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Visual Pigment Molecular Evolution in the Trinidadian Pike Cichlid (*Crenicichla frenata*): A Less Colorful World for Neotropical Cichlids?

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Abstract

The Trinidadian pike cichlid (*Crenicichla frenata*) is a major predator of the guppy (*Poecilia reticulata*), a model system for visual ecology research, and visual predation by the pike cichlid is known to select for male guppies with reduced short-wavelength reflectance. However, an early study of the pike cichlid's visual system suggested a lack of short-wavelength–sensitive cone photoreceptors, a surprising finding as many African cichlids have highly developed short-wavelength vision. In this study, we found evidence for only four expressed cone opsins (LWS, RH2a, SWS2a, and SWS2b), plus one pseudogene (RH2b). Taken together with our microspectrophotometry data, which revealed the presence of three types of cone photoreceptor, including one sensitive to short-wavelength light, this would indicate a broader spectral capacity than previously believed from earlier visual studies of this fish. Relative to the highly diverse African cichlids, however, this Neotropical cichlid appears to have a greatly reduced opsin complement, reflecting both gene loss along the Neotropical lineage (lacking functional RH2b and, possibly, SWS1 opsins) and gene duplication within the African clade (which possesses paralogous RH2aα and RH2aβ opsins). Molecular evolutionary analyses show that positive selection has shaped the SWS2b and RH1 opsins along the Neotropical lineage, which may be indicative of adaptive evolution to alter nonspectral aspects of opsin biology. These results represent the first molecular evolutionary study of visual pigments in a Neotropical cichlid and thus provide a foundation for further study of a morphologically and ecologically diverse clade that has been understudied with respect to the link between visual ecology and diversification.

Key words: *Crenicichla frenata*, *Poecilia reticulata*, *Crenicichla alta*, opsins, d_N/d_S, visual ecology.

Introduction

Understanding the evolution of color patterns is a long-standing goal in evolutionary biology. Determining how natural and sexual selection combine to shape the evolution of color patterns requires an understanding of how these colors are perceived by the eyes of relevant conspecifics and predators (Otte 1974; Endler 1978). The Trinidadian pike cichlid (*Crenicichla frenata*, though often referred to in the literature as *C. alta* (see Coleman and Kutty 2001) is a major predator of the guppy (*Poecilia reticulata*) in streams of Trinidad's Northern Range, and together, these species have served as a model system for the study of natural and sexual selection on coloration in the wild. The guppy is renowned for its exceptionally conspicuous male-limited coloration, a sexually selected trait that is favored by female choice (reviewed in Houde 1997; Magurran 2005); however, at sites where guppies and pike cichlids coexist, male guppies tend to bear relatively drab color patterns (Endler 1978). Comparative and experimental evolutionary studies have shown that predation by the visually oriented pike cichlid opposes the sexually selected promotion of conspicuous patterning in male guppies (Endler 1980; Kemp et al. 2009).

Although guppy color patterns are composed of numerous spots of varying colors, pike cichlid predation appears to impose particularly strong natural selection against males bearing blue and iridescent (structural) spots (Endler 1980; Kemp et al. 2009), suggesting that the pike cichlid employs short-wavelength vision during prey search. However, the pike cichlid has long been thought to be quite insensitive to short-wavelength light, as an early microspectrophotometric study of this fish's retina did not reveal the presence of any cone photoreceptors maximally sensitive to short-wavelength light (Levine J, Lythgoe J, and MacFarland WN, unpublished data cited in Endler 1991). How pike cichlid predation can impose such strong selection against structural spots on male guppies is therefore unclear, and a complete understanding of the role that predation plays in shaping color pattern evolution in this influential study system will be unreachable until this paradox is clarified.

Little is known about cone photoreceptor variation in Neotropical cichlids, but surveys of cone photoreceptors

and visual pigment genes in African cichlids, the sister group to the Neotropical cichlid clade, have shown that short-wavelength vision is generally quite well developed (reviewed in Carleton 2009). African cichlids generally possess three or more spectral classes of cone photoreceptor in their retinas, including at least one maximally sensitive to short-wavelength light. Photon absorption by photoreceptor cells is mediated by visual pigments, photosensitive compounds composed of an opsin protein, and a covalently linked retinal chromophore (Sakmar 2002; Terakita 2005); changing either of these two components can tune the pigment's wavelength of maximally sensitivity (λ_{max}), generating variation in spectral sensitivity (Bowmaker 2008; Yokoyama 2008). Interestingly, divergence in cone opsin sequences and expression levels has been linked to ecologically and sexually selected divergence in the African cichlid adaptive radiations (Seehausen et al. 2008; Maan and Seehausen 2010; Terai and Okada 2011). African cichlids possess seven cone opsins that, when combined with the typical A_1-type retinal chromophore, form pigments maximally sensitive to light ranging from the yellow down to the ultraviolet (UV). Three of these opsins form pigments maximally sensitive to short-wavelength light—SWS2a (blue), SWS2b (violet), SWS1 (UV)—and phylogenetic study of these opsins suggests that all should be present in Neotropical cichlids like the Trinidadian pike cichlid, assuming that none have been lost (Spady et al. 2006).

The limited data on cone photoreceptors in Neotropical cichlids show that most species harbour three cones, including one maximally sensitive to blue-green light ($\lambda_{max} \approx$ 445–480 nm) (Levine and MacNichol 1979; Wagner and Kroger 2005). These findings contrast with the reported data for the Trinidadian pike cichlid's retina—one cone with $\lambda_{max} \approx$ 606 nm, and another with $\lambda_{max} \approx$ 545 nm, both long-wavelength–sensitive pigments (Endler 1991)—and suggest that our knowledge of cone photoreceptors in the Trinidadian pike cichlid may be incomplete. Here, we investigated vision in the Trinidadian pike cichlid through a combination of opsin sequencing, molecular evolutionary analyses, and microspectrophotmetry (MSP). Both our sequencing and MSP results provide convincing evidence for vision at short wavelengths in this fish. However, despite our evidence for increased visual sensitivity at short wavelengths, the pike cichlid appears to have a markedly reduced number of visual pigment genes relative to African cichlids. Molecular evolutionary analyses found significant evidence for positive selection in two of the opsins along the Neotropical lineage, indicating a possible role for adaptive evolution in the vision of this fish.

Materials and Methods

Amplification and Sequencing of *Crenicichla frenata* Opsins

Crenicichla frenata opsin DNA sequences were first obtained by targeted polymerase chain reaction (PCR) using an eye cDNA library as PCR template. Eye tissue was obtained from an unsexed individual from Trinidad's Aripo River. The fish was housed for 2 days in our field station's partially enclosed aquarium facility before euthanization, which occurred mid-day, and eye tissue was preserved in RNAlater (Qiagen) until processing. RNA was extracted from a single whole eye using an RNeasy MiniKit (Qiagen), and the cDNA library was prepared using a SMART cDNA Library Construction Kit (BD Biosciences). Degenerate primer PCR was carried out using the FastStart *Taq* DNA polymerase enzyme (Roche), with primers designed according to conserved regions of teleost fish opsins. African cichlids are known to possess one rod opsin plus seven cone opsins (Carleton 2009): one long-wavelength–sensitive cone opsin (LWS: maximally sensitive to yellow-to-red light), three medium-wavelength–sensitive or rod-like cone opsins (RH2aα, RH2aβ, and RH2b: maximally sensitive to different shades of green light), and three short-wavelength–sensitive cone opsins (SWS2a, SWS2b, and SWS1: maximally sensitive to blue, violet, and UV light, respectively). Multiple forward and reverse primers were designed for each opsin family so as to allow for nested PCR, as needed. Following agarose gel electrophoresis, appropriately sized PCR products were spin column purified using the QiaQuick Gel Extraction Kit (Qiagen), then directly sequenced on an ABI-377 DNA Synthesizer (Applied Biosystems). This initial sequence data was used to design specific primers for use in RACE PCR (Frohman et al. 1988). Genomic DNA was obtained from ethanol-preserved muscle tissue via phenol–chloroform extraction, and the GenomeWalker Universal Kit (Clontech) was used to prepare genomic DNA libraries. All sequencing was carried out in both directions, and sequence chromatograms were evaluated by eye using 4Peaks 1.7 (Griekspoor and Groothuis 2006). As sequence data were obtained from both cDNA and genomic DNA for some opsins, the presence of complete transcripts was confirmed via PCR using primers spanning or situated just beyond the initiation and termination codons. Primer information is provided in supplementary table 1 (Supplementary Material online).

Phylogenetic Analyses

In order to build data sets of teleost opsin sequences, the opsin sequences of Nile tilapia (*Oreochromis niloticus*) (Spady et al. 2006) were used as queries for taxonomically restricted BLASTn searches (Altschul et al. 1990) of the NCBI Genbank "nr" database. Only complete or near-complete sequences were retained, and highly similar sequences were excluded. Within the African cichlid clade, where a great number of highly similar sequences are available, attempts were made to include members of each of the Rift Valley Great Lakes (Lakes Victoria, Malawi, and Tanganyika). Where possible, sequences from each of the rock and sand-dwelling clades of Lake Malawi were included, and sampling took into account the paraphyletic nature of the Lake Tanganyika assemblage (Koblmuller et al. 2008). It should be noted, however, that sequence availability is uneven across different taxonomic groups and opsin families. Opsin family-specific data sets were assembled and aligned using ClustalW (Thompson et al. 1994) as

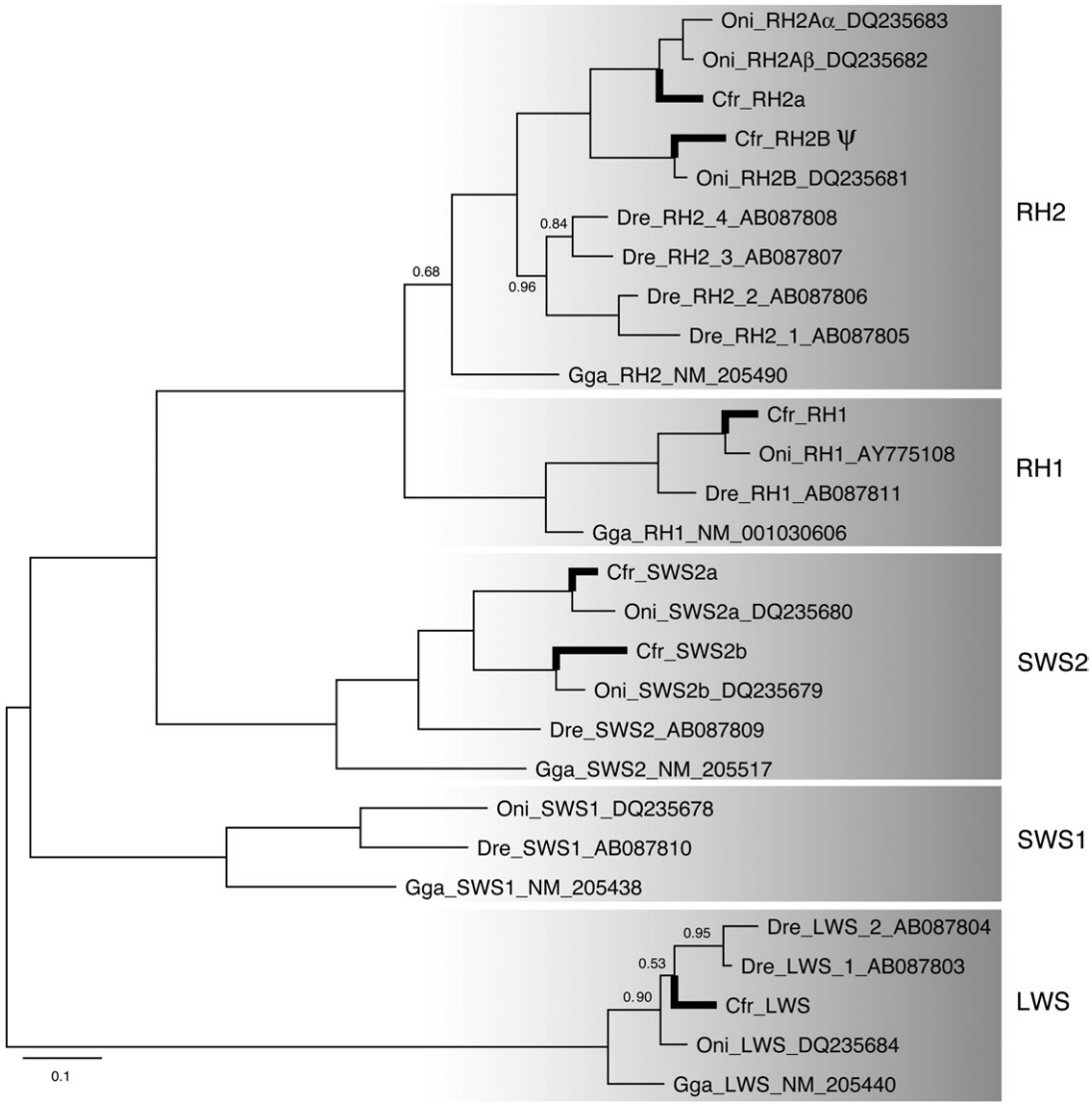

Fig. 1. Bayesian phylogeny of *Crenicichla frenata* rod and cone opsins. The tree describes the relationships among the six *C. frenata* (Cfr) opsin sequences (including the RH2b pseudogene) and the visual opsins of Nile tilapia (*Oreochromis niloticus*, Oni), zebra fish (*Danio rerio*, Dre), and chicken (*Gallus gallus*, Gga). NCBI Genbank accession numbers are provided in the figure. The terminal branches leading to *C. frenata* opsins are indicated in bold, and the five major opsin families are shaded and labeled. Branch length units are amino acid substitutions per site. Branch support values are provided only when the posterior probability was less than 1.00.

implemented in MEGA 4 (Tamura et al. 2007). Alignments were carried out using translated amino acid sequences. The *C. frenata* RH2b opsin, which was found to be pseudogenized (supplementary fig. 1, Supplementary Material online), was manually adjusted to ensure the proper reading frame, as were the *Neolamprologous brichardi* SWS2a and *Tropheus duboisi* SWS2b opsins (see Spady et al. 2005). The extreme N- and C-termini of the alignments were trimmed when necessary due to ambiguous gap placement. An additional data set encompassing all vertebrate visual opsin families (the *All Opsins* data set) was created using opsin data from *C. frenata* and three other vertebrates for which opsins have been extensively studied: Nile tilapia (*O. niloticus*), zebra fish (*Danio rerio*), and chicken (*Gallus gallus*). This data set was aligned and trimmed as described above. Accession numbers are provided in figures 1 and 2. All amino acid numbering follows that of bovine RH1 opsin (rhodopsin).

Gene trees were estimated using a Bayesian approach, as implemented in MrBayes 3.2 (Ronquist and Huelsenbeck 2003). For the *All Opsins* data set, analyses were carried out on amino acid translated data. The empirical amino acid substitution rate matrix of Whelan and Goldman (2001) was employed to account for variation in substitution rates between different amino acids, and a Γ distribution (Yang 1994) was used to model among-site rate variation (WAG + Γ). Other models were employed as well (both amino acid and nucleotide), but the results were qualitatively similar and did not affect our conclusions (results not shown). Bayesian analysis involved four runs, each comprised of four separate chains (three heated, one cold). The analysis was run for 10^6 generations, with sampling occurring every 100 generations. The first 25% of samples were discarded so as to eliminate the "burn-in" phase of the analyses. Adequacy of sampling, run convergence,

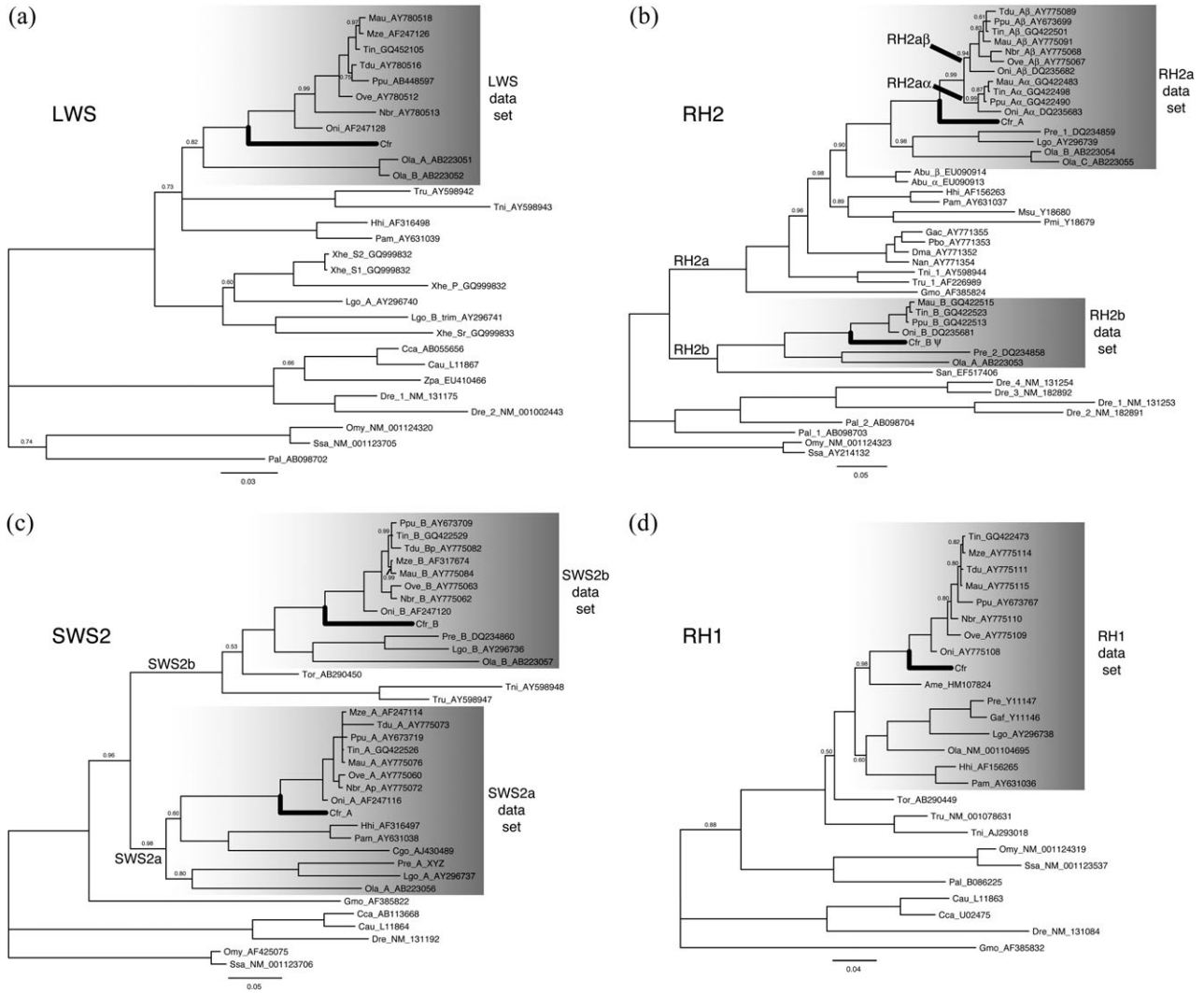

FIG. 2. Bayesian phylogenies of opsin family-specific data sets. (*a*) LWS. (*b*) RH2. (*c*) SWS2. (*d*) RH1. The reduced data sets used for subsequent molecular evolutionary analyses are shaded and labeled. Branch length units are nucleotide substitutions per site. See figure 1 for additional details. Species codes: Abu, *Acanthopagrus butcheri*; Ame, *Amphiprion melanopus*; Cau, *Carassius auratus*; Cca, *Cyprinus carpio*; Cfr, *Crenicichla frenata*; Cgo, *Cottus gobio*; Dma, *Dissostichus mawsoni*; Dre, *Danio rerio*; Gac, *Gymnodraco acuticeps*; Gaf, *Gambusia affinis*; Gmo, *Gadus morhua*; Hhi, *Hippoglossus hippoglossus*; Lgo, *Lucania goodei*; Mau, *Melanochromis auratus*; Msu, *Mullus surmuletus*; Mze, *Metriaclima zebra*; Nan, *Notothenia angustata*; Nbr, *Neolamprologus brichardi*; Ola, *Oryzias latipes*; Omy, *Oncorhynchus mykiss*; Oni, *Oreochromis niloticus*; Ove, *Ophthalmotilapia ventralis*; Pal, *Plecoglossus altivelis*; Pam, *Pseudopleuronectes americanus*; Pbo, *Pagothenia borchgrevinki*; Pmi, *Pomatoschistus minutus*; Ppu, *Pundamilia pundamilia*; Pre, *Poecilia reticulata*; San, *Scopelarchus analis*; Ssa, *Salmo salar*; Tdu, *Tropheus duboisi*; Tin, *Tramitichromis intermedius*; Tni, *Tetraodon nigroviridis*; Tor, *Thunnus orientalis*; Tru, *Takifugu rubripes*; Xhe, *Xiphophorous hellerii*; and Zpa, *Zacco pachycephalus*.

and burn-in assignment were determined by ensuring the following: 1) the standard deviation (SD) of split frequencies was less than 0.01 by the end of the analysis, 2) post-scale reduction factors for all parameter estimates and topology bipartition splits were approximately 1.000, 3) likelihood and parameter estimates were stationary during the post-burn-in phase of estimate-by-generation plots, and 4) the effective sample sizes for all likelihood or parameter estimates were substantially greater than 100. The first two adequacy measures were established directly from the MrBayes output files, whereas the last two were checked using Tracer 1.5 (Rambaut and Drummond 2009). Similar methods were employed for opsin family-specific data sets (LWS, RH2, SWS2, and RH1), except that analyses were run for 5×10^6 generations, and nucleotide, rather than amino acid, sequences were analyzed. Both nonpartitioned and codon-position–partitioned analyses were carried out following Akaike Information Criterion (AIC)-based model selection using MrModeltest 2.2 (Nylander 2004). For the standard (nonpartitioned) approach, AIC model comparisons led us to use a general time reversible model for each data set, with among-site rate variation accommodated by assuming both a category of invariant sites and a Γ distribution of substitution rate variation (GTR + I + Γ) (see Yang [2006] for information on substitution models). Similarly, for the codon-partitioned approach, we used separate GTR + I + Γ models for each codon partition of each opsin-family data set, with three

exceptions; the GTR + Γ model was used for codon positions 2 and 3 of the SWS2 data set, and codon position 3 of the RH1 data set. In each case, Bayes factor (BF) comparisons (Kass and Raftery 1995) revealed the codon-partitioned analyses to be a substantially better fit to the data set compared with the standard analyses ($2 \times ln(BF) > 500$ for each comparison). We therefore only present the results of the codon-partitioned analyses.

Molecular Evolutionary Analyses

Codon substitution models were used to assess among-site and among-branch variation in ω, the ratio of nonsynonymous (d_N) to synonymous (d_S) substitution rates (d_N/d_S). This ratio provides an indication of the form and strength of selection operating on protein coding genes (Yang and Bielawski 2000; Anisimova and Kosiol 2009), with $\omega = 1$ indicating a lack of selective constraint, $0 < \omega < 1$ indicating purifying selection (amino acid substitutions are accumulating slower than synonymous substitutions), and $\omega > 1$ indicating positive selection (amino acid substitutions are accumulating faster than synonymous substitutions). Six reduced data sets, corresponding to the LWS, RH2a, RH2b, SWS2a, SWS2b, and RH1 opsins, were subjected to molecular evolutionary analyses; reduced data sets were constructed by pruning our gene trees to retain only the monophyletic clades including the opsins of *C. frenata* and the medaka (*Oryzias latipes*). Codon substitution models were fit using maximum likelihood (ML) to these pruned, opsin family-specific data sets using the codeml program of the PAML 4.2 package (Yang 2007). We based our analyses on estimated gene trees, rather than trees that reflect consensus opinions of species relationships, as similar analyses, carried out on similar data sets, showed that the use of estimated gene trees results in more conservative tests of selection (Spady et al. 2005). Furthermore, as opsin gene gain and loss appears common in teleosts (Gojobori and Innan 2009), species trees may not accurately reflect evolutionary history for the gene of interest.

Branch analyses (Yang and Nielsen 1998) and branch-site analyses allowing for among-site variation in d_N/d_S (Zhang et al. 2005) were used to compare d_N/d_S between the Neotropical lineage (the terminal branch leading to *C. frenata*; the "foreground" lineage) and the rest of the data set (the "background" lineages). We also carried out additional branch model analyses on the RH2b data set to obtain a rough estimate of when pseudogenization may have occurred. Here, 1) the tree was artificially rooted such that the *C. frenata* terminal branch was bisected, 2) only the "tip-half" of this bisected branch was designated the foreground branch, and 3) d_N/d_S was fixed at $\omega = 1$ for this foreground partition (supplementary fig. 2, Supplementary Material online). Branch lengths, κ, and, for the background partition only, ω were all estimated by ML. This provided us with two partial estimates for the total branch length between *C. frenata* and the African–Neotropical common ancestor, one where $\omega < 1$ (i.e., prior to the removal of constraint) and one where $\omega = 1$ (i.e., following the removal of constraint). We then calculated the proportion of synonymous changes along the total branch that occurred before the removal of purifying selection and transformed this proportion into an estimate of time by assuming that the African–Neotropical common ancestor existed 85 Ma (Genner et al. 2007). A 95% confidence interval (CI) was constructed by varying the position of the artificial root along the bisected branch (leaving all other variables at their ML estimates) and using codeml to estimate overall model likelihood score given the adjusted root position. A Perl script was written to modify the root position (using the "in.codeml" file option and the "−1" option), call the codeml software, and extract likelihood scores from the codeml output file. The 95% CI was calculated by noting when likelihood decreased by >1.92 compared with the ML estimate (Yang 2006). The alignment gaps inserted into the *C. frenata* RH2b sequence to ensure proper reading frame were treated as ambiguous characters, but similar results were obtained when all gaps were deleted prior to analyses (results not shown).

For branch-site analyses, branch-site alternative model (BrS-A) was compared against two simpler models, the branch-site null (BrS-N) model and the M1a random-sites model (Zhang et al. 2005). A likelihood ratio test (LRT) comparing BrS-A against BrS-N constitutes a test for site-specific positive selection along the foreground lineage, whereas an LRT comparing BrS-A against M1a constitutes a test for either positive selection or relaxed constraint. Assignment of specific alignment sites to the positively selected site class was carried out using a Bayes empirical Bayes (BEB) approach (Yang et al. 2005), as implemented in codeml. BEB sites were mapped on to 3D homology models of the opsin protein to aid in structural interpretation; details on the estimation, assessment, and visualization of homology models (Marti-Renom et al. 2000) are presented as supplementary text (Supplementary Material online). It should be noted that a recent paper argued that the branch-site method for detecting positive selection is prone to false positives (Nozawa et al. 2009). However, the conclusions of this study have been challenged (Yang et al. 2009) and subsequent simulation studies have validated the branch-site test's statistical properties (Yang and dos Reis 2011). Nozawa et al. (2009) also argued that the empirical Bayes approach for identifying positively selected sites is unreliable as they did not find good correspondence between lists of inferred positively selected sites and known spectral tuning sites (see also Yokoyama et al. 2008). However, as others have also noted (Yuan et al. 2010), this study suffered from a number of drawbacks, including 1) the use of random-site models to search for positively selected sites rather than the more realistic branch-site models that allow for among-lineage variation in selection, 2) partial reliance on a naive empirical Bayes approach for identifying positively selected sites, and 3) the use of experimentally determined spectral tuning sites as a benchmark list of "adaptive sites," implicitly assuming that all spectral tuning sites are known and contribute to adaptive variation and that nonspectral attributes of visual pigment biochemistry are unimportant for adaptation.

The relative goodness-of-fit of different branch and branch-site models was determined using LRTs (Huelsenbeck and Rannala 1997). LRTs were performed by comparing twice the difference in ln likelihood scores against a χ^2 distribution, with the degrees of freedom equal to the difference in the number of model parameters. The only exception was for the branch-site LRTs for positive selection, where the null model (BrS-N) is formed by constraining a free parameter ($\omega_2 > 1$) from the alternative model (BrS-A) to its lower boundary ($\omega_2 = 1$); here, following Goldman and Whelan (2000) and Yang and dos Reis (2011), a 50:50 mixture distribution of 0 and χ^2_1 was used instead. AIC scores (Akaike 1974), which were also used to evaluate the relative fit of branch-site models, were calculated for each model by adding a penalty of 2*K* to twice the negative ln likelihood score, where *K* equals the number of parameters. Branch lengths and κ, the transition-to-transversion rate ratio, were estimated by ML, whereas stationary codon frequencies were estimated using the F3 × 4 approximation method. Analyses were carried out five times from different initial starting points to detect and avoid local optima; in each case, either the initial ω value or the initial κ value was altered, depending on the model.

Microspectrophotometry

MSP was carried out on two wild-caught *C. frenata* individuals (~12–15 cm long). Both fish were sampled from the Caroni drainage of northern Trinidad: one from the Guanapo River and one from the Aripo River. Both individuals were maintained under laboratory conditions for several months until MSP was performed. After a minimum of 1 h of dark adaptation, fish were euthanized with MS-222 and rapidly enucleated under dim red light. All further isolation and preparation was done using a dissecting scope equipped with infrared illuminators and image converters. The eyes were hemisected and the retinas isolated from the posterior segment under phosphate-buffered saline (pH 7.2) made hypertonic with 6% sucrose. Small pieces of isolated retina were transferred in buffer to cover slips, cut and teased with #11 scalpel blades, and sandwiched with another cover slip edged with silicone vacuum grease. The computer-controlled, single-beam MSP is the same one used by Britt et al. (2001). A 100 W tungsten–halogen lamp together with quartz optics allowed for accurate absorbance measurement down to 350 nm with a rectangular measuring aperture as small as 1.5 μm^2. The selection criteria used for data inclusion into the λ_{max} analysis pool were the same as those used by Loew (1994). Each acceptable spectrum was smoothed prior to normalization using the "smooft" digital filter routine of Press et al. (1987). The smoothed spectrum was overlaid on the original unsmoothed one and checked by eye to make sure that overfiltering or spurious data points had not shifted the apparent maximum; specifically, smoothed curves were only considered reliable if they ran through the "middle" of the unsmoothed data points along the right-hand limb of the raw data, and if the curves were approximately Gaussian around the peak. The peak absorbance used for normalization prior to template fitting was the calculated maximum of the best fit Gaussian to the data points 20 nm either side of the estimated-by-eye absorbance maximum of the alpha band and is referred to as X_{max}. For those curves meeting the selection criteria, the λ_{max} (the wavelength at maximum absorbance for a template-derived visual pigment best fitting the experimental data) of the smoothed, normalized (using X_{max}) visual pigment absorbance spectrum was obtained using the method of Mansfield as presented by MacNichol (1986). The templates used were those of Lipetz and Cronin (1988). The wavelength error of the MSP is ±1.0 nm.

Results

Opsin Sequencing

Five opsins were isolated from *C. frenata* eye cDNA, including two known to form short-wavelength–sensitive visual pigments: SWS2a, SWS2b, RH2a, LWS, and RH1. In addition, an RH2b pseudogene was isolated from genomic DNA. For the RH2a, SWS2a, and SWS2b opsins, it was necessary to isolate the 5′ region of the coding sequence from genomic DNA, and the SWS2b opsin was only found in the eye cDNA library after specific primers were designed from genomic DNA sequence data; these observations may indicate lower relative transcript abundance for these opsins compared with the RH1 and LWS opsins. Examining the genomic sequence data in detail revealed the presence of two divergent RH2a fragments; though effectively identical in the coding sequence region (99.3% identical at the nucleotide level; 100.0% identical at the amino acid level), the fragments were quite divergent upstream of the start codon, and we were unable to reliably align the noncoding portion of these fragments. This may indicate allelic divergence or the presence of very young paralogs. However, since these differences do not alter the amino acid sequence, only a single RH2a-based pigment is available for use in the *C. frenata* retina. The RH2b opsin was obtained only from genomic DNA, and no transcripts were detected from cDNA despite the use of specific primers. Examination of the RH2b opsin sequence revealed that it has pseudogenized, possessing a premature stop codon at the end of exon 1 and two frame-shifting indels in exon 3 (an insertion of 1 bp followed closely by a deletion of 16 bp) (supplementary fig. 1, Supplementary Material online). No evidence was found for an SWS1 opsin, despite repeated attempts with different degenerate primer pairs to amplify this gene from eye cDNA and genomic DNA, under conditions that successfully amplified other visual pigment genes in this species. Although all of our results to date indicate that the SWS1 opsin was lost in this Neotropical cichlid, this conclusion rests on negative evidence and is somewhat surprising given the conserved nature of SWS1 in shallow water fishes. We have recently begun to investigate opsins in related Neotropical cichlids, which will hopefully shed further light on this point. All sequence data have been deposited at NCBI Genbank (accession numbers JN990727–JN990736).

Phylogenetic Analyses

Bayesian phylogenetic analyses of the *All Opsins* data set confirmed the identities of the *C. frenata* LWS, RH2a, RH2b, SWS2a, SWS2b, and RH1 opsins (fig. 1). These *C. frenata* opsin sequences were placed sister to single-copy orthologs from Nile tilapia, *O. niloticus*, with two exceptions. First, the RH2a opsin was placed sister to the duplicated RH2aα and RH2aβ opsins of *O. niloticus*, confirming that these paralogs derive from an African cichlid-specific RH2a opsin duplication event (Spady et al. 2006). Second, instead of being placed sister to the *O. niloticus* LWS opsin as expected, the *C. frenata* LWS opsin was placed sister to duplicated zebra fish (*D. rerio*) LWS opsins. However, this LWS opsin placement was the most poorly supported (PP = 0.53) of all topological splits on the generally well-supported Bayesian gene tree; only two other branches had PP < 0.90. Chicken (*G. gallus*) and zebra fish (*D. rerio*) opsins were placed as expected. For each of the opsin family-specific data sets, including the LWS opsin data set, phylogenetic analyses placed the *C. frenata* opsin sequences in their expected locations, sister to the orthologous African cichlid opsins, with strong support (fig. 2). The sister relationships between *O. niloticus* opsins and those of the lacustrine African cichlids, and the monophyly of haplochromine cichlid opsins, were also recovered, as expected (Koblmuller et al. 2008). Reciprocally monophyletic African cichlid RH2aα and RH2aβ clades were observed, to the exclusion of the *C. frenata* RH2a opsin sequence, providing further confirmation that these paralogs are only present in African cichlids. Deeper relationships within the Acanthomorpha varied depending on data set and were not always strongly supported, though some expected arrangements were consistently found (e.g., the monophyly of puffer fishes, of flounders, and of cyprinodonts [Dettai and Lecointre 2005, 2008; Li et al. 2009]). Interestingly, the phylogenetic placement of the SWS2 and RH2 opsins of the cod (*Gadus morhua*) suggested that the duplication event that resulted in SWS2a/b paralogs is younger than the duplication event that produced the RH2a/b paralogs.

Molecular Evolutionary Analyses

The loss/pseudogenization of SWS1 and RH2b opsins from the *C. frenata* opsin repertoire indicates a unique selective history on its visual system. We thus used ML to fit codon substitution models to the LWS, RH2a, RH2b, SWS2a, SWS2b, and RH1 data sets to determine whether d_N/d_S increased along the *C. frenata* opsin lineages, as might be expected if functional constraint was relaxed or if positive selection drove new amino acid substitutions to fixation. Overall estimates of d_N/d_S for each data set under the M0 model, which does not account for among-site or among-lineage variation in d_N/d_S, were low ($\omega < 0.200$; table 1), indicating the predominant role of purifying selection. Additionally, under this simple M0 model, there were no branches in any of the data sets with estimates of $d_S > 1$, indicating that saturation of synonymous substitutions is unlikely to adversely affect our analyses. Branch models (table 1), which estimated d_N/d_S separately for foreground (*C. frenata* terminal branch) and background (the rest) tree partitions, indicated that d_N/d_S increased significantly along the foreground lineage for the RH2b opsin ($P = 0.007$) and perhaps also for the RH1 opsin ($P = 0.053$); d_N/d_S increased by a factor of at least two in these data sets, though in neither case did it exceed one. Increases were also observed for the other data sets, though in these cases, the increases were not significant according to LRTs ($P > 0.100$).

We used additional branch model analyses to obtain an estimate of when constraint was removed on the now-pseudogenized RH2b opsin. By applying a branch model that assumed that the selective regime switched from strong purifying selection ($0 < \omega < 1$) to neutrality ($\omega = 1$) at a variable point along the *C. frenata* RH2b branch and by considering only synonymous changes, which we assume evolve in a roughly clock-like manner, we estimated that 71.1% of the branch evolved under constraint (95% CI = 21.5–92.8) (supplementary fig. 3, Supplementary Material online). Given a divergence point of 85 Ma (Genner et al. 2007), we therefore calculated that constraint was removed 24.5 Ma (95% CI = 66.7 to 6.1 Ma). From this, we infer that constraint on the RH2b gene was most likely removed relatively recently along the lineage connecting *C. frenata* to its common ancestor with African cichlids and thus that members of distantly related Neotropical cichlid clades likely possess an intact RH2b opsin; future work will address this point through surveys of cichlids from different Neotropical tribes.

As branch models do not allow for among-site rate variation in d_N/d_S, we also analyzed the opsin data sets using branch-site models. Branch-site analyses revealed a site-specific increase in d_N/d_S along the *C. frenata* lineage for some, but not all, of the opsin data sets (table 2). Most notably, LRTs and AIC comparisons indicated that positive selection affected a small proportion ($\omega_2 = 32.519$, $p_2 = 0.014$) of sites along the *C. frenata* SWS2b lineage ($P = 0.004$). Branch-site analyses also suggested the action of positive selection along the *C. frenata* RH1 lineage ($\omega_2 = 9.552$, $p_2 = 0.018$), though in this case the LRT *P* value approached, but did not exceed, the 5% significance threshold ($P = 0.053$). However, the BrS-A model did significantly improve on the M1a null model ($P = 0.017$), indicating a significant increase in ω along the *C. frenata* RH1 branch possibly due to either positive selection or relaxed constraint. This mirrors the findings of our branch model analyses, which also suggested a large and nearly statistically significant increase in ω along the *C. frenata* RH1 branch (table 1). Furthermore, the BrS-A model, which allows for positive selection, was found to be the best fitting of the three models by a slight margin according to AIC scores. Given these combined results, we tentatively conclude that positive selection shaped a small proportion of sites along the *C. frenata* RH1 opsin lineage.

When applied to the RH2b data set, the BrS-A model (table 2) estimated that a large proportion of sites ($p_2 = 0.218$) switched from the purifying selection site class along the foreground lineage but that these sites did not

Table 1. Likelihood Scores, Parameter Estimates, and Likelihood Ratio Test *P* Values from Branch Analyses of LWS, RH2a, RH2b, SWS2a, SWS2b, and RH1 Opsin Data Sets with the *Crenicichla frenata* Branch Designated the "Foreground" Lineage.

Data Set	Model	*ln*L	n.p.	κ	ω Background	ω Foreground	LRT *P* Value
LWS	Branch	−2801.2559	22	2.235	0.177	0.238	0.395
	M0	−2801.6181	21	2.236	0.189	—	—
RH2a	Branch	−3833.6032	31	1.756	0.145	0.225	0.204
	M0	−3834.4109	30	1.726	0.151	—	—
RH2b	Branch	−2888.4232	14	1.745	0.122	0.377	0.007
	M0	−2892.0999	13	1.739	0.141	—	—
SWS2a	Branch	−4600.3504	28	2.071	0.178	0.329	0.115
	M0	−4601.5950	27	2.069	0.184	—	—
SWS2b	Branch	−3317.5926	24	1.989	0.154	0.230	0.197
	M0	−3318.4231	23	1.990	0.164	—	—
RH1	Branch	−3819.1026	31	2.373	0.169	0.361	0.053
	M0	−3820.9696	30	2.375	0.175	—	—

NOTE.—*ln*L, natural log likelihood score; n.p., number of parameters; κ, transition-to-transversion rate ratio; ω, nonsynonymous-to-synonymous rate ratio (d_N/d_S).

experience positive selection ($\omega_2 = 1.000$). This model significantly improved on the M1a null model ($P = 0.023$) but not on the BrS-N null model ($P = 0.500$); with $\omega_2 = 1.000$, the BrS-A model effectively collapsed to the simpler BrS-N model. The BrS-N model was the best fitting RH2b model according to AIC comparisons. A large proportion of sites thus appear to have switched from purifying to neutral site classes along the pseudogenized *C. frenata* RH2b opsin lineage. Accordingly, BEB analysis suggested that 12 sites (listed in table 3) switched from the purifying to neutral site classes along this lineage (PP > 0.60); several of these sites are highly conserved among RH2 opsins, or even broadly among vertebrate visual opsins, suggesting that these substitutions would not be tolerated in a functional opsin. For the LWS opsin data set, the BrS-A model estimates (table 2) suggest that a moderate proportion of sites ($p_2 = 0.077$) experienced weak positive selection ($\omega_2 = 1.442$) along the foreground lineage but, whereas this model was a significantly better fit than the M1a null model ($P = 0.033$), it did not improve on the BrS-N model ($P = 0.889$). Of the three models, AIC comparisons favored the BrS-N model. These results are consistent with either the relaxation of purifying selection along the *C. frenata* LWS opsin lineage, as occurred for the RH2b opsin lineage, or perhaps with the action of very weak positive selection. There was no evidence for a change in d_N/d_S along either the *C. frenata* RH2a or SWS2a opsin lineages; for both data sets, the M1a model was favored by both LRTs and AIC comparisons (table 2).

Positively Selected Sites

BEB analysis identified several sites (at least one from each data set) as probable (PP > 0.50) members of the positively selected site class along the Neotropical lineage (table 3 and fig. 3). Comparing these sites to the results of past functional studies (primarily carried out on bovine rhodopsin) shows that many are likely to affect opsin biochemistry, influencing both spectral (i.e., λ_{max}) and nonspectral

Table 2. Likelihood Scores, Parameter Estimates, Likelihood Ratio Test *P* Values, and AIC ΔValues from Branch-Site Analyses of LWS, RH2a, RH2b, SWS2a, SWS2b, and RH1 Opsin Data Sets with the *Crenicichla frenata* Branch Designated the "Foreground" Lineage.

					Site Class 0		Site Class 1		Site Class 2			
Data Set	Model	*ln*L	n.p.	κ	ω_0	p_0 (%)	ω_1	p_1 (%)	ω_2	p_2 (%)	LRT *P* Value	AIC Δ
LWS	BrS-A	−2744.3097	24	2.164	0.000	76.3	1	15.9	1.442	7.7	—	1.98
	BrS-N	−2744.3194	23	2.160	0.000	74.3	1	15.5	1	10.2	0.445	—
	M1a	−2747.7172	22	2.168	0.005	80.7	1	19.3	—	—	0.033	4.80
RH2a	BrS-A	−3750.7241	33	1.767	0.023	82.0	1	17.6	2.314	0.4	—	3.95
	BrS-N	−3750.7325	32	1.768	0.023	81.8	1	17.6	1	0.6	0.448	1.97
	M1a	−3750.7471	31	1.768	0.023	82.3	1	17.7	—	—	0.977	—
RH2b Ψ	BrS-A	−2864.0333	16	1.774	0.050	69.1	1	9.1	1.000	21.8	—	2.00
	BrS-N	−2864.0333	15	1.774	0.050	69.1	1	9.1	1	21.8	0.500	—
	M1a	−2867.8205	14	1.764	0.065	88.5	1	11.5	—	—	0.023	5.57
SWS2a	BrS-A	−4498.8988	30	2.146	0.049	75.5	1	24.5	1.000	0.0	—	4.00
	BrS-N	−4498.8988	29	2.146	0.049	75.5	1	24.5	1	0.0	0.500	2.00
	M1a	-4498.8988	28	2.146	0.049	75.5	1	24.5	—	—	1.000	—
SWS2b	BrS-A	−3276.5762	26	2.077	0.068	84.4	1	14.3	32.519	1.4	—	—
	BrS-N	−3280.7854	25	2.048	0.064	81.5	1	14.7	1	3.8	0.002	6.42
	M1a	−3281.1288	24	2.056	0.065	84.0	1	16.0	—	—	0.011	5.11
RH1	BrS-A	−3674.0617	33	2.339	0.019	82.7	1	15.5	9.552	1.8	—	—
	BrS-N	−3675.3684	32	2.313	0.019	78.0	1	14.5	1	7.5	0.053	0.61
	M1a	−3678.1467	31	2.330	0.021	83.7	1	16.3	—	—	0.017	4.17

NOTE.—*p*, proportion; other abbreviations are as in table 1. "—" Indicates the best-fitting models for the AIC △ column.

Table 3. Sites Identified by BEB Analysis as Members of the Positively Selected Site Class (site class 2 of the BrS-A model; table 2) along *Crenicichla frenata* Opsin Lineages.

Data Set	BEB Sites and Observed Amino Acid Substitutions (posterior probability)
LWS	A232S (0.847), T315S (0.619)
RH2a	F109A (0.625)
RH2b Ψ	N8D (0.638), P12L (0.642), L19I (0.672), A80T (0.644), C167S (0.681), R252C (0.623), A285P (0.697), S297T (0.679), Q312E (0.638), N315S (0.650), *G322R* (0.633), *G325S* (0.659)
SWS2a	L85I (0.529)
SWS2b	Q64T (0.922), G188S (0.514), A238S (0.955), V276T (0.533)
RH1	I123M (0.524), V130A (0.578), L172V (0.632), C210V (0.977)

NOTE.—Site numbering follows Bovine RH1 opsin. Italics indicate approximate site numbering due to ambiguous alignment.

(e.g., activation or deactivation kinetics) properties. Interestingly, recent work has shown that opsins form dimers and higher level oligomers, and some of the BEB-identified sites are located in regions of the protein known to contribute to oligomerization interfaces (Fotiadis et al. 2006). Extended details on these sites are provided in the supplementary materials online, but here only general patterns from the SWS2b and RH1 analyses are mentioned, as it is for these genes that we have the strongest evidence for positive selection. Homology models were constructed for these two opsins to help visualize and interpret the BEB-identified sites. In the case of the SWS2b opsin, it appears likely that substitutions at BEB identified sites affect non-spectral aspects of opsin biochemistry. Two of the four sites (sites 64 and 238) are located in the cytoplasmic loops, which are known to undergo structural rearrangement following light-activation (supplementary fig. 4, Supplementary Material online), allowing for the binding and activating of the downstream G protein, transducin (Franke et al. 1992; Scheerer et al. 2008). Furthermore, these two sites both project outwards into a proposed between-dimer interface (fig. 3), and the stability of such opsin–opsin interactions has been suggested to influence transducin activation capacity (Fotiadis et al. 2006). Finally, past biochemical analyses suggest that substitutions at these sites could possibly also affect G protein-coupled receptor kinase docking, which contributes to opsin deactivation following light absorption (Shi et al. 1995). These substitutions could thus influence sensitivity to light under dim-light conditions by altering the stability and activity of the activated visual pigment. For the RH1 opsin, conversely, it seems likely that spectral aspects (i.e., λ_{max}) were also affected. First, mutating site 123 has been shown to affect λ_{max} (as well as other nonspectral properties) in bovine rhodopsin (Garriga et al. 1996). This site does not directly contribute to the opsin's retinal binding pocket, but the adjacent residue at site 122 does and, as such, it presumably affects the chromophore indirectly; site 122 is known to have major effects on both spectral and non-spectral properties of visual pigments (Imai et al. 1997). Similarly, BEB site 210 does not directly impinge on the chromophore, but adjacent site 211 does and it is known to influence λ_{max} in SWS2 opsins (Takahashi and Ebrey 2003), as does site 209 in RH2 opsins (Chinen et al. 2005). It is interesting that, despite these substitutions, the pike cichlid RH1 opsin likely produces an A_1-type pigment with $\lambda_{max} \approx 502$ nm (see below), which is typical of many vertebrate RH1 pigments; whether this opsin converged on the "typical" λ_{max} value following earlier divergence to a higher or lower value or whether it retained this λ_{max} value since diverging from the RH1 opsins of African cichlids and other fishes remains to be seen, but it is a point worthy of further study. Finally, as found for the SWS2b sites, some of the RH1 BEB sites (172 and 210) may contribute to opsin–opsin interfaces (Fotiadis et al. 2006), though here the sites project into the within-dimer rather than between-dimer interface (fig. 3).

Microspectrophotometry

MSP of 67 photoreceptor cells from the retinas of two *C. frenata* individuals revealed the presence of three spectrally distinct types of cone photoreceptor arranged either as single or double cones. Most notably, short-wavelength–sensitive single cones were observed in the pike cichlid retina ($\lambda_{max} = 480 \pm 7$ nm, $n = 9$; mean ± SD), contrary to previous findings (see Endler 1991). Double cones either possessed long-wavelength–sensitive pigments in both cells, or they possessed a long-wavelength–sensitive pigment in one cell and a medium-wavelength–sensitive pigment in the other (long: $\lambda_{max} = 614 \pm 5$ nm, $n = 33$; medium: $\lambda_{max} = 547 \pm 4$ nm, $n = 11$); these findings are largely consistent with the previous study of the pike cichlid's retina (Endler 1991). Rod cells were also observed and assayed ($\lambda_{max} = 520 \pm 3$ nm; $n = 14$). Example MSP absorbance curves for each of these photoreceptor types are provided in figure 4.

The λ_{max} values of the rod and long-wavelength–sensitive cones were extremely red shifted, well beyond the norm for A_1-type RH1 and LWS visual pigments; this indicates the predominant use of the A_2-type chromophore, 3′4′-didehydroretinal (Bowmaker 2008). The magnitude of the red shift caused by switching from the typical A_1-type chromophore to the A_2-type chromophore has been empirically determined to be a simple function of the initial A_1-type pigment's λ_{max} (Parry and Bowmaker 2000). Assuming that our MSP-derived λ_{max} values reflect pure A_2-type visual pigments, these MSP results indicate the presence of an A_1-type rod pigment with $\lambda_{max} \approx 502$ nm, and A_1-type cone pigments with $\lambda_{max} \approx 568$ nm, 522 nm, and 470 nm. Given known λ_{max} values for A_1-type pigments from African cichlids and other closely related fishes (Yokoyama 2008; Carleton 2009), these estimates imply the usage of RH1, LWS, and RH2a pigments in the rod, long-wavelength–sensitive, and medium-wavelength–sensitive photoreceptors, respectively, and either a blue-shifted RH2b pigment or a red-shifted SWS2a pigment in the short-wavelength–sensitive cone. However, as the RH2b opsin is pseudogenized in this fish, the short-wavelength–sensitive cone photoreceptors

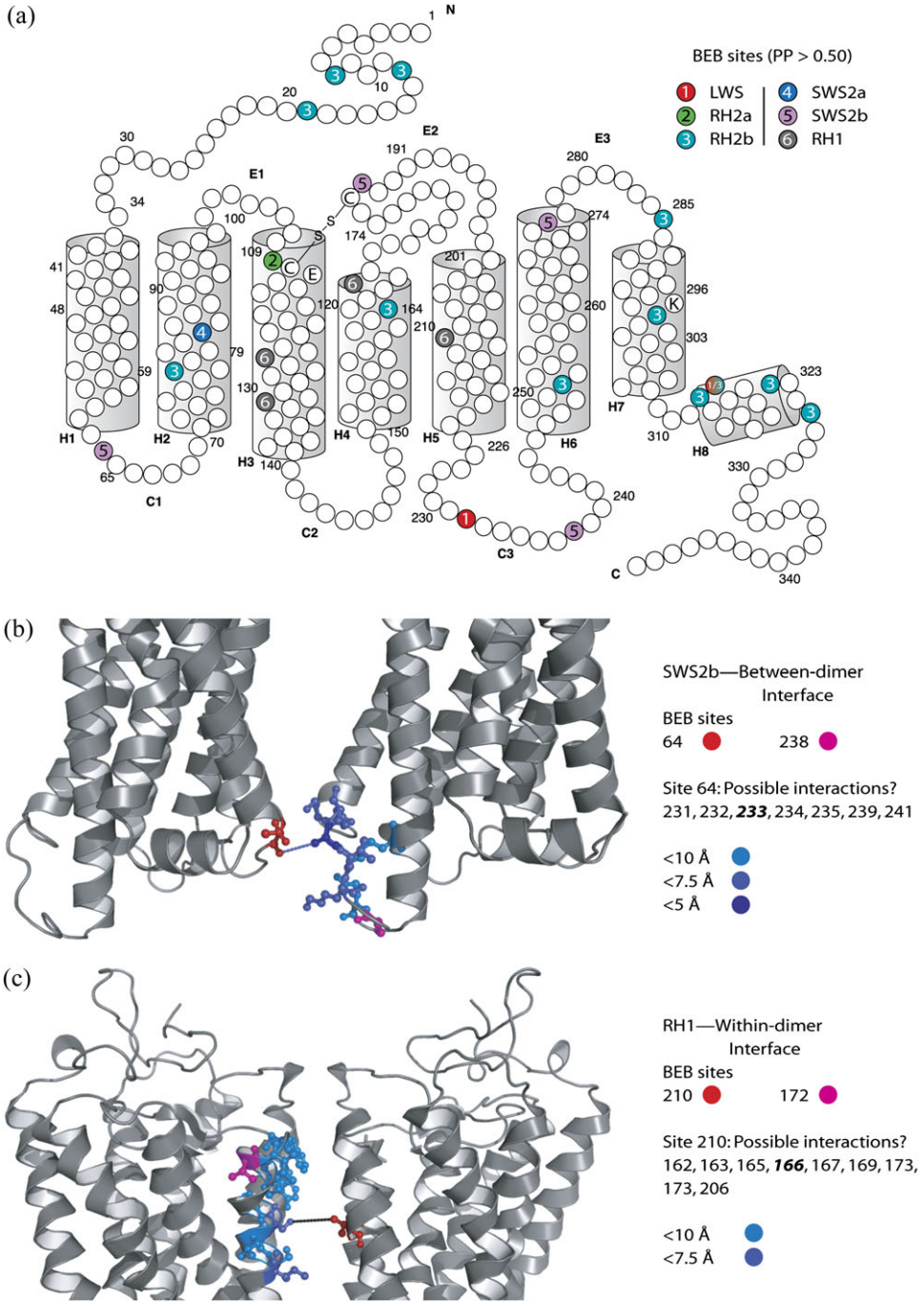

FIG. 3. Structural location of BEB-identified sites. (*a*) Snake-plot diagram of bovine rhodopsin (RH1 opsin) indicating the location of BEB-identified sites for each analyzed data set. The eight helixes (H), three cytoplasmic loops (C), and three extracellular loops (E) are labeled and numbered. BEB-identified sites stem from analyses of six different opsin data sets (LWS, RH2a, RH2b, SWS2a, SWS2b, and RH1), as indicated by color and number (see inset). Critical opsin residues are indicated by one-letter amino acid code: K296 (chromophore Schiff base link); E113 (Schiff base counter ion); C110 and C187 (disulfide bond sites). (*b*) SWS2b homology models (gray) aligned to show the putative between-dimer interface. BEB sites 64 (red) and 238 (purple) are indicated, as are sites within approximately 10 Å across the interface from site 64 (blue). The closest site to 64 across the interface is indicated via a dashed line and provided in bold italics in the list. (*c*) RH1 homology models (gray) aligned to show the putative within-dimer interface. BEB sites 210 (red) and 172 (purple) are indicated, as are sites within approximately 10 Å across the interface from site 210 (blue). The closest site to 210 across the interface is indicated via a dashed line, and provided in bold italics in the list.

must therefore employ the SWS2a opsin. Some cichlids are known to use mixtures of both A_1- and A_2-type pigments within single photoreceptor cells (Carleton 2009); if that is the case here, the pure A_1-type and A_2-type visual pigment λ_{max} values will be somewhat underestimated.

Interestingly, the individual sampled from the Aripo River had slightly red-shifted λ_{max} values compared with the individual sampled from the Guanapo River for all four photoreceptor classes (supplementary table 2, Supplementary Material online), suggesting intraspecific variation in λ_{max}

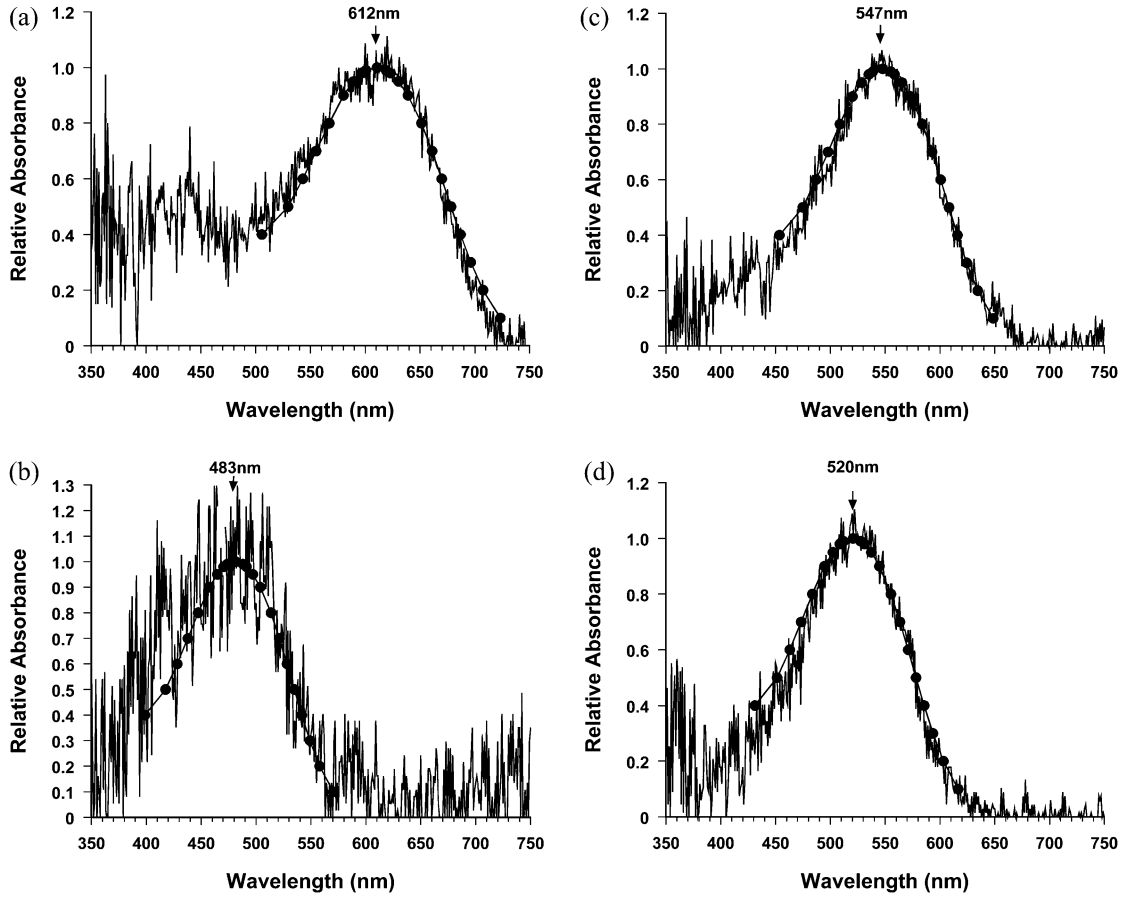

Fig. 4. Example absorbance spectra for visual pigments of the rod and cone photoreceptor cells of *Crenicichla frenata*. The raw absorbance spectra, derived by MSP, are overlain with smooth curves calculated from the best-fitting A_2-type chromophore visual pigment curve. (*a*) Double cone member, long-wavelength sensitive: λ_{max} = 614 ± 5 nm (n = 33). (*b*) Double cone member, medium-wavelength sensitive: λ_{max} = 547 ± 4 nm (n = 11). (*c*) Single cone: short-wavelength sensitive: λ_{max} = 480 ± 7 nm (n = 9). (*d*) Rod: λ_{max} = 520 ± 3 nm (n = 14).

for *C. frenata* due to variation in opsin sequence, expression, and/or chromophore usage. Variation in chromophore usage may be the simplest explanation for intraspecific variation in λ_{max} that affects all photoreceptor types simultaneously, but this hypothesis cannot fully explain the observed patterns. The effect of shifting between A_1 and A_2 chromophores on λ_{max} is known to be much greater for long-wavelength–sensitive pigments than for short-wavelength–sensitive pigments (Parry and Bowmaker 2000); however, we observed nearly equal differences in λ_{max} for the pike cichlid's long- and short-wavelength–sensitive cones (supplementary table 2, Supplementary Material online). Both A_1 and A_2 chromophore template curves were fit to the data (as described in Lipetz and Cronin 1988), but the data were too noisy to allow for conclusions to be made about chromophore mixtures. Spectrophotometric study of visual pigments from more individuals, sampled from a wider geographic range, will be necessary to fully address these possibilities, which could have implications for future research on color pattern variation in the pike cichlid's guppy prey.

Discussion

Through targeted opsin sequencing, molecular evolutionary analysis, and MSP, we have shown that the Trinidadian pike cichlid (*Crenicichla frenata*) possesses a larger set of visual pigments than previously believed based on prior MSP analysis (Endler 1991). However, compared with the closely related African cichlids, which are quite opsin rich (Carleton 2009), the pike cichlid appears to have a dramatically reduced opsin complement, lacking an SWS1 opsin entirely and possessing a pseudogenized RH2b opsin. Furthermore, positive selection was detected along the *C. frenata* opsin lineages for the SWS2b and RH1 opsins. Here, we discuss the implications of our findings for evolutionary ecological research on the pike cichlid and its prey fish, the Trinidadian guppy (*Poecilia reticulata*), and for comparative study of cichlid opsins.

According to MSP data cited in Endler (1991), the Trinidadian pike cichlid possesses a retina bearing only two cone photoreceptors, both maximally sensitive to long-wavelength light. A retina of this design would be very insensitive to shorter wavelengths of light (less than approximately 500 nm). At the molecular level, those MSP data imply the presence of LWS and RH2 opsin-based visual pigments in the pike cichlid retina but not SWS1 or SWS2 opsin-based pigments (Bowmaker 2008; Yokoyama 2008). Our results, however, revealed the expression of SWS2a and SWS2b opsins in a pike cichlid eye cDNA library

and established the presence of a cone photoreceptor maximally sensitive to short-wavelength light in the pike cichlid retina. These findings indicate that the pike cichlid has the capacity for trichromatic color vision including the blue portion of the spectrum. Recognizing these expanded sensory capabilities has important consequences for visual ecology research on this fish as pike cichlid predation is known to select against guppies with large/numerous blue and iridescent spots (Endler 1980; Kemp et al. 2009); by establishing that the pike cichlid possesses a visual system capable of detecting short-wavelength light during prey search, our results provide a mechanistic explanation for this pattern.

Interestingly, our results also suggest that the pike cichlid may lack an SWS1 opsin and, therefore, that this fish may be relatively insensitive to UV light and unable to discriminate hues in the lower part of the visual spectrum. If this is the case, guppies could potentially use UV light as a private communication channel, as they do possess an UV-sensitive cone (Archer and Lythgoe 1990). Consistent with this idea, recent work found that male guppies from populations that coexist with the pike cichlid reflect more UV light, on average, than those that do not (Millar and Hendry 2011). Of course, predation could also influence prey color pattern evolution indirectly by directly affecting aspects of female mate choice (e.g., Gong and Gibson 1996; Bierbach et al. 2011), but to our knowledge, the relative importance of direct predation-mediated selection on color patterns and indirect selection via predation on mate choice has not been explored in this system. The nature of selection on guppy color patterns depends on a variety of ecological parameters, both biotic and abiotic (Endler 1995); establishing that one of the guppy's most dangerous predators can detect a wide swath of the spectrum represents a critical step toward fully understanding the nature of selection on color patterns in this system.

There are several possible explanations why the previous MSP analysis (see Endler 1980) of the pike cichlid's retina did not report a cone maximally sensitive to short-wavelength light. First, since the single cones that bestow short-wavelength sensitivity are the rarest type of photoreceptor within cichlid retinas (Fernald 1981; Braekevelt 1992; Braekevelt et al. 1998; Wagner and Kroger 2005; Lisney and Hawryshyn 2010), it may simply be that short-wavelength–sensitive cones were missed; indeed, it is possible that other cone classes, for instance a violet (SWS2b) cone class, exist in the pike cichlid retina, present only at very low levels or in restricted regions of the retina, though we note that most cichlid retinas appear to be trichromatic, not tetrachromatic (Carleton 2009). Ontogenetic variation among individuals could also have played a role; studies on African cichlids have reported large changes in the set of expressed opsins through development (Spady et al. 2006). Finally, geographic variation may be important; gene flow between populations in different rivers and drainages in the Northern Range of Trinidad is likely quite low, and we cannot exclude the possibility that cichlids from some populations are in fact blind to short-wavelength light. This kind of variation has been observed elsewhere; for example, SWS2b opsins comprise 13.65 $\pm$ 7.68% (mean $\pm$ SD) of total cone opsin expression in wild-caught *Metriaclima zebra* cichlids from the "Zimbawe Rock" population in Lake Malawi, but only 0.80 $\pm$ 0.88 % of total opsin expression in wild-caught cichlids from the nearby "Thumbi West" population (Smith et al. 2011). Future work on *C. frenata* is needed to explore these possibilities, but regardless of the reason, our results serve as a caution against the use of potentially incomplete or imprecise data on photoreceptor spectral sensitivities in visual perception models. Visual perception models (Endler and Mielke 2005) are increasingly being used in studies of animal and plant color pattern evolution (e.g., Darst et al. 2006; Forrest and Thomson 2009), and obtaining reliable predictions from visual perception models requires accurate estimates of visual system parameters (e.g., cone λ_{max} or relative cone abundance) (Lind and Kelber 2009; Renoult et al. 2010). The accidental absence of entire cone classes will likely have dramatic effects on the accuracy and precision of these predictions. Our decision to study the pike cichlid's retina via MSP was motivated by our preliminary findings of SWS2 opsins expressed in eye cDNA, demonstrating the benefit of combining direct analysis of visual pigments by MSP with sequence-based analysis of opsin sequences. Of course, with regard to the pike cichlid–guppy system, it remains to be seen whether all *C. frenata* retinas are spectrally equivalent, or if perceptual variation exists due to, say, phenotypic plasticity or opsin coding and regulatory sequence polymorphisms.

Although our results revealed that the Trinidadian pike cichlid possesses more cones and more cone opsins than early MSP results suggested, this fish still possesses substantially fewer cone opsins than expected based on knowledge of the visual systems of closely related fishes. African cichlids, most importantly, possess seven cone opsins (Carleton 2009), yet we only confirmed the presence of four intact cone opsins in this Neotropical cichlid. Specifically, our results suggest that two instances of gene loss occurred along the Neotropical lineage since diverging from the African clade approximately 85 Ma (Genner et al. 2007), with the pike cichlid possessing a pseudogenized RH2b opsin and, it appears, lacking an intact SWS1 opsin. The reasons why these two opsins might have been lost along the Neotropical lineage are not obvious, though past comparative studies of vertebrate opsins and photoreceptors suggest a few possibilities. First, the loss of cone photoreceptors and opsins appear to be common consequences of the invasion of light-limited niches (Davies 2011). Early studies of this phenomenon focused on species living at great depths, for example, the cottoid fishes of Lake Baikal, where both the intensity and spectral breadth of the downwelling light are reduced (Bowmaker et al. 1994). Interestingly, the Trinidadian pike cichlid's RH1 opsin possesses an asparagine (N) residue at site 83 instead of the more typical aspartate (D); Sugawara et al. (2010) recently demonstrated that the D83N substitution increases the formation of the visual pigment's active state following photon absorption and

that this substitution has occurred multiple times in vertebrate lineages subject to dim-light environments, indicating recurrent adaptation to light-limited habitats. Although there are a few lakes of sufficient depth in the Neotropics to create such an environment, many Neotropical rivers are rich in tannins and suspended particulate and are quite light limited as a result (Furch and Junk 1997). Furthermore, turbid freshwater habitats typically transmit long wavelengths of light much more effectively than shorter wavelengths, and this could have favored increased reliance on longer wavelength opsins, which would provide a better sensitivity match to the environment than their shorter wavelength paralogs (Lythgoe 1979). Our results are consistent with this scenario. First, of the three SWS opsins, the one with the lowest λ_{max} (SWS1) appears to have been lost and the one with the highest λ_{max} (SWS2a) appears to be expressed more than the other remaining SWS opsin (SWS2b). Second, of the two RH2 opsins found in the Trinidadian pike cichlid, the one with the lower λ_{max} (RH2b) is pseudogenized. Finally, unlike the sympatric guppy and many African cichlids, the Trinidadian pike cichlid appears to primarily employ the red-shifting A_2-type chromophore.

A reduced light environment could also explain why we found evidence for positive selection along both the Neotropical lineage for the RH1 (rod) and SWS2b (cone) opsins and why some of the inferred targets of selection are more easily related to nonspectral aspects of opsin biology than to λ_{max}. Looking at African cichlid opsins, Spady et al. (2005) found that d_N/d_S varied among lineages in a manner consistent with adaptation to turbid environments, and our results may likewise indicate that the Neotropical lineage spent a critical period in such an environment. Intriguingly, some of the amino acid sites identified in this study as positively selected have been shown to affect nonspectral opsin properties such as transducin binding and activation or opsin phosphorylation (discussed above). Changes at these sites could thus alter visual pigment activation and deactivation kinetics in ways that affect perceptual sensitivity in a light-limited environment. Indeed, Sugawara et al. (2010) recently demonstrated that African cichlid opsins may adaptively vary in ways beyond λ_{max}, showing that the rod pigments of deep water species form the active meta II state—the state that binds the downstream G protein, transducin—more quickly than those of shallow water species. The SWS2b and RH1 sites identified by BEB analysis as targets of positive selection along the Neotropical lineage are prime candidates for biochemical studies of mutagenized pigments, especially if the focus is on properties beyond λ_{max}, the most commonly studied attribute. It is interesting that selection was detected for the SWS2b opsin given that we did not observe any cone cells likely to contain an SWS2b-based visual pigment (though we did detect low-level expression in the eye cDNA library). It is possible that SWS2b cones exist at low numbers or at different ontogenetic stages. Alternatively, selection on the SWS2b opsin may reflect past environmental pressures that no longer apply to the pike cichlid species we examined.

Other factors could have selected for or otherwise precipitated a reduced number of cone opsins in the Neotropical lineage as well. One possibility is that the evolution of intraocular filters, such as lenses that cut off short wavelength light, may have rendered the SWS1 cone unnecessary. The lenses of Lake Malawi cichlids, for example, can either transmit or block UV light, and species with UV-blocking lenses tend to be less reliant on UV-sensitive pigments (Hofmann et al. 2010). Finally, adaptive evolution related to foraging could play a role. Foraging mode varies greatly among Neotropical cichlids, but *Crenicichla* spp. generally prey on other fish and/or on benthic invertebrates (Montana and Winemiller 2009). Recent studies of Lake Malawi and Lake Tanganyika cichlids found that opsin expression covaries with diet; species with diets similar to *Crenicichla* spp. are less likely to express SWS1 opsins in their single cones (Hofmann et al. 2009; O'Quin et al. 2010). Of course, establishing the visual environment experienced when the SWS2b and RH1 opsins were targeted by positive selection and when the SWS1 and RH2b opsins were lost will require further study of the visual biology and phylogenetic relationships of Neotropical cichlids. Our analyses suggest that the RH2b pseudogenization event may have occurred fairly recently. Assuming that the African and Neotropical lineages diverged approximately 85 Ma (Genner et al. 2007), our ML estimate of the date of loss of function is approximately 25 Ma, which indicates that RH2b pseudogenization may be specific to cichlids of the Geophagini tribe or perhaps even just the large *Crenicichla* genus. The putative loss of the SWS1 opsin gene may represent a more ancient event; although only a few Neotropical cichlids have been examined to date, none have been found to possess retinas with UV cones or lenses transparent in the UV, suggesting that a lack of UV vision may be an ancestral state within the Neotropical cichlid clade (Levine and MacNichol 1979; Thorpe et al. 1993; Wagner and Kroger 2005). It should be noted, however, that the survey by Levine and MacNichol (1979) of Neotropical cichlid cone λ_{max} values occurred prior to the discovery of UV cone photoreceptors in vertebrates (Harosi and Hashimoto 1983). More data are needed to estimate ancestral visual phenotypes and visual niches in order to test these predictions.

The difference in the number of functional cone opsins between African cichlids (seven) and the Trinidadian pike cichlid (four) is not entirely due to gene loss along the Neotropical cichlid lineage; gene gain within the African cichlids played a role as well. Phylogenetic analyses revealed that the RH2aα and RH2aβ opsins, first described in the Lake Malawi cichlid *M. zebra* (Parry et al. 2005), are derived from an African cichlid–specific gene duplication event as the pike cichlid RH2a sequence fell sister to separate RH2aα and RH2aβ clades. Although divergence date estimates vary, this duplication event likely occurred between 46 and 85 Ma (Genner et al. 2007); examination of more African cichlids, especially those beyond the East African Rift valley, will be needed to narrow this range further. Since duplication, spectral sensitivity has diverged between the

two paralogs, with RH2aα and RH2aβ visual pigments differing in λ_{max} by ~10 nm (Carleton 2009). Whether or not these duplicate genes are continuing to diverge functionally and whether they differ in nonspectral ways as well remains to be seen. It is also not clear if this divergence in λ_{max} reflects functional evolution in both lineages or only in one lineage (i.e., with one retaining the ancestral spectral phenotype). We are currently carrying out molecular evolutionary analyses to explore postduplication evolutionary dynamics among African cichlid RH2a opsins.

African cichlids are celebrated for their exceptional and recent adaptive radiations; in contrast, Neotropical cichlids have not speciated as dramatically or as quickly, and much of their diversity stems from older radiations (Smith et al. 2008; Hulsey, Hollingsworth, and Fordyce 2010; Hulsey, Mims, et al. 2010; Lopez-Fernandez et al. 2010). If the reduced number of cone opsins found in the Trinidadian pike cichlid is typical of Neotropical cichlids, this may help explain the relative differences in diversity of the Neotropical and African clades. Studies of African lacustrine cichlids indicate that speciation in this system is often associated with divergence in habitat, trophic mode, and/or male color patterning, and visual system divergence has been linked to each of these axes of diversification (Streelman and Danley 2003; Kocher 2004). Possessing three fewer cone opsins might mean that Neotropical cichlids like *C. frenata* are less capable of invading new visual niches. Although speculative, a similar hypothesis was recently proposed to link the duplication of an opsin gene to the diversification of *Heliconius* butterflies (Briscoe et al. 2010). Obviously, many biological and geological factors contribute to differences in diversification rates among clades (Coyne and Orr 2004), and further study of Neotropical cichlid opsins and visual niches are needed before this hypothesis can be reliably evaluated. However, the multifaceted effects of the visual system on habitat use, feeding, and mating suggests that it could have played a critical role in the reduced diversification of Neotropical cichlids. Our results provide a useful foundation for future research on this system.

Supplementary Material

Supplementary tables 1 and 2, figures 1–4, and text are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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