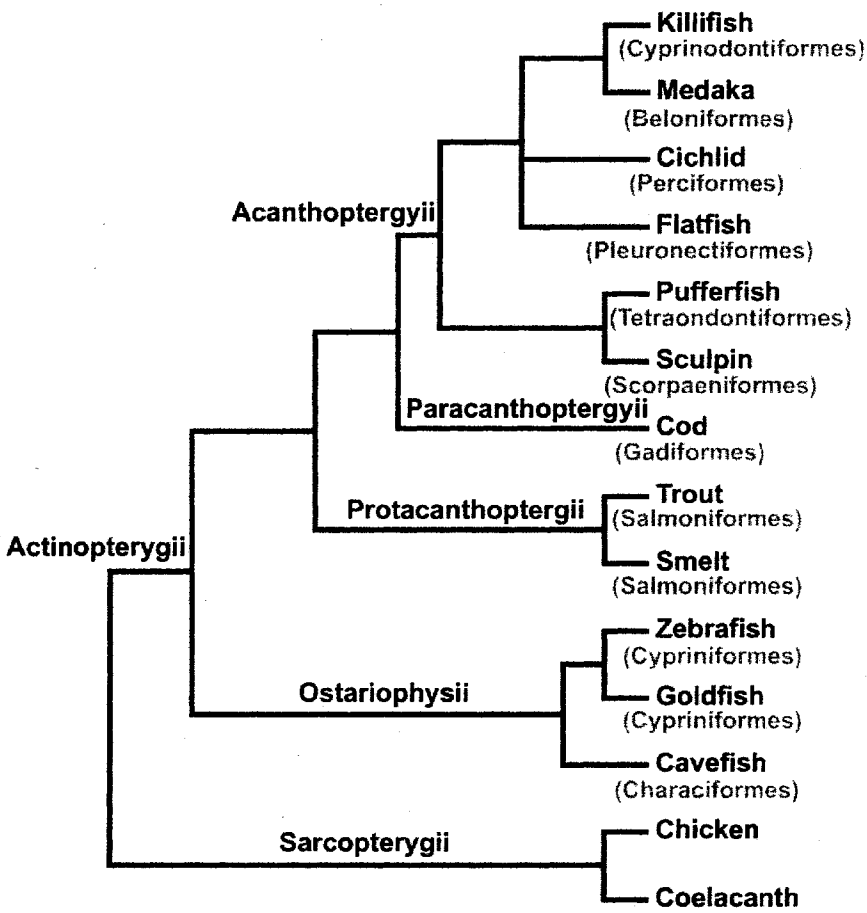


Figure 4.2
Fish opsin phylogenies with gene duplications highlighted. NJ trees were constructed for each opsin class, using gamma corrected Tamura-Nei distances. Duplications events are highlighted as open circles and given alphabetical designations. Trees were constructed for fish LWS (A), Rh2 (B), SWS2 (C) SWS1 (D), using gamma corrected Tamura-Nei distances. Bootstrap values are indicated when greater than 50%. Scale bars indicate the number of substitutions per 100 sites. The following sequences were included: cavefish (LWS g103, U12025; LWS g101, U12024; LWS R007, M90075), zebrafish (LWS1, AB087803; LWS2, AB087804; Rh21, AB087805; Rh2 2, AB087806; Rh2 3, AB087807; Rh2 4, AB087808; SWS2, BC062277; SWS1, AB087810), goldfish (LWS, L11867; Rh2 1, L11865; Rh2 2, L11866; SWS2, L11864; SWS1, D85863), smelt (LWS, AB098702; Rh2 1, AB098703; Rh2 2, AB098704; SWS1 1, AB098705; SWS1 2, AB098706), trout (LWS, AF425073; Rh2, AF425076; SWS2, AF425075; SWS1, AF425074), halibut (LWS, AF316498; Rh2, AF156263; SWS2, AF316497; SWS1, AF156264), flounder (LWS, AY631039; SWS2, AY631038), turbot (LWS, AF385826), pufferfish (LWS, AY598942; Rh2a, AF226989;SWS2, AY598947), medaka (LWS, AB001604; Rh2a, BJ495952, BJ491781; Rh2b, AB001603; SWS2, AB001602; SWS1, AB001605), killifish (LWSa, AY296740; LWSb, AY296741; Rh2, AY296739; SWS2a, AY296737; SWS2b, AY296736; SWS1, AY296735), Lake Malawi cichlid (LWS, AF247126; Rh2a α, DQ088651; Rh2a β , DQ088650; Rh2b, DQ088652; SWS2a, AF247114; SWS2b, AF317674; SWS1, AF191222), cod (Rh2, AF385824; SWS2, AF385822), bullhead (SWS2, CGO430489), and tilapia (LWS, AF247128; Rh2a α, DQ235683; Rh2a β, DQ235682; Rh2b, DQ235681; SWS2a, AF247116; SWS2b, AF247120; SWS1, AF191221). The outgroups were chicken (LWS, M62903; Rh2, M92038; SWS2, M92037; SWS1, M92039) and coelacanth (Rh2, AH007713).

Figure 4.2

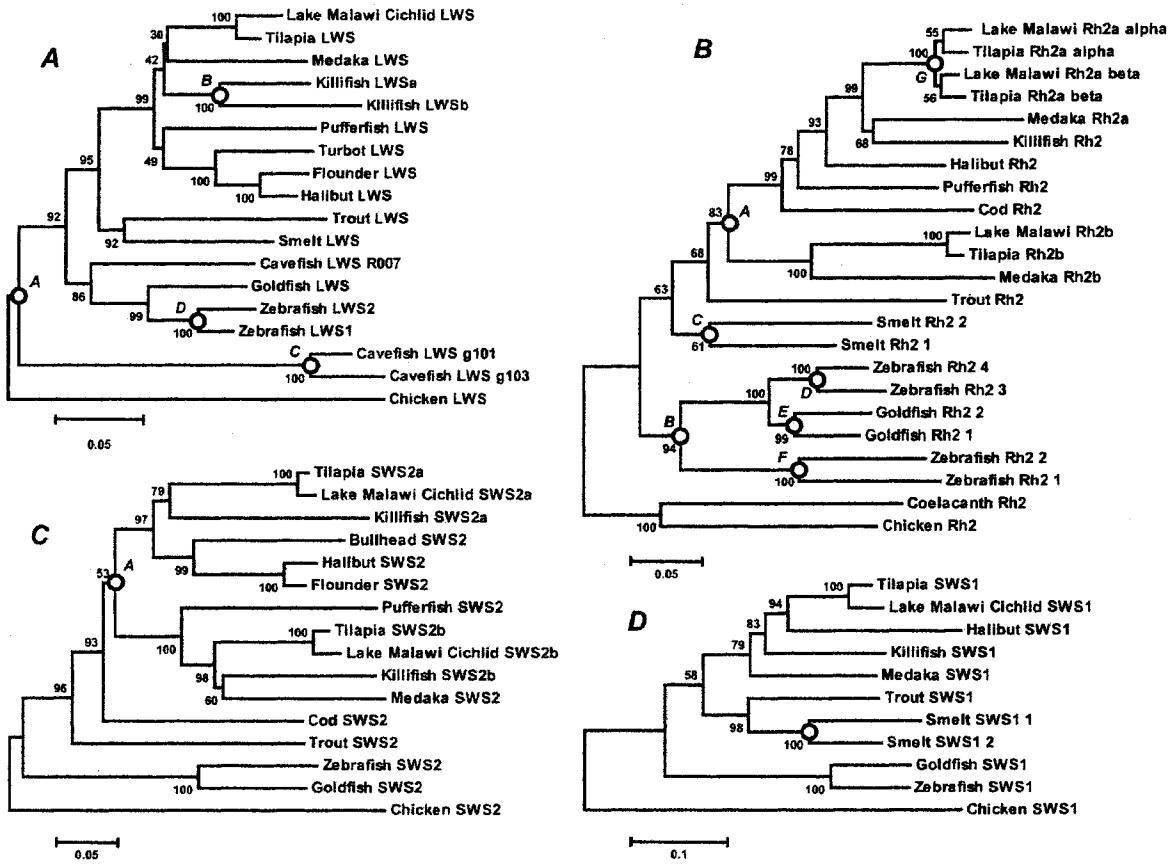


Figure 4.3
Molecular clock calibrations. For each opsin class, species divergence times were estimated based on three molecular clock calibrations. Sarcopterygii vs. Actinopterygii (SA; triangles), Ostariophysii vs. Acanthopterygii/Paracanthopterygii/Protacanthopterygii (OA; squares), and tilapia vs. haplochromine cichlids (TH; diamonds) based divergence estimates (various dotted lines) are compared to previously published divergence estimates (x's - solid line)(Kocher et al. 1995; Kumazawa et al. 1999).

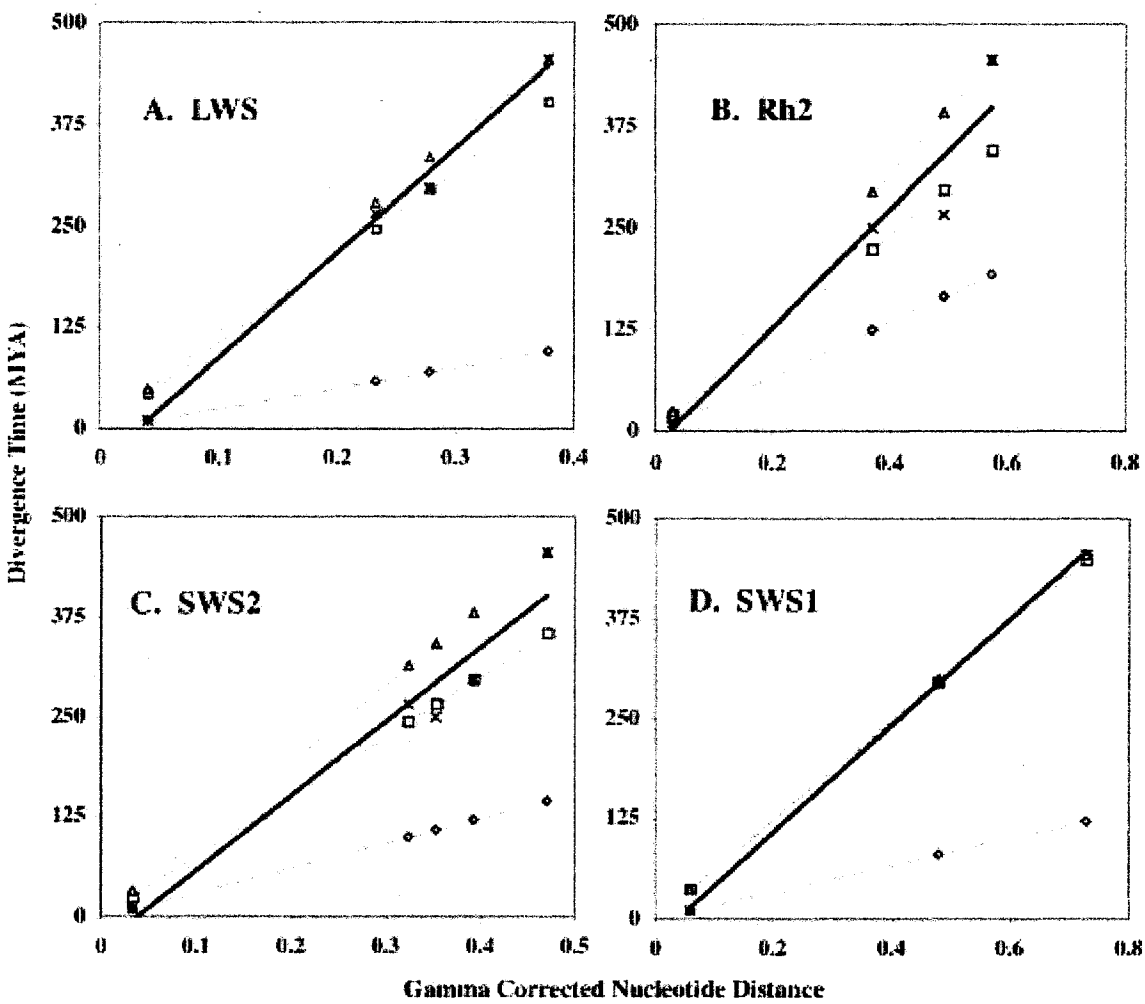
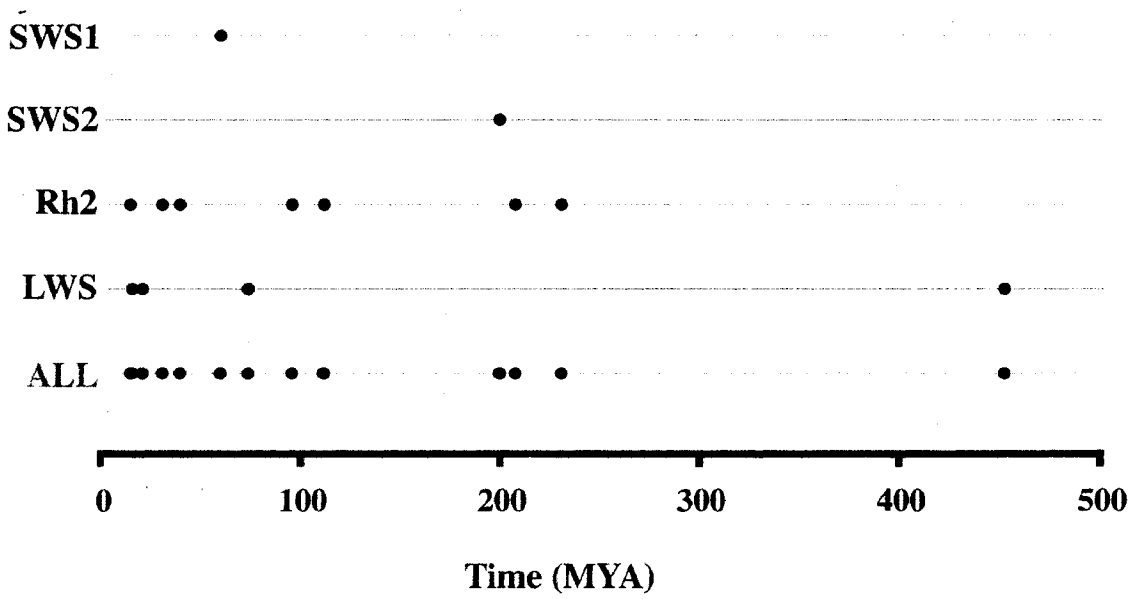


Figure 4.4
The chronological distribution of fish cone opsin duplication events. Duplication events are shown for each opsin class individually, as well as for the sum of all classes.



CHAPTER V

WHAT CAN CICHLIDS REVEAL ABOUT THE RELATIONSHIP OF COLOR VISION AND ECOLOGY?

East Africa's adaptive radiations of lacustrine cichlids are renowned for their extraordinary diversity of form and ecology (Fryer and Iles 1977; reviewed in Kocher 2004). The cichlid radiations have been likened to a natural mutant screen, a mutant screen enriched for evolutionary adaptive genetic changes. Studies of cichlid oral jaw morphology, for example, have identified several genomic regions important in the adaptation of cichlid oral jaws to specific foraging strategies (Albertson et al. 2003a; 2003b; 2005). Another major strength of the cichlid system is the presence of replicate radiations. These radiations span in age from ten million years to tens of thousands of years (Kocher et al. 1995; Johnson et al. 1996), allowing for the examination of the timescales of genetic change in response to changing ecological conditions. One would predict that adaptation to various foraging strategies and photic conditions would include the tuning of visual sensitivity to specific needs (Spady et al. 2005, Chapter 1; Carleton et al. in press).

Seven cone opsin genes have been sequenced from Lake Malawi cichlids (Parry et al. 2005). The same seven genes have also been found in the outgroup to the East African lacustrine radiations, the Nile tilapia, suggesting that of the common ancestor to these species also had seven cone opsin genes (Spady et al. in review, Chapter 2). Differential expression of subsets of the opsin genes, in cichlids, has given rise to the

diverse color visual systems (Levine and MacNichol 1979; Fernald and Liebman 1980; van der Meer and Bowmaker 1995; Carleton et al. 2000; Carleton et al. 2001; Carleton et al. 2005; Parry et al. 2005, Carleton et al. in press; Chapter 3).

Comparisons of expressed cone opsin subsets among closely related species may reveal how ecological differences have driven the evolution of differential gene expression. The first Lake Malawi species examined by both MSP and real-time RT-PCR, *Metriaclima zebra* and *Dimidiochromis compressiceps*, were found to differ dramatically in visual sensitivity (Levine and MacNichol 1979; Carleton et al. 2000; Carleton et al. 2001), despite being found sympatrically (personal observation). This would suggest that factors other than habitat or location are responsible for the observed differences. However, these species may have diverged in allopatry and subsequently come to inhabit the same areas.

A more compelling factor in explaining the color vision differences between these species is the divergent foraging strategies employed by each. *M. zebra* is a planktivore. *D. compressiceps* is a piscivore. From a visual perspective, these foraging strategies pose very different challenges. Visual adaptations to piscivory have received relatively little attention, but comparative analyses among the cichlids may help to identify those adaptations if they exist. In contrast to the piscivore, the plankivore is challenged with detecting very small, largely translucent (to the human eye) particles against a bright background. Ultraviolet visual sensitivity enhances plankton foraging by increasing the contrast of strongly ultra violet absorbing plankton against the bright ultraviolet background of down or side welling light (Browman et al. 1994). Ultraviolet visual sensitivity is thought to be a common sensory adaptation to planktivory (McFarland and

Loew 1994; Losey at al 1999). Consistent with this trend, the cichlid planktivore highly expresses the ultraviolet sensitive SWS1 visual pigment, unlike the piscivore.

In chapter 3, intrageneric differences in cone opsin gene expression were described among *Labidochromis* species. Neither differences in depth distribution nor feeding ecology explain the observed variation. This highlights the need for further characterization of cone opsin gene expression in an ecologically diverse group of cichlids to reveal trends in the relationship of ecology and visual sensitivity.

Just as the retention/differential expression of opsin genes has been important in shaping visual sensitivity (Chapter 4), so too has the loss of opsin genes through nonfunctionalization. The ancestor to mammals, for example, lost both Rh2 and SWS2 opsin classes likely as a result of an emergent nocturnal lifestyle (reviewed in Jacobs 1993). Since then, several lineages have also independently lost the SWS1 opsin class. Nocturnal lorisiform prosimians have accumulated multiple deletions that result in the formation of a premature stop codon in SWS1 (Kawamura and Kubotera 2004). Similarly, pinnipeds (Peichl et al. 2001) and whales/dolphins (Fasick et al. 1998, Peichl et al. 2001; Levenson and Dizon 2003) have both independently lost their functional SWS1 gene, although the exact ecological causative agent(s) remains unclear.

Among fishes there is also evidence of multiple independent losses of opsins thru nonfunctionalization. Minamoto and Shimizu (2005) and Neafsey and Hartl (2005) both provide evidence of the ancient loss of SWS2 and SWS1, in smelt and pufferfish respectively. Both genes have either been excised from the genome or decayed to the point of being unrecognizable as opsins. The same is thought to have occurred with an ancient LWS gene copy that among extant fishes has only been found in cavefish

(Yokoyama et al. 1993). On a more recent evolutionary scale, different pufferfish lineages have independently lost functional Rh2b genes via transposon insertion induced frameshift or frameshift/gene truncation (Neafsey and Hartl 2005).

Although the duplication and loss of opsin genes is an established phenomenon, the influence of ecological factors has largely been unclear. The cichlid system has enormous illustrative potential in identifying those ecological forces, beyond gross differences in photic environment.

In tilapia, the retention of all the seven cone opsin genes may have been result of ontogenetic subfunctionalization, via differential gene expression (Spady et al. in review, Chapter 2). This is perhaps the product of the generalist ecological strategy employed by tilapia. Ontogenetic differential opsin gene expression has not been observed among the Lake Malawi species that have been examined to date (Carleton pers. com.). Many of the species of the East African lacustrine adaptive radiations have highly specialized feeding strategies (Fryer and Iles 1972). I hypothesize that ecological specialization leads to opsin gene loss. The Lake Tanganyika, Lake Malawi, and Lake Victoria radiations are <10, 1, and 0.1-0.01 MYO, respectively (Kocher et al. 1995; Johnson et al. 1996). In the youngest lineages of the east African lacustrine radiations, those of Lake Malawi and Lake Victoria, there has been no evidence of cone opsin nonfunctionalization (Carleton et al. 2001; Terai et al. 2002; Spady et al. 2005, Chapter 1; Carleton et al. 2005). However in the oldest lineages, those of Lake Tanganyika, Spady and coworkers (2005, chapter 1) found evidence of three nonfunctionalization events in two of three species sampled, two (SWS2a and SWS1) in a microinvertebrate predator and one in an herbivore (SWS2b). Further, all nonfunctionalization events appear to have been relatively recent, which is in

line with the expectation that the genes should have lost functionality after colonization of the lake and the initiation of ecological specialization. It is of note that most documented cases of gene loss/nonfunctionalization occurred in SWS2 and SWS1 and that these are the same gene classes where nonfunctionalization has been reported among highly specialized lacustrine cichlids.

The evolution of the opsin gene family is a dynamic process. I have very little understanding of the role of ecological factors. The cichlid system provides an attractive model to examine questions of the role of ecology in the evolution of cone opsin genes. Many cichlids possess functional copies of all the same major classes of cone opsin genes as other fishes. The key strengths of the cichlid system are the high levels of ecological diversity and the replicate nature of the lacustrine adaptive radiations. Future studies should focus on the detailed analysis of patterns of cone opsin gene expression across East African cichlid fishes to examine correlations between the suite of cone opsin genes expressed and foraging and environmental conditions. Thus cichlids will likely provide a useful resource in understanding how ecology shapes the evolution of the opsin gene palette and visual sensitivity.

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APPENDIX

November 18, 2004

Carleton, Karen
Hubbard Center for Genomic Studies
Gregg Hall
35 Colovos Road
Durham, NH 03824

IACUC #: 031201
Original Approval Date: 12/03/2003 **Next Review Date:** 12/03/2005
Review Level: C

Project: Genetics of Cichlid Visual System

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a report with regard to the involvement of animals in this study before that date. If your study is still active, you may apply for extension of IACUC approval through this office.

The appropriate use and care of animals in your study is an ongoing process for which you hold primary responsibility. Changes in your protocol must be submitted to the IACUC for review and approval prior to their implementation.

Please Note:

1. All cage, pen, or other animal identification records must include your IACUC # listed above.
1. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Van Gould at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Robert G. Mair, Ph.D.
Chair

cc: File

UNIVERSITY of NEW HAMPSHIRE

June 9, 2005

Karen Carleton
Hubbard Center for Genomic Studies
Gregg Hall
35 Colovos Road
Durham, NH 03824

IACUC #: 031201
Category: C **Approval Expiration Date:** 12/03/2005
Project: Genetics of Cichlid Visual System

To Whom It May Concern:

Please note that the individuals listed below are currently-affiliated staff on this project approved by the UNH Institutional Animal Care and Use Committee (IACUC).

Karen Carleton	Tyrone Spady
Thomas Kocher	Christie Klisz
Michael Kidd	Janis Watson

Please do not hesitate to contact me at 603-862-2003 if you have any questions.

For the IACUC,

Julie F. Simpson
Manager

cc: File
SURF/UROP