Evolution of the Cichlid Visual Palette through Ontogenetic Subfunctionalization of the Opsin Gene Arrays

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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, we present data on the riverine cichlid, *Oreochromis niloticus*, which is an ancestral outgroup to the cichlid adaptive radiations in the Great African lakes. We identify 7 cone opsin genes with several instances of gene duplication. We 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 polymerase chain reaction at 3 ontogenetic time points demonstrates that although only 4 genes (*SWS2a*, *Rh2a* α and β, and *LWS*) are expressed in adults, mRNAs for the other genes are all expressed during ontogeny. Therefore, subfunctionalization through differential ontogenetic expression may be 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, Poopalasundaram, Wilkie, Bowmaker, Hunt 2002; Cowing, Poopalasundaram, Wilkie, Robinson 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 5 major evolutionary classes of vertebrate opsins: rod opsin (*Rh1*) and 4 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). Extensive gene duplication has also been described among invertebrate opsins (Briscoe 2001; Hill et al. 2002; Spaethe and Briscoe 2004).

The duplication of opsin genes has been a common occurrence among teleost fishes. Duplications have been observed in all 4 cone opsin classes including *LWS* (cavefish: Yokoyama R and Yokoyama S 1990; Register et al. 1994; zebrafish: Chinen et al. 2003; killifish: Fuller et al. 2004; and medaka: Matsumoto et al. 2006), *Rh2* (cichlids: Carleton and Kocher 2001; zebrafish: Chinen et al. 2003; goldfish: Johnson et al. 1993; herring: AF385829 and AF385830; turbot: AF385827 and AF385828; smelt: Minamoto and Shimizu 2005; puffer fish: Neafsey and Hartl 2005; and medaka: Neafsey and Hartl 2005; Matsumoto et al. 2006), *SWS2* (cichlids: Carleton and Kocher 2001; killifish: Fuller et al. 2004; and medaka: Matsumoto et al. 2006), and *SWS1* (smelt: Minamoto and Shimizu 2005).

Cichlids had previously been thought to have 5 spectrally distinct cone opsin gene classes: *LWS*, *Rh2*, *SWS2a*, *SWS2b*, and *SWS1* (Carleton and Kocher 2001). Recent sequencing of bacterial artificial chromosome (BAC) clones containing the opsin genes from *Oreochromis niloticus* (Nile tilapia, referred to as tilapia for the remainder of the paper) has revealed the presence of 2 other *Rh2* genes (KL Carleton and JL Boore, unpublished data). Opsin genes were detected at 3 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; see also commentary by Trezise and Collin 2005), these data indicate that tilapia has a total of 7 cone opsin genes, not 5 as had been previously thought.

The revelation that tilapia might have 7 cone opsin genes is interesting because opsin gene expression has so far only been detected for a subset of the 5 genes originally reported (Carleton and Kocher 2001); why the remaining seemingly functional cone opsin genes would

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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 2 paths. They argue that subfunctionalization is a transitional state in the process of neofunctionalization. This model is well supported by the work in butterfly *LWS* opsin duplicates where duplication of the *Rh2* gene leads to both spatial subfunctionalization and spectral neofunctionalization (Briscoe 2001).

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. We 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. We then sampled larval, juvenile, and adult tilapia to examine the ontogeny of opsin gene expression and determine if temporal subfunctionalization had occurred. Finally, we compared the tilapia visual pigments with those used by the cichlids of the African lacustrine radiations to learn how gene preservation through subfunctionalization sets the stage for new adult phenotypes.

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, Carlsbad, CA). Retinal RNA preparations were then reverse transcribed with a poly T primer and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA).

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-*Eco*RI), GGCGGGTCGACCAGGAGCCACAGAGGAGACC (LWS-*Sal*I), GGCGGGAATTCCACCATGAGGGGTAATCGTGATATGG (SWS2a-*Eco*RI), GGCGGGTCGACCAGGCCCAACTTTGG (SWS2a-*Sal*I).

The expression primers and DyNAzyme EXT DNA polymerase (MJ Research, Waltham, MA) were used to amplify full-length cone opsin cDNA's. Polymerase chain reaction products were digested with *Eco*RI (NE Biolabs, Beverly, MA) and *Sal*I (NE Biolabs, Beverly, MA) 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 with 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, Madison, WI). 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 (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, Whitehouse Station, NJ). Thirty 90-mm plates were used per experiment. Cells were harvested 48 h posttransfection and washed 4 times with phosphate-buffered saline (PBS) (pH 7.0), and the cell pellets were 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 (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. Peak absorbance values quoted in the text are taken from the difference spectra to avoid distortion by the underlying absorbance and scatter of the protein. Difference spectra were generated by subtracting hydroxylamine or acid-treated spectra from the untreated absorbance spectra. The peak absorbance values were determined by fitting to visual pigment templates (Govardovskii et al. 2000).

Quantitative Real-Time PCR

Real-time reverse transcriptase (RT)–PCR was used to quantify relative cone opsin mRNA levels. Isolated whole retinas or whole eyes for larval animals were prepared as described above to generate total retinal RNA

for each individual (see cDNA Synthesis and Expression Constructs). Total retinal RNA (1 μg) was reverse transcribed using a poly T primer and Superscript III (Invitrogen, Carlsbad, CA) at 42 °C to create a retinal RT cDNA mixture (50-ul reaction). Real-time amplifications using 25-ul reactions containing 0.5 ul of the retinal RT cDNA mixture were then run (corresponds to 0.01 μg of total retinal RNA). Primers and probes were designed to amplify short (60–90 bp) fragments for each gene using Primer Express 1.5 (Applied Biosystems, Foster City, CA), as previously described by Carleton and Kocher (Carleton and Kocher 2001). Because the *Rh2a* α and β genes were so similar, we first analyzed the sum of these 2 in comparison to the other 5 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 2 *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 6 opsin genes (with *Rh2a* α and β combined) as a fraction of the total cone opsin genes expressed for an individual, (Carleton and Kocher 2001), according to

$$T_i/T_{\text{all}} = (1/(1+E_i)^{\text{Ct}_i}) \Big/ \sum (1/1+E_i)^{\text{Ct}_i},$$

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 2 *Rh2a* genes to calculate their ratio, and this ratio was then used to get 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 6 primer/probe sets was measured using a novel tool developed for this work. A construct containing amplicons for each of the 6 opsin genes (including the fragment of *Rh2a* common to both *Rh2a* α and β) was used to normalized template amounts to a 1:1 ratio for all genes. The concatenated amplicon construct (CAC; fig. 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

$$(1+E_{\text{Rh2a}})^{C_{\text{tRh2a}}}/(1+E_i)^{\text{Ct}_i} = 1,$$

where C_{tRh2a} represents the critical cycle number for the *Rh2a* gene. The relative efficiencies were averaged between all of the replicates and standard errors were determined.

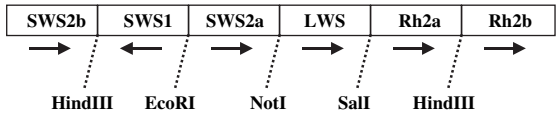

FIG. 1.—The CAC is a novel tool developed to obtain relative PCR efficiencies for real-time RT-PCR comparisons. Cichlid opsin cDNA fragments were directionally ligated using the indicated restriction sites. The fragments correspond to cDNA regions of the primers/probe used in quantitative RT-PCR experiments. Arrows have been used to indicate fragment directionality.

To determine the absolute efficiency of *Rh2a*, critical cycle number was measured for a series of 9 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

$$\text{absolute } E_i = (\text{relative } E_i \times \text{absolute } E_{Rh2a}).$$

Efficiencies for *Rh2a* α and β were also determined using the slope from a dilution series plot.

Tilapia specimens were laboratory bred and reared under standard conditions. Four to five individuals from each of the 3 age classes, larval (14–18 days postfertilization [dpf]), juvenile (43–64 dpf), and adult (>150 dpf), were sampled. Two replicates were performed for each individual.

Results

Tilapia Opsin Gene Sequences

Complete opsin-coding sequences were obtained for all 7 tilapia cone opsin genes (*LWS*, *Rh2a* α, *Rh2a* β, *Rh2b*, *SWS2a*, *SWS2b*, and *SWS1*). There were a small number of nucleotide differences among the expression constructs when compared with the previous tilapia individual. (Carleton et al. 2000; Carleton and Kocher 2001). All except 2 of these substitutions were synonymous. The 2 exceptions were both in the *SWS1*-coding sequence, although only one encoded an amino acid change (F-214-I; bovine rhodopsin numbering) within a transmembrane region (IV). 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. Opsin gene sequences obtained have been deposited in the GenBank database (DQ235678–DQ235684).

Phylogenetic Relationships of Fish Opsin Genes

Figure 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 have occurred (Nelson 1994; Kumazawa et al. 1999; Miya et al. 2003; Saitoh et al. 2003; Chen et al. 2004).

LWS genes from the 2 cichlid species cluster together with 100% bootstrap support (fig. 2*A*). Acanthopterygian

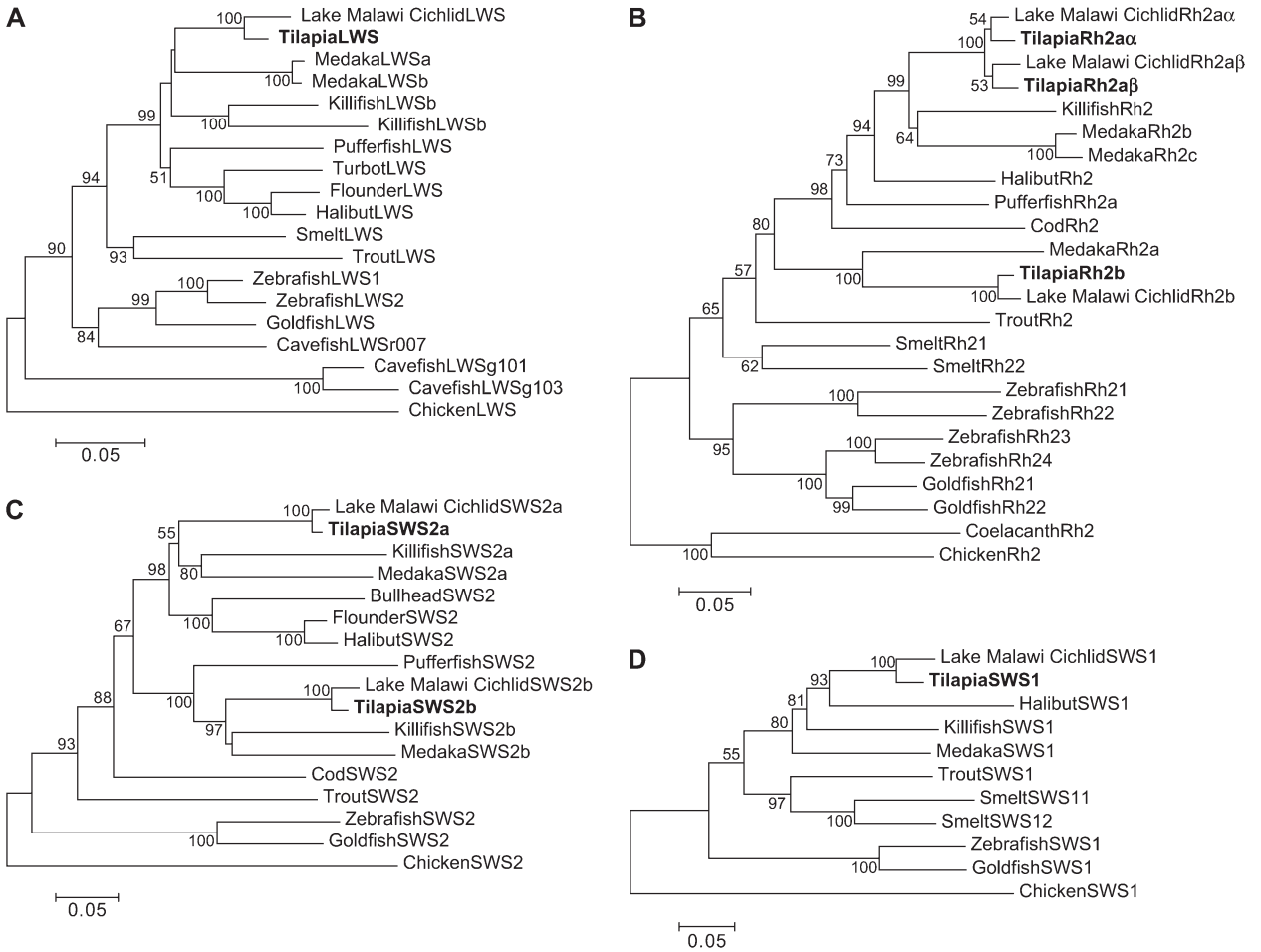

FIG. 2.—Neighbor-joining trees were constructed for fish *LWS* (*A*), *Rh2* (*B*), *SWS2* (*C*), and *SWS1* (*D*), using gamma-corrected Tamura–Nei distances. Bootstrap values are indicated when greater than 50%. Scale bars indicate the number of substitutions per 100 sites. The following sequences were 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), puffer fish (*LWS*, AY598942; *Rh2a*, AF226989; *SWS2*, AY598947), medaka (*LWSa*, AB223051; *LWSb*, AB223052; *Rh2a*, AB223053; *Rh2b*, AB223054; *Rh2c*, AB223055; *SWS2a*, AB223056; *SWS2b*, AB223057; *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).

LWS genes form a clade supported by a 99% bootstrap score. The remainder of the tree is in agreement with previous studies of fish *LWS* duplications and other vertebrate *LWS* genes (Chinen et al. 2003; Fuller and Travis 2004; Matsumoto et al. 2006).

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 (fig. 2*B*). 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 the tilapia *Rh2* genes (Parry et al. 2005), suggesting that the duplication event that generated cichlid *Rh2a* paralogs occurred before the divergence of tilapia from the rapidly speciating lacustrine cichlids. Alternatively, the *Rh2a* duplicates of both tilapia and the lacustrine lineages could have arisen independently, although this is less likely. Gene conversion between the duplicated *Rh2* genes may have reduced the level of divergence and thereby reduced the apparent antiquity of the duplication event, as found for the L and M *LWS* duplicate opsin genes of Old World primates (Ibbotson et al. 1992). This is however unlikely as the tilapia *Rh2a* duplicates are in different genomic orientations (KL Carleton and JL Boore, unpublished data). It is difficult to envisage a mechanism for conversion between genes in a head-to-head configuration. The branching pattern of the medaka *Rh2b*/*Rh2c* and tilapia *Rh2a* duplicates could also be consistent with gene conversion, which could inflate the number of apparent duplication events. However, medaka *Rh2b* and

Rh2c are in a head-to-tail configuration (Matsumoto et al. 2006), whereas tilapia *Rh2a* α and *Rh2a* β are in a head-to-head configuration (KL Carleton and JL Boore, unpublished data), almost certainly therefore the products of independent duplication events. The tilapia *Rh2a*/*Rh2b* split is far older. Tilapia *Rh2b* clusters with medaka *Rh2a* with 100% bootstrap support to the exclusion of all other Acanthopterygii (e.g., cichlids) and Paracanthopterygii (e.g., cod) *Rh2* genes, which form a clade with 98% bootstrap support. Similarly, the tilapia *Rh2a* genes cluster with medaka *Rh2b*/*Rh2c* with 99% 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) and Matsumoto et al. (2006), which suggest that other Paracanthopterygii and Acanthopterygii may have an ortholog to tilapia *Rh2b*, giving them at least 2 *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 (fig. 2*C*). Cichlid *SWS2a* and *SWS2b* opsin genes cluster with killifish/medaka *SWS2a* and *SWS2b* with bootstrap support of 55% and 97%, respectively. 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*/*Rh2b* split, although the modest bootstrap value (67%) 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; Matsumoto et al. 2006).

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). We are aware that gene conversion could have had an impact on the number of apparent gene duplication events and hence on the branching patterns of many of the noncichlid duplicates in *SWS1*, *LWS*, and *Rh2* trees, although we did not test for this.

Spectral Characteristics of Tilapia Visual Pigments

Expression and in vitro reconstitution of the 7 tilapia cone opsin genes gave 7 photosensitive pigments, confirming that all genes are indeed functional. The pigment set covers the entire visible spectrum, with each pigment showing a spectrally distinct peak absorbance value: LWS 561 nm; Rh2a α 528 nm; Rh2a β 518 nm; Rh2b 472 nm; SWS2a 456 nm; SWS2b 425 nm; and SWS1 360 nm (fig. 3). The 3 *Rh2* genes cover a large range from 472 to 528 nm, making this class spectrally very broad. The peak absorbance values obtained for these pigments agree well with those observed for closely related species (Parry et al. 2005; Jordan et al. 2006).

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*). These values do not consider possible differences in the reverse transcription efficiencies, although preliminary comparisons of tilapia photoreceptor cell counts and mRNA expression are generally correlated (KL Carleton and JL Boore, unpublished data), as have been observed in killifish (Fuller et al. 2004). 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 3 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 2 genes are so similar in sequence. Cross amplification was 0.06 and 0.007 for *Rh2a* α and β, respectively. However, this level of cross amplification is sufficiently low to distinguish these genes.

Gene expression changed considerably through the 3 ontogenetic stages examined (fig. 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 60% or more of the total cone opsin gene expression for juvenile and adult age classes (fig. 4). In the larval class, all opsins are expressed except *SWS2a*. By the juvenile age class, *SWS2a* is expressed while *Rh2a* α, *Rh2b*, and *SWS1* expression falls dramatically. By the adult age class, LWS opsin expression makes up 80% of the total cone opsin expression. *SWS2b* expression falls from 11% (juvenile) to less than 2% of the total cone opsin expression. In contrast, *SWS2a* expression increases from undetected (larval) to 8.8%.

These results demonstrate that each of the opsin gene codes for a functional visual pigment and that its mRNA is expressed within the retina at some developmental stage. This suggests that visual system sensitivities change considerably from larvae to adults. 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 may benefit from a shorter wavelength sensitivity.

Discussion

The duplication of the opsin genes is widespread, occurring in invertebrates (Briscoe 2001; Hill et al. 2002; Spaethe and Briscoe 2004) and vertebrates alike (Nathans et al. 1986; Jacobs et al. 1996; Dulai et al. 1999). Phylogenetic analysis of fish retinal opsin gene sequences in both current and previous studies supports this (Yokoyama R and Yokoyama S 1990; Johnson et al. 1993; Register et al. 1994; Carleton and Kocher 2001; Chinen et al. 2003; Fuller and Travis 2004; Minamoto

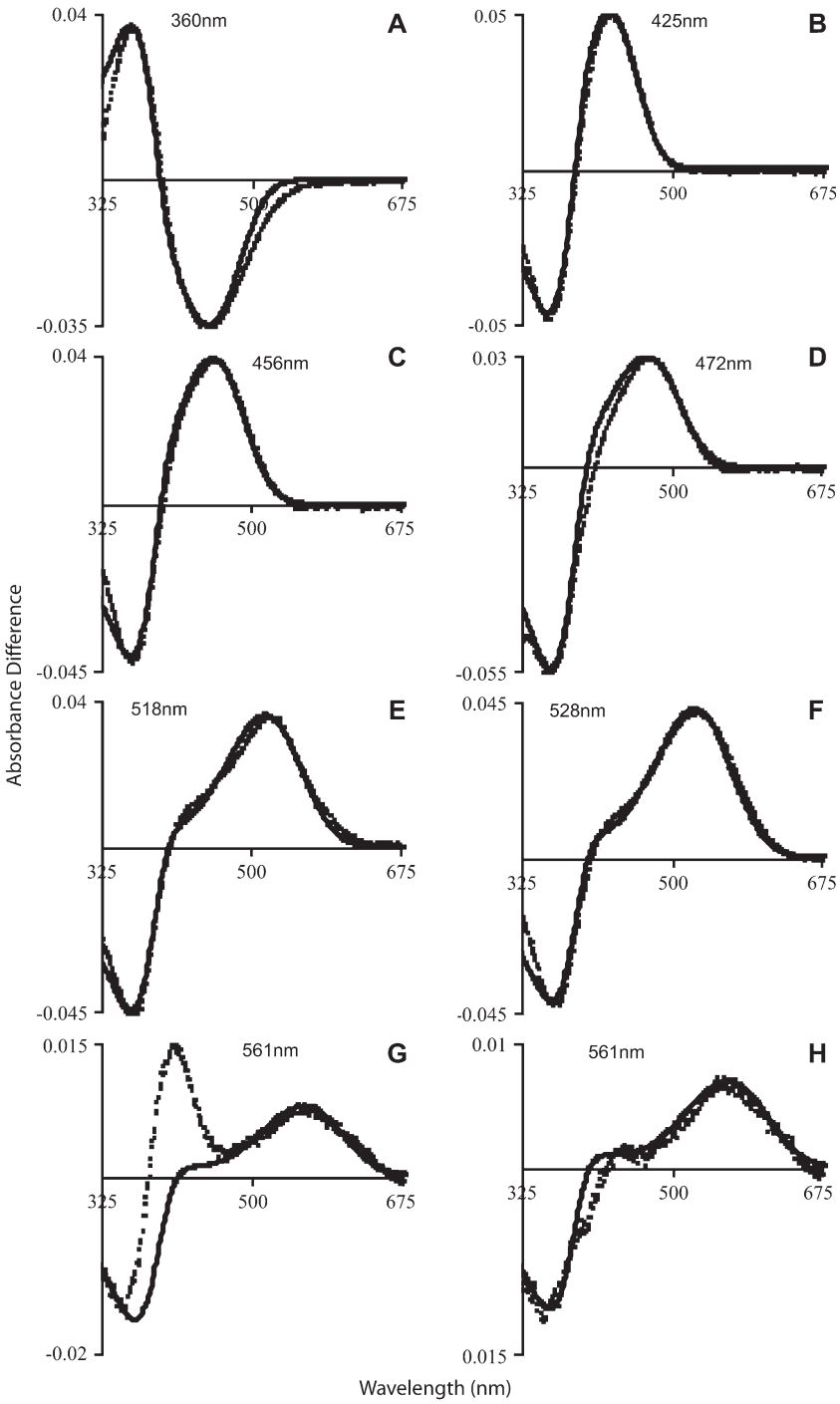

FIG. 3.—Difference spectra of reconstituted tilapia visual pigments are shown. Absorbance spectra were measured before the pigment was denatured with acid (*A*) or hydroxylamine treated (*B–G*). The latter spectra were subtracted from the former and resulting difference spectra fit using visual pigment templates (Govardovskii et al. 2000). Visual pigment peak absorbances were 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 360 nm (*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*).

and Shimizu 2005; Neafsey and Hartl 2005; Matsumoto et al. 2006). 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 all may have at least 2 *SWS2* and 2 *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

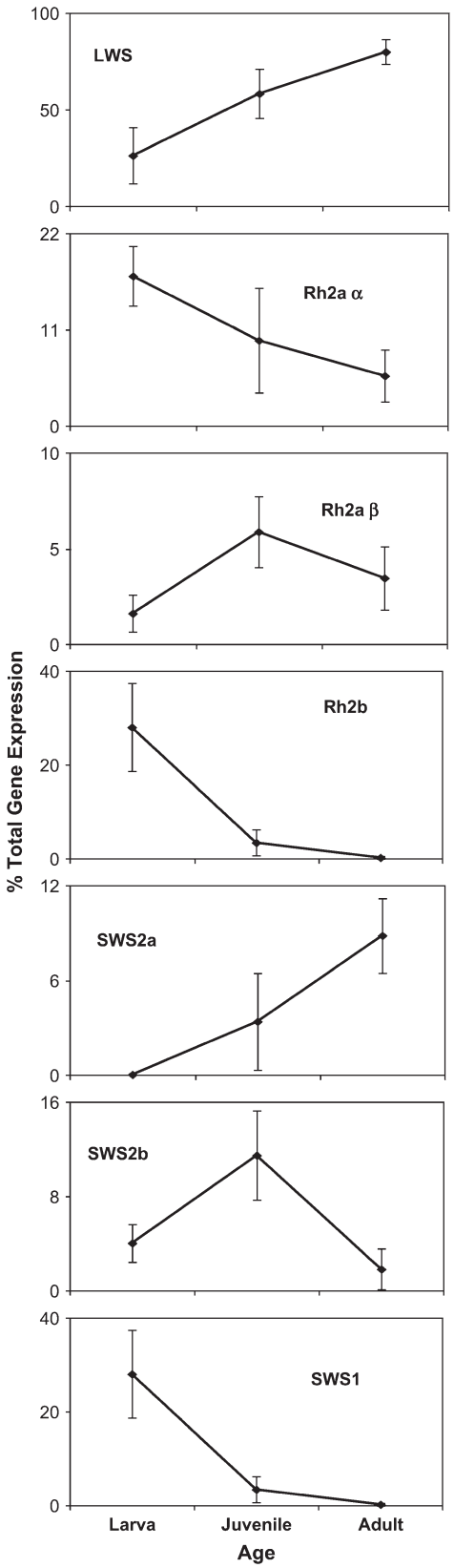

FIG. 4.—Relative cone opsin expression profiles for larval, juvenile, and adult age classes were determined using real-time RT-PCR analysis of the equivalent of 0.01 μg total retinal RNA per reaction. Expression levels are given as percentages of the total cone opsin genes expressed for a given age class. Error bars are ±1 standard deviation.

260 MYA (Kumazawa et al. 1999). Yet, only in puffer fish, medaka, and cichlids have these genes been sequenced and only in medaka and cichlids have their expression been confirmed (Neafsey and Hartl 2005; Parry et al. 2005; Matsumoto et al. 2006). 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 11 nm. 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 microspectophotometry (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 life stages, and this may be true for other species. Alternatively, the possibility remains that extra cone opsin genes may be expressed outside the photoreceptors, for example, in noncone neural tissues/cell types (Forsell et al. 2001, 2002) and skin (Ban et al. 2005), although none have been shown to be expressed exclusively outside the cones. In situ hybridization studies are in progress to examine the exact cellular location of cone opsin transcripts within the retina.

Genes that are not expressed are expected to evolve free of the constraints of selective pressure. 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 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 (Deutschlander et al. 2001), 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.

Comparisons can be made between 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*. These

Table 1
Comparison of tilapia and Lake Malawi cichlid cone visual pigments

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

[a] Parry et al. 2005.
[b] Peak absorbances are from MSP recordings of *Melanochromis vermivorus* (556 nm) and *Tramitochromis intermedius* (455 nm). All other peak absorbances are from in vitro expression and reconstitution experiments.

show that peak absorbances are largely similar among these species (table 1), as have been predicted from sequence comparisons (Carleton and Kocher 2001). This is in spite of the 10 Myr 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. 2005). Although there is a 12-nm difference in peak absorbance of Rh2b and an 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 these same species.

Photopigment complements are quite different between East African cichlids studied by MSP. 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). Among Lake Malawi species, 3 different cone pigment combinations have been observed. The most long wavelength 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 2 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., *M. zebra* with a 368-nm pigment [Carleton et al. 2000]). These 2 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. 2005).

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 (fig. 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. For example, for the Lake Tanganyika species, *A. burtoni*, the *SWS2a*, *Rh2a β*, and *LWS* opsin genes were sequenced from retinal cDNA (Halstenberg et al. 2005), however, the presence of *Rh2a α* cDNA was not assayed and therefore cannot be ruled out. 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 (Carleton et al. 2005; Parry et al. 2005).

The cichlids studied from all 3 lakes show that the full set of cichlid opsin genes have been used across species to generate at least 3 different photopigment combinations. The riverine tilapia is an outgroup to the lacustrine cichlid

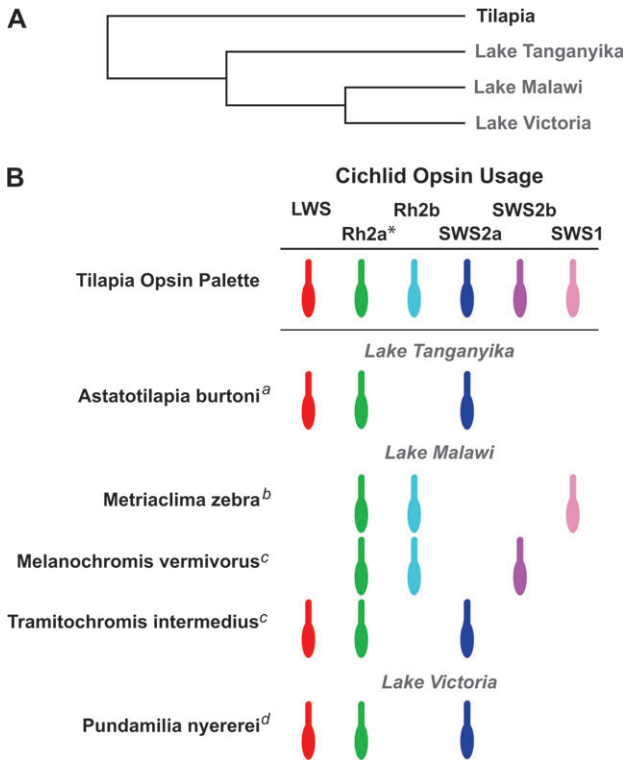

FIG. 5.—Tilapia opsin genes are correlated to lacustrine cichlid photopigment usage. (*A*) The basal phylogenetic relationship of tilapia relative to the lacustrine cichlids is shown (Kocher et al. 1995). (*B*) Photoreceptor markers are used to indicate opsin gene usage. Opsin gene usage was inferred 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 (Carleton et al. 2005; Parry et al. 2005). a, Fernald and Liebman 1980; b, Carleton et al. 2000; Levine and MacNichol 1979; c, Parry et al. 2005; d, Carleton et al. 2005.

species and is in many ways representative of the ancestral state. This suggests that all genes 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 we have characterized in tilapia therefore 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.

Supplementary Material

Sequences reported in this paper have been deposited in the GenBank database (accession numbers: DQ235678–DQ235684).

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