Molecular Biology and Evolution

Parallel Evolution of Opsin Gene Expression in African Cichlid Fishes

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Associate editor: Adriana Briscoe

The DNA sequences generated here have been deposited in GenBank under the accession nos. HM135105–HM135138, HM135141–135146, HM135148–HM135152, and HM135155.

Abstract

Phenotypic evolution may occur either through alterations to the structure of protein-coding genes or their expression. Evidence for which of these two mechanisms more commonly contribute to the evolution of a phenotype can be garnered from examples of parallel and convergent evolution. The visual system of East African cichlid fishes is an excellent system with which to address this question. Cichlid fishes from Lakes Malawi (LM) and Victoria together exhibit three diverse palettes of coexpressed opsins and several important protein-coding mutations that both shift spectral sensitivity. Here we assess both opsin expression and protein-coding diversity among cichlids from a third rift lake, Lake Tanganyika (LT). We found that Tanganyikan cichlids exhibit three palettes of coexpressed opsins that largely overlap the short-, middle-, and long-wavelength–sensitive palettes of LM cichlids. Bayesian phenotypic clustering and ancestral state reconstructions both support the parallel evolution of the short- and middle-wavelength palettes among cichlids from LT and LM. In each case, these transitions occurred from different ancestors that expressed the same long-wavelength palette. We also identified similar but distinct patterns of correlated evolution between opsin expression, diet, and lens transmittance among cichlids from LT and LM as well. In contrast to regulatory changes, we identified few functional or potentially functional mutations in the protein-coding sequences of three variable opsins, with the possible exception of the *SWS1* (ultraviolet) opsin. These results underscore the important contribution that gene regulation can make to rapid phenotypic evolution and adaptation.

Key words: opsin, cichlids, parallel evolution.

Research article

Introduction

Phenotypic evolution may occur either through alterations to the structure of protein-coding genes or their expression. Mutations that alter the structure of protein-coding genes have long been known to underlie adaptive phenotypic differences between populations and species (e.g., Jessen et al. 1991; Hoekstra et al. 2006; Protas et al. 2006). However, recent work has provided abundant new evidence that mutations that alter the regulation or expression of genes also contribute to adaptive phenotypic evolution (e.g., Wittkopp et al. 2003; Shapiro et al. 2004). Evidence for which of these two mechanisms more commonly contribute to the evolution of a phenotype can be garnered from examples of repeated evolution either through parallelism or convergence (Gompel and Prud'homme 2009). For example, the parallel loss of pelvic spines among adaptively radiating sticklebacks has been achieved through recurrent mutations in the *cis* regulatory region of *Pitx1* (Chan et al. 2010). This observation suggests that the evolution of pelvic spine loss in sticklebacks is biased toward regulatory mutations. Similar examples for protein-coding mutations also exist. For example, reduced pigmentation phenotypes have evolved repeatedly among vertebrates. In many cases, these convergent phenotypes arose through independent mutations within the protein-coding region of *Mc1r* (reviewed in Mundy 2005; Gompel and Prud'homme 2009).

The visual system of African cichlids is an excellent model with which to study the roles of protein-coding and regulatory mutations during phenotypic evolution. Both protein-coding mutations and regulatory changes contribute to spectral sensitivity in these fishes (Hofmann and Carleton 2009; Hofmann et al. 2009). Spectral sensitivity—or sensitivity to different wavelengths of light—is determined by the coding sequence and expression of several duplicated opsin genes. These opsins are expressed within distinct photoreceptor cells in the retina and, when combined with a light-sensitive chromophore, confer sensitivity to light (Wald 1935). Cichlids have 8 opsin genes, 7 used for bright light, or photopic, vision, and one used for dim-light, or scotopic, vision (Carleton 2009). These opsins are *SWS1* (ultraviolet [UV]), *SWS2b* (violet), *SWS2a* (blue), *RH2b* (blue–green), *RH2aα* and *RH2aβ* (green), *LWS* (red), and *RH1* (dim light) (Spady et al. 2006). Among cichlid fishes from Lake Malawi (LM), closely related species can differ in their maximal short- and long-wavelength spectral sensitivity by as much as 100 nm (Jordan et al. 2006; Carleton 2009; Hofmann et al. 2009). These differences are highly

correlated with discrete changes in opsin gene expression (Carleton and Kocher 2001; Hofmann et al. 2009). LM cichlids collectively coexpress three distinct opsin gene palettes, which generate visual pigment sets broadly sensitive to short- (*SWS1-RH2b-RH2a*), middle- (*SWS2b-RH2b-RH2a*), and long (*SWS2a-RH2a-LWS*)-wavelength spectra. The differential expression of these palettes is in part an adaptive response to divergent foraging preferences or diet (Hofmann et al. 2009). In contrast, cichlids from Lake Victoria (LV) collectively express only a single-opsin palette, the long-wavelength set (*SWS2a-RH2a-LWS*). However, these species do vary slightly in the expression of the *SWS2b* and *LWS* opsins. These smaller, continuous changes are an adaptive response to local differences in the light environment (Carleton et al. 2005; Hofmann et al. 2009).

Opsin protein–coding mutations are also associated with the adaptive evolution of spectral sensitivity in cichlids and other vertebrates (Yokoyama S and Yokoyama R 1996; Spady et al. 2005). In some cases, these protein-coding mutations are even associated with population divergence and speciation. For example, among cichlids from LV, polymorphisms in the protein-coding sequence of the *LWS* opsin are adaptively associated with local variation in the light environment, male color, and speciation (Terai et al. 2006; Seehausen et al. 2008). Among cichlids from both LM and LV, the majority of functional or potentially functional opsin sequence polymorphisms are found in the two opsins sensitive to the ends of the visible light spectrum (*SWS1* and *LWS*). This pattern is due to the inability of changes in gene expression to further tune spectral sensitivity outside of the spectral range of these two opsins as defined by their coding sequences. In contrast, shifts in gene expression predominately tune sensitivity across the middle portion of the visible light spectrum, where opsins of longer or shorter spectral sensitivity can be replaced with one another. Therefore, coding mutations are the only way to further shift spectral sensitivity at the ends of the visible light spectrum (Hofmann et al. 2009). Despite this observation, virtually all the cichlid opsins exhibit molecular signatures of natural selection (Sugawara et al. 2002; Spady et al. 2005), including those sensitive to the middle portion of the visible light spectrum; however, it is possible that in some cases, these estimates are too liberal (Yokoyama et al. 2008). Additionally, these polymorphisms are correlated with much smaller differences in spectral sensitivity, typically on the order of 5–15 nm (Carleton 2009; Hofmann et al. 2009).

Thus, African cichlids provide a unique system with which to investigate the relative contribution that opsin regulatory and protein-coding mutations make to phenotypic evolution. However, cichlids from LM and LV form reciprocally monophyletic groups that are composed entirely of species from a single lineage, the Haplochromini (Salzburger et al. 2002; Koblmuller et al. 2008; see fig. 1). Cichlids from these lakes share very few opsin protein–coding polymorphisms in common, but at least some species share similar opsin expression profiles (*SWS2a-RH2a-LWS*) (Hofmann et al. 2009). But, due to the sister

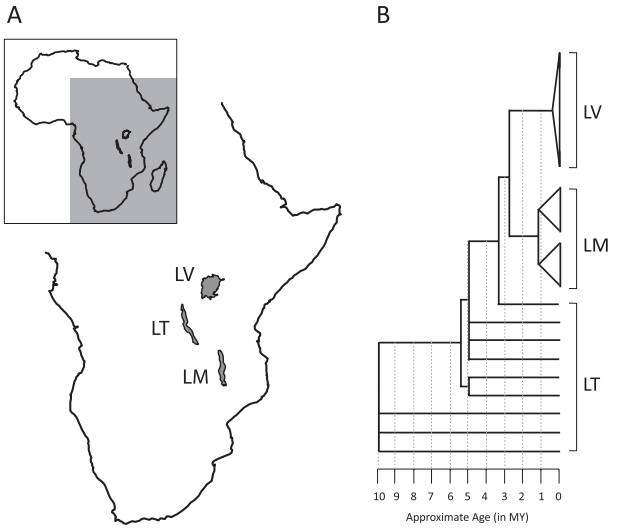

Fig. 1. Schematic of the East African Great Lakes and the phylogenetic structure of their associated cichlid species flocks. (*A*) Map of the African continent with the location of the three Great Lakes—LT, LM, and LV—shown in gray. (*B*) Representative phylogeny of cichlids from each of the Great Lakes, with approximate dates of divergence (modified from Kocher 2004; Koblmuller et al. 2008). Map modified from the R package "maps" (Becker et al. 2010).

relationship of these two groups, it is unclear if this similarity is due to repeated evolution or shared ancestry. Therefore, here we assess opsin gene expression in 28 cichlids from a third nearby lake, Lake Tanganyika (LT). Cichlids from LT are both phylogenetically and phenotypically more diverse than cichlids from either LM or LV (Huber et al. 1997; Salzburger et al. 2002; Pollen et al. 2007). LT contains cichlids from many diverse lineages and tribes, including many older taxa that are ancestral to the LM and LV cichlid species flocks (Sturmbauer 1998; Takahashi 2003) (fig. 1); thus, cichlids from LT should provide a tractable system for identifying potential examples of repeated evolution in opsin gene expression. However, little is known of the spectral sensitivity of cichlids in LT. Although visual acuity has been documented for four species from the tribe Ectodini (Dobberfuhl et al. 2005), actual retinal sensitivities have been measured for only a single LT cichlid, *Astatotilapia burtoni* (Fernald and Liebman 1980). Hence, it is unclear whether retinal sensitivities are evolving primarily through opsin protein–coding mutations or regulatory changes among cichlids in LT. We hypothesize that similar opsin expression palettes will be present among cichlids from both LM and LT because both these lakes have clear, spectrally broad waters (Carleton et al. 2006) and both contain cichlids with parallel morphological and ecological adaptations (Kocher et al. 1993; Kassam et al. 2003).

In addition to repeated phenotypic evolution, we also assess phenotypic correlations between opsin expression and two factors associated with opsin expression divergence in cichlids, diet (Hofmann et al. 2009), and lens transmittance (Hofmann, O'Quin, Marshall, and Carleton 2010). Among cichlids from LM, the *SWS1* (UV) opsin is

upregulated among species that forage on zooplankton and other microorganisms (Hofmann et al. 2009). This adaptation increases the ability of cichlids and other teleosts to find and capture zooplankton (Browman et al. 1994; Jordan et al. 2004). Also among cichlids in LM, lens transmittance is positively correlated with both relative *SWS1* expression and the estimated sensitivity (λ_{max}) of single-cone photoreceptors (Hofmann, O'Quin, Marshall, and Carleton 2010). This correlation reveals that cichlids do not express opsins sensitive to wavelengths of light that their lenses ultimately filter before reaching the retina.

In summary, our goals were to 1) test the hypothesis that similar opsin gene expression palettes have evolved repeatedly among African cichlids in LT and LM and 2) test for the presence of similar phenotypic correlations among opsin expression, diet, and lens transmittance. The repeated evolution of these opsin palettes would suggest that regulatory mutations have played an important role in the evolution of spectral sensitivity among African cichlids. Additionally, the independent evolution of one or more phenotypic correlations would implicate natural selection as one driver of opsin expression evolution in these fishes (e.g., Schluter 2000).

Materials and Methods

Sampling Tanganyikan Cichlids

We sampled 85 individual fish representing 28 different LT cichlid species. Half of these samples were collected as adult fish from LT near Kigoma, Tanzania, in 2004. The remaining species were purchased as wild-caught adult fish from a commercial supplier. Additionally, we also sampled adult fish from a laboratory strain of *A. burtoni*. All eyes were collected at midday from full spectrum light-adapted animals. We noted the primary diet of each species following a survey of relevant literature sources (Taborsky et al. 1986; Yamaoka et al. 1986; Brichard 1989; Salzburger et al. 2002; Takahashi 2003; Duftner et al. 2005; Koblmuller et al. 2007). A complete list of the species sampled and their dominant diet is presented in table 1.

Real-Time Quantitative Polymerase Chain Reaction

We measured opsin gene expression in each cichlid via real-time quantitative polymerase chain reaction (RT-qPCR). Our methods for RNA extraction and subsequent RT-qPCR analysis generally followed those previously used to analyze opsin expression in cichlids from LM and LV (Spady et al. 2006; Carleton et al. 2008; Hofmann et al. 2009). Binding sites for the Taqman primers and probes used in these studies were sequenced for all seven cone opsins from one individual of each species. Primers used to generate these sequences are listed in the supplementary table S1 (Supplementary Material online). Many LT species had opsin sequences that perfectly matched the primers created previously for LM and LV cichlids. In these cases, we used the primers and probes from these previous studies. However, where these sequences differed, we created new LT-specific primers and probes. These new primers and probes, along with the original LM primers used, are listed in table 2. In all, we identified 15 unique primer/probe combinations needed to match the different LT species sampled (supplementary table S2, Supplementary Material online; see also table 1). We performed all RT-qPCR reactions on a LightCycler 480 (Roche). We normalized all RT-qPCR reaction efficiencies against a construct of cichlid opsins specially developed for the normalization of cichlid opsin RT-qPCR (Spady et al. 2006). In some LT species, however, the primer/probe-binding region did not match the sequence of the normalization construct. For these species, we normalized reaction efficiencies against known concentrations of a relevant cDNA sample or a ~120 bp oligomer encoding the primer- and probe-binding site. As in previous studies (Spady et al. 2006; Carleton et al. 2008; Hofmann et al. 2009), our measurement of *RH2a* expression combined the genetically and functionally similar *RH2aα* and *RH2aβ* opsins. We quantified opsin expression twice for all individuals and averaged the results. We then averaged individual results to obtain one final, species-specific mean and variance of opsin expression.

Predicting Maximal Retinal Sensitivity from Opsin Gene Expression

Cichlid cone opsins are expressed within the retina in two distinct cell types: single-cone photoreceptors and double-cone photoreceptors (Bowmaker 1995; reviewed in Carleton 2009). We predicted the wavelength of maximal sensitivity (PS_{max}) of each species' single- and double-cone photoreceptors from the results of our RT-qPCR analysis. We used the results of these estimates to infer how retinal sensitivities may vary as a result of changes in opsin gene expression (Carleton et al. 2008; Hofmann et al. 2009). These estimates provide a useful descriptive statistic for how multivariate shifts in opsin expression may alter spectral sensitivity, but they are not meant to imply that we find fish with cones that exhibit these exact λ_{max} values (although the results can be quite similar). Following previous studies (Carleton et al. 2008; Hofmann et al. 2009), we used equation (1)

$$PS_{max,C} = \sum(f_i\lambda_i) / \sum f_i \quad (1)$$

to calculate the predicted maximal sensitivity (PS_{max}) of cichlid single and double cones, where C is either the single- or the double-cone photoreceptor, f_i is the percent expression of the ith opsin out of the total, and λ_i is the corresponding peak absorbance of that opsin in *Oreochromis niloticus* (from Spady et al. 2006). Based on comparison of the λ_{max} of each cichlid opsin with the λ_{max} of single- and double-cone photoreceptors from several cichlid species—both measured physiologically using microspectrophotometry (reviewed in Carleton 2009)—we used the expression of the *SWS1*, *SWS2b*, and *SWS2a* opsins when estimating the PS_{max} of single cones and *RH2b*, *RH2a*, and *LWS* when estimating the PS_{max} of double cones. In previous analyses, we refer to the descriptive statistic PS_{max} as "predicted single/double-cone λ_{max}" (Hofmann

Table 1. LT Cichlid Species Used in This Study.

Species	*n*	Tribe[a]	Foraging	*ND2*	*CYTB*	*D-LOOP*	Combination[b]
Benthochromis tricoti	3	Benthochromini	Benthic invertebrates[c]	AY682515	AF428164	AY682477	15
Cyprichromis leptosoma	5	Cyprichromini	Zooplankton[c,d]	AY740343	AB280682	AY740320	3
Paracyprichromis nigrapinnis	3	Cyprichromini	Zooplankton[c,d]	AY740339	AY740204	AY740282	4
Asprotilapia leptura	2	Ectodini	Epilithic algae[c]	AY337772	AY337801	AF400701	5
Enantiopus melanogenys	3	Ectodini	Benthic inverts[c]	AY682517	AY337813	AY682480	2
Ophthalmotilapia ventralis	3	Ectodini	Zooplankton[c]	AY337774	AY337805	AY615479	14
Xenotilapia bathyphila	3	Ectodini	Benthic inverts[c]	AY337789	AY337844	AY339027	2
X. boulengeri	3	Ectodini	Benthic inverts[c]	HM135111	AY337823	AY339029	10
X. flavipinnis	2	Ectodini	Benthic inverts[c]	AY337794	AY337825	AY339030	2
X. ochrogenys	4	Ectodini	Benthic inverts[c]	AY337767	Z21772	Z21750	2
X. spiloptera	3	Ectodini	Benthic inverts[c]	AY337788	AY337841	AY339040	2
Eretmodus cyanostictus	4	Eretmodini	Epilithic algae[c]	AF398220	Z97477	EF035326	4
Tanganicodus irsacae	3	Eretmodini	Epilithic algae[c,e]	AF398219	Z97557	Y15134	3
Astatotilapia burtoni	1	Haplochromini	Benthic inverts[c]	AF317266	Z21773	Z21751	5
Chalinochromis brichardi	2	Lamprologini	Phytoplankton[c]	HM135112	Z29991	Z30006	11
Julidochromis regani	2	Lamprologini	Phytoplankton[c,f]	EF462228	EF470898	U01106	6
Neolamprologus brichardi	4	Lamprologini	Zooplankton[c]	DQ055015	AF438804	Z30021	7
N. cunningtoni	4	Lamprologini	Fish[c]	HM135113	HM135105	HM135109	3
N. furcifer	3	Lamprologini	Benthic inverts[c,g]	EF462249	Z29999	Z30026	5
N. mondabu	3	Lamprologini	Benthic inverts[c,g]	EF462242	HM135106	HM135110	8
N. tretocephalus	3	Lamprologini	Benthic inverts[c,g]	DQ055026	HM135107	—	13
Greenwoodichromis christyi	5	Limnochromini	Benthic inverts[d]	AY682528	HM135108	AY682489	12
Perissodus microlepis	2	Perissodini	Fish[c,h]	DQ055006	AF428167	EF437536	9
Lobochilotes labiatus	1	Tropheini	Benthic inverts[c]	U07254	AY301932	U01110	1
Petrochromis famula	2	Tropheini	Epilithic algae[c]	HM135114	AY301937	AY301963	1
Simochromis diagramma	5	Tropheini	Phytoplankton[c]	AY930087	AY301951	AY574628	1
Tropheus moori "muzi"	4	Tropheini	Epilithic algae[c]	AB018975	AB018990	Z12069	1
Tropheus sp. *mpimbwe*	3	Tropheini	Epilithic algae[c]	AY930086	EF470900	Z12054	1

[a] Takahashi (2003).
[b] Primer/probe combination used for RT-qPCR.
[c] Brichard (1989).
[d] Duftner et al. (2005).
[e] Yamaoka et al. (1986).
[f] Salzburger et al. (2002).
[g] Taborsky et al. (1986).
[h] Koblmuller et al. (2007).

et al. 2009) or simply "single/double-cone λ_{max}" (Carleton et al. 2008).

Opsin Sequence Divergence

Our estimation of photoreceptor PS_{max} assumes that all species exhibit opsin-coding sequences that are functionally identical to those of *O. niloticus*. This assumption is generally supported by microspectrophotometry results that demonstrate little variation in the spectral absorption of cones from different cichlid species that express the same opsin palette (Jordan et al. 2006; see also table 1 of Carleton 2009). Additionally, several studies have generally found little variation in the protein-coding sequence of each opsin across several cichlid species. These studies include sequences from 4 LT cichlids (Halstenberg et al. 2005; Spady et al. 2005), 16 LM cichlids (Parry et al. 2005; Spady et al. 2005; Hofmann et al. 2009), and 12 LV cichlids (Carleton et al. 2005; Hofmann et al. 2009). Although mutations within opsin-coding sequences can play an important role in cichlid spectral adaptation (Sugawara et al. 2005; Terai et al. 2006), these shifts are generally small (5–15 nm). However, in order to further test this assumption, we sequenced the three most variable cichlid opsins—*SWS1*, *RH2aβ*, and *LWS*—in a subset of the LT species sampled. We then compared the coding regions of these opsins with those from *O. niloticus*. The primers used to sequence these opsins are listed in supplementary table S1 (Supplementary Material online). This analysis also provides an important estimate of the contribution that protein-coding mutations make to the evolution of spectral sensitivity in LT cichlids.

Phylogenetic Analysis

For our comparative analyses of opsin expression evolution, we reconstructed the phylogenetic relationships among the LT species sampled using three mitochondrial loci, *ND2* (1047 bp), *CYTB* (401 bp), and *D-loop* (364 bp). These sequences were accessed through GenBank or else sequenced directly using previously published primers and protocols (Meyer et al. 1990; Taberlet et al. 1992; Kocher et al. 1995; Lee et al. 1995). Table 1 lists the accession numbers of these sequences for each species, and supplementary table S1 (Supplementary Material online) lists the primers used for PCR. Sequences were concatenated and aligned in MAFFT (Katoh et al. 2002), and we used Modeltest v3.7 (Posada and Crandall 1998) to choose an appropriate model of sequence evolution for the alignment. Phylogenetic reconstruction was performed using both

Table 2. Sequence of All Primers and Probes Used to Measure Cichlid Opsin Gene Expression.

Opsin	Primer	Sequence
SWS1	UV.Cic.Forward[a]	5′-GGCTGTGCCTGCCCAC-3′
	UV.Tang.Forward[b]	5′-GGCTGCGCCTGCCCAC-3′
	UV.Tang.Ov.Forward[b]	5′-TGCTGCGCCTTCCCAC-3′
	UV.Cic.Reverse[a]	5′-AGGAGCAGCCCAGACCTTC-3′
	UV.Cic.Probe[a]	5′-TTTCTTTGGCTGGAGCAGGTACATCCC-3′
SWS2b	B2.Cic.Forward[a]	5′-TTTGGTGCGCTAGCATGC-3′
	B2.Cic.Reverse[a]	5′-AAGGGACCACAGGCTTACCAT-3′
	B2.Cic.Probe[a]	5′-AGATCGAAGGTTTCATGGTAACACTCGGTG-3′
SWS2a	B1.Cic.Forward[a]	5′-TTTGGTGCGCTAGCATGC-3′
	B1.Tang.Reverse[b]	5′-CTTGCAAATCACAAGCCATC-3′
	B1.Cic.Probe[a]	5′-AGATCGAAGGTTTCATGGTAACACTCGGTG-3′
	B1.Tang.Probe[b]	5′-AGATCGAAGGTTTCATGGCAACACTCGGTG-3′
	B1.Tang.Nb.Probe[b]	5′-AGATCGAAGGTTTCATGGCAACACTTGGTG-3′
	B1.Tang.Nm.Probe[b]	5′-AGTTCGAAGGTTTCATGGCAACACTCTGTG-3′
	B1.Tang.Pm.Probe[b]	5′-AAATCGAAGGTTTCATGGCAACACTCGGTG-3′
	B1.Tang.Xeno.Probe[b]	5′-AGATCGAAGGTTTCTTGGCAACACTCGGTG-3′
RH2b	G3.Cic.Forward[a]	5′-TGCTGCCCCCCCATTG-3′
	G3.Cic.Reverse[a]	5′-AGGTCCACAGGAAACCTGAA-3′
	G3.Cic.Probe[a]	5′-TGGCTGGTCAAGGTACATTCCTGAGGGA-3′
RH2a	G.Tang.Forward[b]	5′-TTAATGGCTACTTCATTCTTGGA-3′
	G.Cic.Reverse[a]	5′-CCAGGACAACAAGTGACCAGAG-3′
	G.Cic.Probe[a]	5′-TGGCCACACTAGGAGGTGAAGTTGC-3′
	G.Til.Probe[a]	5′-TGGCCACACTTGGAGGTGAAGTTGC-3′
	G.Tang.Gc.Probe[b]	5′-TGGCCACACTTGGAGGTGAAGTTTC-3′
	G.Tang.Ov.Probe[b]	5′-TGGCCACACTAGGAGGTCAAGTTGC-3′
LWS	R.Cic.Forward[a]	5′-CTGTGCTACCTTGCTGTGTGG-3′
	R.Cic.Reverse[a]	5′-GCCTTCTGGGTTGACTCTGACT-3′
	R.Tang.Nb.Reverse[b]	5′-GCTTTCTGGGTTGACTCTGACT-3′
	R.Tang.Nt.Reverse[b]	5′-GCCTTTTGGGTTGACTCTGACT-3′
	R.Tang.Xb.Reverse[b]	5′-GCCTTCTGGGTTGACTCTGATT-3′
	R.Cic.Probe[a]	5′-TGGCCATCCGTGCTGTTGCC-3′

[a] Spady et al. (2006)
[b] This study.

Bayesian inference (BI) and maximum likelihood (ML) methods in the programs MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) and PAUP v4.0b10 (Swofford 2003). For the BI analysis, the best-fit model of sequence evolution chosen by Modeltest (general time reversible [GTR] + Γ + I) was used to construct and run four Markov chain Monte Carlo (MCMC) sampling chains, each run for 1,000,000 generations with a swap frequency of once every 10 generations. Trees were sampled every 1,000 generations after discarding the first 10% as burn-in. We additionally discarded as burn-in the first 250 trees when calculating posterior probability values for the final 50% majority-rule consensus tree. For the ML analysis, we used the heuristic tree search method with random addition of sequences and tree bisection and reconnection branch swapping. In addition to the GTR + Γ + I model, for this analysis, we also specified several additional model parameters estimated by Modeltest. These parameters were base frequencies (A = 0.2862, C = 0.3267, G = 0.1140, T = 0.2731), substitution rates (A–C = 0.7613, A–G = 11.8437, A–T = 1.2821, C–G = 0.6842, C–T = 6.5861, G–T = 1.000), proportion of invariable sites (0.4546), and the gamma distribution shape (0.9362). We used 100 ML bootstrap replicates to calculate nodal support for the final 50% majority-rule consensus tree. We rooted this tree with sequences from *O. niloticus* (AB018974, AF550020, and AF328847). We used this tree in all comparative analyses of opsin expression with diet and lens transmittance among LT cichlids.

For the analyses of repeated evolution among cichlids from different East African rift lakes, we combined this mitochondrial tree with four published amplified fragment length polymorphism (AFLP) phylogenies of LM and LV cichlids (Albertson et al. 1999; Allender et al. 2003; Seehausen et al. 2003; Kidd et al. 2006). Due to the young age of the LM and LV cichlid species flocks (1.0 My and <0.2 My, respectively), the interrelationships of these taxa can only be resolved with genome-wide scans of many AFLP or single nucleotide polymorphism genotypes; mitochondrial DNA is not sufficient to resolve the phylogenies of these two groups. However, the monophyly of the LM and LV radiations, the structure of the LT radiation, and the interrelationships among taxa between the three major lakes have all been confidently resolved using mitochondrial loci (Meyer et al. 1990; Kocher et al. 1993; Salzburger et al. 2002; Kocher 2004; see fig. 1). The overall structure of our combined tree is consistent with the purported relationships of taxa in these three lakes (Salzburger et al. 2002; Koblmuller et al. 2008). The interrelationships among taxa from LM and LV reported here use only those nodes supported by ≥60% bootstrap support in their respective studies. We set all branch lengths of this composite phylogeny to one.

Analysis of Parallel Evolution

To test the hypothesis that the various opsin gene expression palettes have evolved repeatedly among African cichlids from different rift lakes, we used two methods. First, we performed multivariate Bayesian clustering (Fuentes and Casella 2009; Gopal et al. 2009) to statistically group the 28 LT cichlids sampled here with 65 additional species from LM and LV (Hofmann et al. 2009), as well as the Nile tilapia, *O. niloticus* (Carleton et al. 2008). Because the cichlid species flocks of LM and LV both form monophyletic groups, we do not expect them to more closely resemble cichlids from LT unless they have evolved similar patterns of opsin gene expression in parallel. Bayesian clustering groups observations not by a distance-based metric but by a Metropolis search algorithm that attempts to maximize the marginal probability of $(\mathbf{Y}|\omega_k)$, where $\mathbf{Y}$ is a matrix of response variables (e.g., opsin expression values for each species) and ω is the partitioning of $\mathbf{Y}$ into a prespecified number of k clusters. This method then tests the statistical significance of the resulting clusters using a Bayes factor to estimate the empirical posterior probability (PP) of the null hypothesis

$$H_0: \text{ No clusters } (k = 1) \text{ versus } H_1: k \text{ clusters.} \quad (1)$$

In order to generate a frequentist probability value for this test, we performed a second search of the PP space under the null hypothesis in order to generate a null distribution of quantiles for these values; we then compared the final PP value with this distribution (Fuentes and Casella 2009). For our analysis, we specified the presence of $k = 3$ clusters, representing the three opsin gene expression palettes so far observed in African cichlids (Fernald and Liebman 1980; Carleton et al. 2000, 2005, 2008; Parry et al. 2005; Jordan et al. 2006; Hofmann et al. 2009). However, we also performed this analysis with k equal to 4 and 5 clusters as well. We performed Bayesian clustering in the R package "bayesclust" (Gopal et al. 2009). We used 1,000,000 simulations to estimate both the optimal clustering of the taxa and the PP of the null hypothesis. We used 10,000 simulations when generating the null distribution of PP values.

Second, we reconstructed the ancestral state of each major cichlid tribe using both Bayesian and ML methods. Using a composite phylogeny of 47 cichlids from all 3 lakes, we first estimated the posterior probability that the ancestor of each tribe expressed the opsin palette represented by $k = 3$, 4, and 5 clusters in the program BayesTraits (Pagel et al. 2004; Pagel and Meade 2007). BayesTraits infers ancestral states using a reversible-jump MCMC search algorithm. For this analysis, we specified a reversible-jump hyperprior derived from the exponential distribution but seeded from a uniform (uninformative) distribution of values ranging from 0 to 30. We also specified a rate deviation parameter equal to one. Together, these parameters produced acceptance rates of newly proposed values equal to ~24%, which is within the desired range for this type of analysis (Pagel and Meade 2007). We ran the RJ-MCMC for 20,020,000 generations, discarded the first 20,000 generations as burn-in, and sampled the chains every 300 generations. All reconstructions were performed using the "BayesMultiState" module with the "AddNode" command.

Finally, we also reconstructed the ancestral state of each cichlid tribe following a ML analysis of each opsin's expression value in the R package APE v2.5 (Paradis et al. 2004). This analysis allowed us to reconstruct the ancestral state of each opsin individually, without forcing a discrete cluster assignment to each species' palette or the reconstructed ancestral states. However, we note that continuous character state reconstructions have been shown to perform poorly over adaptive radiations (Schluter et al. 1997).

For all ancestral state reconstructions, we rooted our tree of African cichlids from LT, LM, and LV with the tilapine cichlid *O. niloticus*. Both physiological measurements of retinal sensitivity as well as predictions made from opsin expression values indicate that *O. niloticus* expresses the long-wavelength palette (Spady et al. 2006; Carleton et al. 2008). These physiological and opsin expression values are representative of all additional riverine outgroups for which spectral sensitivities have been measured, including the tilapine *Sarotherodon* and the distantly related Neotropical cichlids (Levine and MacNichol 1979; Spady et al. 2006; Carleton et al. 2008; Carleton 2009). Thus, we present *O. niloticus* as a representative member of *Oreochromis* and other outgroups to the cichlids we include here.

Comparative Analyses with Diet and Lens Transmittance

We tested the hypothesis of correlated evolution among opsin expression, diet, and lens transmittance using the phylogenetic comparative method (Felsenstein 1985). For the analysis of opsin expression with diet, we used phylogenetic analysis of variance (ANOVA) (Garland et al. 1993) to compare the mean expression of each opsin among species grouped into five foraging levels (table 1). We implemented phylogenetic ANOVA in the programs PDSIMUL v2.0 (Garland et al. 1993) and PHYLOGR (Diaz-Uriarte and Garland 2007). We performed 1,000 simulations of each opsin variable across the LT phylogeny using a Brownian motion model of character evolution. These simulations were used to generate phylogenetically corrected null distributions of our test statistics for phylogenetic ANOVA. However, prior to performing these simulations, we first transformed several opsin variables to better meet the ANOVA assumptions of homogeneity of variances and normality of errors. These transformations were performed using Box–Cox powers estimated in the R package "car" (Fox 2008) and are presented in table 3. We added 1.5 as a constant to each observation in order to maintain the order of the means before transformation. Additionally, for the comparison of the *SWS2a* opsin, we transformed the branch lengths of the mitochondrial tree using Grafen's (1989) rho ($\rho = 0.1$) and excluded *Neolamprologus tretocephalus* as an outlier from this analysis. We ultimately compared the probability values from these analyses with the Bonferroni-corrected significance

Table 3. Results of Phylogenetic ANOVA Comparing Opsin Gene Expression with Foraging Preference and Post Hoc Comparisons of *SWS1* Expression between Foraging Levels.

Opsin	Pagel's λ	Box–Cox Power	Degrees of Freedom	*F* or *t* Value	*P* Value[a]
SWS1	**0.3988**	**−1.772**	**4, 23**	**2.587**	**0.099**
Zooplankton versus Algae	**—**	**—**	**1, 23**	**1.823**	**0.115**
Zooplankton versus Phytoplankton	**—**	**—**	**1, 23**	**1.945**	**0.071**
Zooplankton versus Fish	**—**	**—**	**1, 23**	**1.462**	**0.129**
Zooplankton versus Invertebrates	**—**	**—**	**1, 23**	**3.174**	***0.004***
SWS2b	**0.1644**	**—**	**4, 23**	**1.465**	**0.337**
SWS2a	**<0.0001**	**−1.541**	**4, 22**	**0.541**	**0.820**
RH2b	**0.1301**	**—**	**4, 23**	**0.959**	**0.557**
RH2a	**<0.0001**	**0.188**	**4, 23**	**0.209**	**0.952**
LWS	**0.1796**	**0.350**	**4, 23**	**0.989**	**0.528**

Note.—Value in italic is statistically significant after Bonferronni correction for 10 comparisons.

[a] Bonferonni-corrected significance threshold following 10 comparisons is $\leq$ 0.5/10 tests = 0.005.

threshold for 10 comparisons (α = 0.05/10 comparisons = 0.005; see table 3). Finally, for each opsin, we also estimate Pagel's (1999) λ via ML in the R package "geiger" (Harmon et al. 2009). Pagel's λ provides an important measure of association between the phylogeny and variance for a given trait.

For the analysis of opsin expression with lens transmittance, we extracted lenses for approximately half of the species sampled. We measured the transmission of these lenses using an Ocean Optics USB4000 spectrometer and a pulsed Xenon lamp (PX2, Ocean Optics). Our measurements followed the previously published protocols of Siebeck and Marshall (2001). Transmission values were normalized to 1 at 600 nm and used to determine the wavelength of 50% transmission (T50). Because light must first pass through the lens before reaching the retina, lens transmittance can limit the wavelengths of light reaching the photoreceptors. This is particularly true for wavelengths at the short-wavelength end of the visible light spectrum (Siebeck and Marshall 2001). Because short-wavelength sensitivity is mediated by the single-cone photoreceptors in cichlids (Fernald and Liebman 1980; Jordan et al. 2006; Carleton 2009), we tested the hypothesis of correlated evolution between lens T50 and the predicted maximal sensitivity (PS_{max}) of cichlid single-cones (see eq. 1). For this analysis, we used phylogenetically independent contrasts (PICs; Felsenstein 1985) implemented in the PDAP:PDTREE module (Midford et al. 2003) of the program Mesquite v1.12 (Maddison WP and Maddison DR 2001). We set all branch lengths to one and log transformed single-cone λ_{max} values to meet the assumptions of the independent contrasts method and normality of errors.

Results and Discussion

Tanganyikan Opsin Expression Diversity

Figure 2 illustrates the results of our RT-qPCR analysis for the 28 LT cichlids sampled. The expression values measured for each opsin ranged from 0% to 73% of total opsin expression (supplementary table S3, Supplementary Material online). Despite previous analyses that reveal small but statistically significant differences in opsin expression between retinas extracted while in the field and those extracted after rearing for one generation in a laboratory setting (Hofmann, O'Quin, Smith, and Carleton 2010), we found no discernable differences between the retinas of wild-caught Tanganyikan cichlids processed in the field and those shipped to our laboratory (data not shown). The majority of species simultaneously expressed 3 or 4 of the 6 opsins measured. Importantly, these expression patterns generally matched those observed among cichlids from LM and LV, which are *SWS1-RH2b-RH2a* (short-wavelength sensitive), *SWS2b-RH2b-RH2a* (middle-wavelength sensitive), and *SWS2a-RH2a-LWS* (long-wavelength sensitive) (Hofmann et al. 2009). However, many species also expressed appreciable amounts (between 5% and 18%) of a fourth opsin—typically *LWS*—which is also observed among some LM and LV species (Hofmann et al. 2009). In general, most of the species we sampled expressed opsins from either the middle- or the long-wavelength palettes. But, in contrast to many LM cichlids that express the long-wavelength palette, LT cichlids with this palette generally expressed *SWS2b* in place of *SWS2a*. Also in contrast to LM and LV cichlids, a few species expressed high levels of either *SWS1* or *SWS2a* (fig. 2). Finally, at least two species exhibited opsin expression profiles that had not been previously described in cichlids from LM and LV. *Paracyprichromis nigrapinnis* expressed high levels of *SWS1* in conjunction with *RH2a* and *LWS*, and *N. tretocephalus* expressed high levels of *SWS2a* in conjunction with *RH2b* and *RH2a* (fig. 2).

The approximate spectral sensitivity estimated for cichlids with these opsin expression palettes is illustrated in figure 2. The average predicted maximal sensitivity (PS_{max}) for single-cones ranged nearly 100 nm, from 366 to 453 nm. The average joint double–cone PS_{max} for these species had a slightly narrower range, from 487 to 552 nm (supplementary table S3, Supplementary Material online). The distribution of LT cichlids across the combined predicted sensitivity of these two photoreceptors reveals that LT cichlids likely exhibit spectral sensitivities that overlap those observed or predicted for cichlids from LM and LV (fig. 2). Among taxa with the middle-wavelength palette, several LT species also exhibited opsin expression profiles that were subtly divergent from those previously observed. Members of the tribe

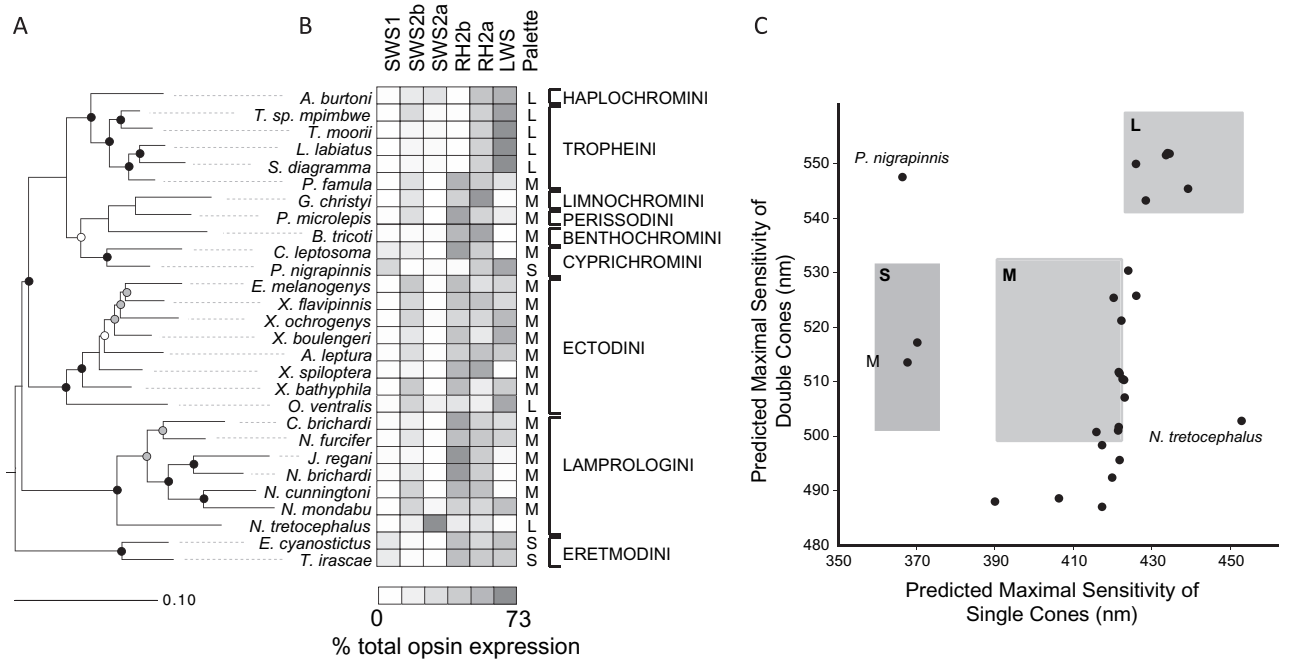

Fig. 2. Opsin expression diversity in 28 cichlid species from LT. (*A*) Mitochondrial phylogeny of the species sampled. Filled circles indicate nodes with >80% bootstrap and posterior probability support; gray circles, nodes with >50% bootstrap and posterior probability support; open circles, nodes with >50% posterior probability support only. (*B*) Heat map of relative opsin gene expression. The tribe to which each species belongs is shown on the left along with the visual palette estimated from the opsin expression profile. The assignment of each species' palette is based on Bayesian clustering of taxa into $k = 3$ clusters (see text). (*C*) Predicted maximal sensitivity (PS_{max}) of single- and double-cone photoreceptors estimated from the opsin expression results. The distribution of photoreceptor sensitivities estimated for cichlids from LM and LV are indicated by gray boxes (Hofmann et al. 2009). These boxes show the approximate distribution of single- and double-cone sensitivities for taxa expressing the short- (S), middle- (M), and long (L)-wavelength opsin sets. The results demonstrate that cichlids from LT exhibit opsin expression profiles that are very similar to cichlids from the monophyletic LM and LV radiations.

Lamprologini, including *Julidochromis regani*, *N. brichardi*, *N. furcifer*, and *Chalinochromis brichardi*, exhibited single-cone PS_{max} that were short-wavelength shifted relative to other species with this palette, and members of the tribe *Ectodini*, including *Enantiopus melanogenys* and *Xenotilapia ochrogenys*, exhibited double-cone PS_{max} that were long-wavelength shifted (fig. 2). The novel opsin expression palettes of *P. nigrapinnis* and *N. tretocephalus* were predicted to confer visual pigment sensitivities with pigment spacings that were broader and narrower, respectively, compared with the three more common palettes. Once again, the results of our analysis of estimated photoreceptor sensitivities are not meant to imply that these species have photoreceptors with these exact absorbance values; rather they provide a useful summary statistic for estimating how multivariate changes in opsin gene expression may shift spectral sensitivity. However, the results of our opsin expression and photoreceptor PS_{max} analyses both suggest that visual system diversity is similar among African cichlids in LT and LM but that this diversity is potentially greater among the more phenotypically and phylogenetically diverse LT cichlids (Salzburger et al. 2002).

Opsin Sequence Diversity

Our analysis of opsin-coding sequences supports the assumption that LT cichlids possess opsins with λ_{max} similar to those of *O. niloticus*. We sequenced the *SWS1* opsin in 10 species and found that it was the most variable of the 3 opsins examined (supplementary table S4, Supplementary Material online). We identified 25 polymorphic amino acid sites among these taxa; however, only six of these sites occurred in regions likely to affect chromophore binding and, therefore, spectral sensitivity. Of these 6 sites, 5 exhibited replacements that considerably alter the physical or chemical properties of the amino acid substituted (A52T, A97S, I290T/S, and A298S). However, only one substitution (I290T/S) was absolutely fixed between *O. niloticus* and the LT cichlids. Substitutions at the remaining sites were shared between *O. niloticus* and other species, and no substitutions were found in sites already known to influence *SWS1* absorption (Yokoyama 2008). We then sequenced *RH2aβ* in 14 species. Here we found 18 polymorphic sites, but only one of which occurred in a chromophore-binding region (supplementary table S4, Supplementary Material online). This polymorphism, F203Y, varies in amino acid polarity but has not yet been shown to impact spectral tuning. However, it is possible that such a polarity shift could slightly impact the spectral absorption of the *RH2aβ* opsin (Chang et al. 1995). Finally, we sequenced *LWS* in 11 species and found 10 variable sites. But, once again, we found only one site that occurred in a chromophore-binding region (supplementary table S4, Supplementary Material online). This polymorphism, A164S, does change the amino acid polarity and has been shown to cause a 7-nm increase in *LWS* absorbance in humans (Asenjo et al. 1994) and

LV cichlids (Terai et al. 2006). In summary, we found only one polymorphism in each opsin that was likely to produce a shift in the sensitivity of that gene relative to *O. niloticus*. Therefore, we conclude that LT cichlids have opsins with spectral sensitivities similar to those of *O. niloticus*, which justifies our use of *O. niloticus* opsin λ_{max} in the estimation of photoreceptor sensitivities. We emphasize that the sequence differences we observe would only produce small shifts (5–15 nm; Carleton 2009; Hofmann et al. 2009) in spectral sensitivity relative to the large shifts (30–100 nm) caused by changes in opsin gene expression. Therefore, none of the sequence substitutions we observe would alter the placement of LT species into different visual palette groups.

Interestingly, several of the sites we identified as polymorphic in the opsins of LT cichlids are also polymorphic among cichlids from LM and LV (e.g., *SWS1* site 217; *Rh2aβ* sites 107, 151, and 218; and *LWS* site 264; supplementary table S4, Supplementary Material online) (Hofmann et al. 2009). These mutations could indicate parallel mutations within opsin-coding sequences but more likely reflect ancestral polymorphisms (Spady et al. 2005; Terai et al. 2006). We also found that the *SWS1* opsin exhibited the largest number of putatively functional replacements among cichlids from LT. Although we did not examine all opsins, this pattern is consistent with a specific role for protein-coding evolution within opsins sensitive to the ends of the visible light spectrum (Hofmann et al. 2009). However, only one site (*SWS1*-217) was variable in both these groups; the rest were unique to cichlids from each lake. This observation could suggest that there has been convergent functional evolution of the *SWS1* opsin in cichlids from LT and LM. This pattern is likely not the result of the rapid accumulation of deleterious alleles because none of the sequences we examined were pseudogenes, although we acknowledge that the *SWS1* opsin is not highly expressed among the adults of most LT species examined (fig. 2*A*). However, lack of *SWS1* expression among adults does not rule out its use earlier during development (e.g., Carleton et al. 2008). The small number of putatively functional substitutions we identify in the remaining two opsins suggests that opsin protein–coding mutations likely contribute very little to divergence of spectral sensitivity among LT cichlids, with the possible exception of the *SWS1* opsin. However, we note that even small shifts in spectral sensitivity can impact female choice and even speciation (Seehausen et al. 2008). The much larger shifts in spectral sensitivity associated with changes in opsin expression could have an even greater impact on divergence among cichlids from LM and LT.

Phylogenetic Analysis

The final 50% majority-rule consensus trees produced by our Bayesian and ML analyses were highly resolved and widely congruent. In each case, the positions of the major tribes were identical, and the trees differed only slightly in their branch lengths and support for certain nodes. Figure 2*A* illustrates the final consensus tree for both analyses incorporating BI-estimated branch lengths. Despite weak support for five nodes, our phylogeny is highly concordant with those previously reported for these or closely related species (e.g., Salzburger et al. 2002; Duftner et al. 2005; Day et al. 2007).

Parallel Evolution of Opsin Gene Expression

The results of our Bayesian cluster analyses using $k = 3$, 4, and 5 clusters generated clustering schemes with empirical posterior probabilities (PP) equal to 1.07e-21, 2.35e-19, and 3.05e-16, respectively. All clustering schemes produced PP values that were a statistically better fit to the observed data than the null hypothesis of $k = 1$ clusters or no differences between species ($P < 0.0001$ in all three cases). Unfortunately, the implementation of Bayesian clustering we use here cannot currently estimate the optimal number of k clusters, and the PP values of different tests cannot be compared for this purpose because each PP is unique to the model of k clusters specified (Fuentes and Casella 2009). However, because previous estimates support $k = 3$ as the optimal number of opsin expression clusters among LM cichlids (Hofmann et al. 2009), we were primarily concerned with the results of this analysis.

The clustering scheme for $k = 3$ grouped cichlids from LT, LM, and LV into clusters that chiefly reflected the three opsin expression palettes previously identified in these fishes (supplementary table S5, Supplementary Material online). Group 1 consisted of three LT cichlids that express the short-wavelength opsin palette (*Eretmodus cyanostictus*, *Tanganicodus irsacae*, and *P. nigrapinnis*) as well as most members of the LM rock-dwelling (mbuna) lineage and also some members of the LM sand-dwelling (utaka) lineage (fig. 3; supplementary table S5, Supplementary Material online). Group 2 consisted of taxa that express the middle-wavelength palette and included the majority of the LT cichlids sampled. Species in this group include members of the tribes Neolamprologini and Ectodini, *Benthochromis tricoti*, *Cyprichromis leptura*, *Petrochromis famula*, *Greenwoodichromis christyi*, and several members of both the LM mbuna and utaka lineages. Finally, group 3 consisted of seven LT cichlids that express the long-wavelength opsin palette, including *A. burtoni*, *Ophthalmotilapia ventralis*, *N. tretocephalus*, most members of the tribe Tropheini, many members of the monophyletic LM utaka lineage, and all members of the monophyletic LV cichlid species flock. The results for $k = 4$ and 5 simply subdivided the short- and long-wavelength–sensitive clusters, respectively (Supplemental fig. S1 and table S5, Supplementary Material online). Grouping taxa into $k = 4$ clusters split species that express the two short- and middle-wavelength palettes into a third group of species that exhibit additional *SWS2b* and *LWS* expression. Grouping taxa into $k = 5$ clusters split taxa that express the long-wavelength palette into two groups based on those with additional *SWS2b* expression (supplementary table S3, Supplementary Material online; see also table S1 in Hofmann et al. 2009). Finally, we note that the clustering results of Bayesian clustering of $k = 3$ groups are very similar to the clustering scheme identified by principle component analysis and *k*-means

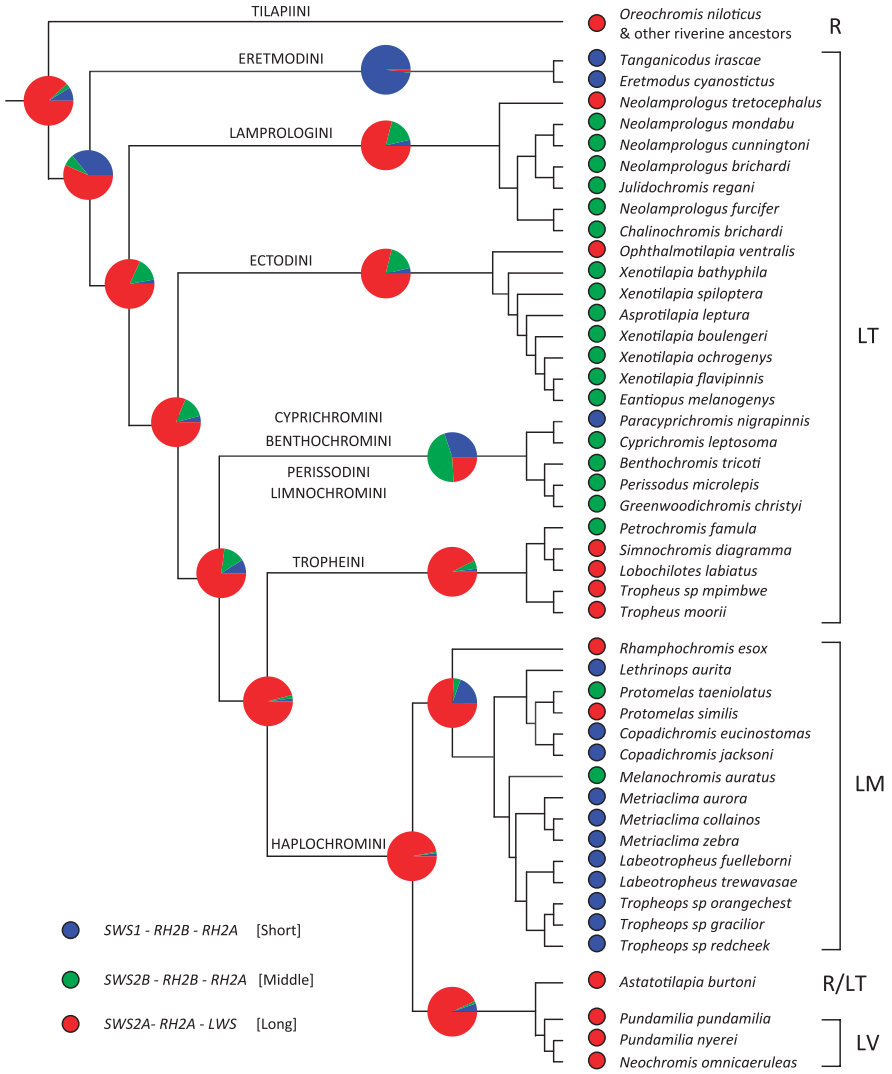

Fig. 3. Parallel evolution of opsin gene expression in 47 African cichlid fishes from LT, LM, and LV, as well as the rivers (R). Pie charts illustrate the results of Bayesian ancestral reconstruction and show the relative posterior probability that the ancestor expressed each of three opsin expression palettes determined by clustering taxa into $k = 3$ clusters. The long-wavelength (red) palette is supported as the ancestral state for most African cichlid lineages, including the Haplochromini (LM and LV). States at the tips indicate several parallel shifts to the short- (blue) and middle (green)-wavelength palettes among cichlids in LT and LM from ancestors that each expressed the long-wavelength palette (red).

clustering (data not shown). Thus, our results are robust to the analytical method used to group individuals based on opsin gene expression. In all cases, the statistically significant clustering of species from different, monophyletic lineages within LT, LM, and LV strongly supports the repeated evolution of opsin gene expression among African cichlids.

Reconstruction of the evolutionary history of these clusters on the phylogeny of African cichlids also supports the repeated evolution of multiple opsin expression palettes. Figure 3 illustrates the posterior probability that the ancestor of each major tribe expressed the palettes represented by $k = 3$ clusters following Bayesian ancestral state reconstruction. With only two exceptions, this reconstruction overwhelmingly supports the long-wavelength palette as the ancestral state for most major clades, including the haplochromine tribes of LM and LV. The two exceptions are the joint ancestor of the tribes Cyprichromini, Benthochromini, Perissodini, and Limnochromini, which likely expressed the middle-wavelength palette, and the ancestor of the Eretmodini, which expressed the short-wavelength palette. This reconstruction therefore indicates several transitions to the short- and middle-wavelength palettes among members of the various African cichlid lineages in LT and LM. Specifically, the short-wavelength palette arose twice within LT and then again among members of the LM cichlid radiation; the middle-wavelength palette arose at least four times among cichlids from LT and at least twice among cichlids from LM; and, because the long-wavelength palette is ancestral to most tribes, its evolution does not appear to have occurred in parallel among the cichlids from LT, LM, and LV. However, this palette may have re-evolved at least once within the LM utaka clade (fig. 3). Reconstructions of $k = 4$ and 5 clusters on the cichlid phylogeny also overwhelmingly support a long-wavelength palette (group 3 or 5, colored red and yellow in supplementary fig. S1, Supplementary Material online) as the ancestral state for most

African cichlid tribes. Reconstruction of $k = 5$ clusters suggests that the ancestors of each lineage gradually developed a violet (as opposed to blue)-shifted long-wavelength palette leading up to the LV radiation. However, the reconstructions of both $k = 4$ and 5 clusters also indicate numerous transitions to the short- and middle-wavelength palettes among members of the LT tribes Eretmodini, Lamprologini, Ectodini, as well as LM cichlids (supplementary fig. S1, Supplementary Material online). Once again, these transitions occurred among species and lineages with different ancestors that each expressed the long-wavelength palette. Hence, even though we cannot distinguish between the optimality of $k = 3$, 4, or 5 clusters, the ancestral reconstruction of each of these scenarios all supports the parallel evolution of the short- and middle-wavelength palettes among cichlids from LT and LM from ancestors that expressed the long-wavelength palette.

Finally, we also used continuous character state reconstructions via ML to infer ancestral states of each opsin's expression pattern independently. This continuous character reconstruction produced estimates of ancestral states that were highly uncertain. Ninety-five percent confidence intervals for the inferred ancestral states overlapped for expression values at many nodes. Among internal nodes, only states at the base of the clades Eretmodini, Lamprologini, and Benthochromini deviated significantly from the states of their direct ancestor along the base of the tree (indicated by pluses and minuses in supplementary fig. S1, Supplementary Material online). In contrast, many species (tips) had 95% confidence intervals that did deviate significantly from the expression values predicted for the ancestor at the base of their respective clade. This pattern could be due to the inherent uncertainty in the ancestral states of nodes further from the tips of the phylogeny; however, we believe this pattern indicates that most shifts in opsin expression have occurred near the tips of the cichlid phylogeny, not at its base. To account for this possible bias, we also identified shifts in opsin expression of greater than 10% (indicated by greater than and less than symbols in supplementary fig. S1, Supplementary Material online). This analysis illustrates the same pattern: few large shifts in expression at internal nodes, except for the base of the clades Eretmodini, Lamprologini, Benthochromini, and Tropheiini. Once again, most shifts in opsin expression of more than 10% were concentrated at the tips of the phylogeny, indicating that this observation is not merely the result of statistical uncertainty in the ancestral states of internal nodes. Most shifts in opsin expression that were statistically significant (e.g., where 95% confidence intervals between an ancestor and descendent node did not overlap) were $\geq$10%. Additionally, our analysis based on percent expression is necessary where multiple observations of a particular species are not available to generate confidence intervals, which was the case for most LM cichlids. However, analyses of both confidence intervals and percentage units infer many parallel shifts in opsin expression. Most shifts represent increases in expression of the *SWS1*, *RH2b*, and *RH2a* opsins among cichlids from LT and LM (supplementary fig. S1, Supplementary Material online). We also find evidence for many independent shifts to lower expression levels for the *SWS2a* and *LWS* opsins. Both observations are consistent with the parallel evolution of the short (SWS1-*RH2b*-*RH2a*) and middle (*SWS2b*-*RH2b*-*RH2a*) palettes. Most importantly, the results of our continuous character state reconstructions are highly concordant with the results of our Bayesian reconstructions of $k = 3$ opsin palettes (fig. 3). By estimating the spectral sensitivity of each ancestor via estimated single- and double-cone PS_{max}, we demonstrate that most ancestors exhibit inferred opsin expression values consistent with the long-wavelength palette (inset in supplementary fig. S1, Supplementary Material online). The only nodes that deviate from the long-wavelength palette are nodes at the base of the clades Eretimodini and Benthochromini. This observation is perfectly consistent with our reconstruction of $k = 3$ clusters (fig. 3). Thus, we conclude that similar opsin expression profiles among cichlids from LT and LM are due to parallel evolution from ancestors that each expressed the long-wavelength–sensitive opsin palette.

We refer to the repeated evolution of similar opsin expression profiles among cichlids from LT and LM as due to parallelism because, in each case, these transitions have occurred independently among taxa with different ancestors that shared the same ancestral state. However, we cannot infer how the palettes evolved in parallel with our current data. One hypothesis is that the presence of similar opsin expression profiles among cichlids in LT and LM is simply due to the sorting of ancestral polymorphism that affects adult variation in opsin expression. We do not believe this is the case because the presence of alternate opsin expression palettes has not been reported among the adults of any one cichlid population or species. This observation suggests that the ancestral groups likely did not exhibit this much population-level variation either. A second hypothesis is that these palettes evolved independently among an ancestral group of haplochromine cichlids that subsequently produced a hybrid swarm (e.g., Seehausen 2004). These palettes could then have been sorted coincident with the formation of new species. This hypothesis would produce the appearance of ancestral polymorphism; however, it would still indicate that the short- and middle-wavelength palettes evolved in parallel among LT and LM cichlids, only with a much earlier origin than our current phylogeny suggests (near the base of the LM clade instead of near the tips). Both the sorting of ancestral polymorphisms and a hybrid swarm scenario are consistent with what has been shown for the evolution of pigmentation blotching in LM cichlids (Roberts et al. 2009) and mitochondrial loci (Moran and Kornfield 1993). However, we favor a third hypothesis that the presence of similar opsin expression profiles among unrelated cichlids in LT and LM is the result of parallel heterochronic shifts in opsin expression from ancestors that expressed the entire complement of opsin palettes during development (e.g., Carleton et al. 2008). Both the basal cichlid *O. niloticus* (Carleton et al. 2008) and the derived

haplochromine cichlid *A. burtoni* (O'Quin KE, Smith A, Sharma A, Carleton KL, unpublished data) express the short- and middle-wavelength palettes as fry and juveniles, respectively, but then consistently express the long-wavelength palette as adults. We believe that the presence of ontogenetic variation in opsin expression among both basal and derived cichlids indicates that intermediate ancestral species (e.g., nodes b–g in supplementary fig. S1, Supplementary Material online), which are predicted to express the long-wavelength palette, probably did so following a similar developmental progression. If this is indeed the case, the presence of similar short- and middle-wavelength palettes among cichlids in LT and LM would be due to independent, heterochronic shifts in opsin expression from these ancestors (Carleton et al. 2008; O'Quin KE, Smith A, Sharma A, Carleton KL, unpublished data). However, additional sampling of LT cichlids with all three palettes at different ontogenetic stages will be necessary to conclusively test this hypothesis. We note also that our results rely on the observation that basal riverine cichlids express the long-wavelength opsin palette, of which *O. niloticus* is representative. Although this appears to be the case for all known African and Neotrophical outgroups so far surveyed (Levine and MacNichol 1979; Carleton et al. 2008), sampling of additional genera such as *Tylochromis* and *Tilapia* may strengthen this conclusion.

Comparative Analyses with Diet and Lens Transmittance

To determine whether diet is associated with opsin expression divergence in LT cichlids, we compared the mean expression of each opsin among LT species divided into five foraging groups (table 1). The ML estimates of Pagel's (1999) λ for each opsin generally indicate a weak association with phylogeny for the *SWS1* opsin and little or no association for the remaining opsins (table 3). Despite these weak associations, we still use appropriate phylogenetic comparative methods for all comparisons. In our overall phylogenetic ANOVA, we found no statistically significant association between diet and mean relative expression for any of the opsins examined (table 3). However, we did identify a similar trend of increased *SWS1* expression among zooplanktivorous cichlids in both LT and LM (fig. 4). Among cichlids from LM, diet is an important predictor of mean *SWS1* expression as well as actual and predicted single-cone λ_{max} (Jordan et al. 2004; Hofmann et al. 2009). LM cichlids that forage on zooplankton, algae, and phytoplankton on average exhibit higher levels of *SWS1* expression than cichlids that forage on fish or benthic invertebrates (Hofmann et al. 2009; fig. 4). *SWS1* expression increases sensitivity to UV light, which has been shown to increase the ability of teleost fish to detect and feed on zooplankton because the UV-absorbing zooplankton appear as dark objects against the bright UV background (Browman et al. 1994). Therefore, this trend motivated us to perform a post hoc Dunnett's test contrasting mean *SWS1* expression among LT cichlids that forage on zooplankton with the remaining foraging groups (table 3).

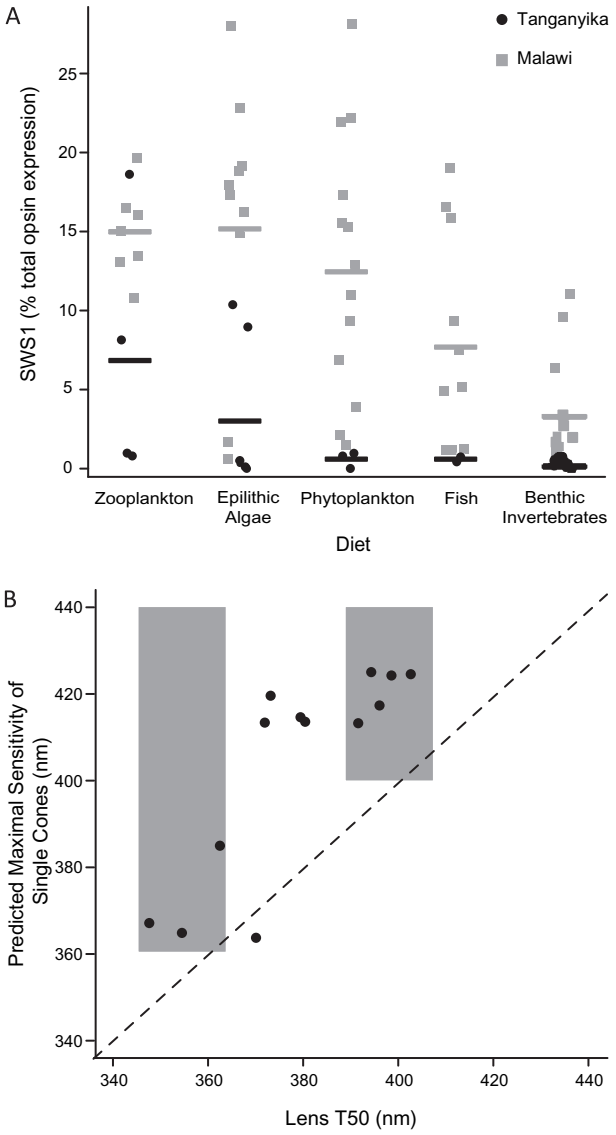

Fig. 4. Comparative analysis of opsin gene expression with foraging preference and lens transmittance. (*A*) Mean *SWS1* (UV) opsin expression is higher among zooplanktivorous cichlid species than benthivorous ones (means are indicated by gray and black bars). This pattern is observed among cichlids from both LT and LM. Opsin expression data for LM from Hofmann et al. (2009). (*B*) Regression of predicted maximal sensitivity of single-cone photoreceptors (PS_{max}) and lens transmittance (T50). Dotted line indicates $x = y$ such that single-cone PS_{max} equals lens T50. The distributions of lens T50 and single-cone PS_{max} among LM cichlids are indicated with gray boxes. Lens transmittance data for LM from Hofmann, O'Quin, Marshall, and Carleton (2010).

We found that mean *SWS1* expression was significantly higher among zooplanktivorous species versus benthivores (phylogenetic *t*-test; $t = -3.174$, $P = 0.004$); however, we found no difference in mean *SWS1* expression between zooplanktivores and the remaining foraging groups. This weak but interesting correlation suggests that similar associations between *SWS1* expression and diet may have evolved independently among cichlids from both LT and LM. The evolution of the same phenotypic correlation

among unrelated cichlids in LT and LM could implicate natural selection in the parallel evolution of opsin expression among these species (Schluter 2000). Future studies of additional zooplanktivorous cichlids in LT may bolster this conclusion.

In addition to diet, we also examined the correlated evolution of single-cone PS_{max} with lens transmittance. Lens transmittance (T50) values from LT cichlids were continuously distributed and ranged from 348.5 to 409 nm (supplementary table S4, Supplementary Material online). Lens T50 was positively correlated with predicted single-cone PS_{max} (PICs: $r^2 = 0.417$, $F_{1,11} = 6.717$, $P = 0.013$; fig. 4). Additionally, lens transmittance wavelengths were always lower than predicted single-cone PS_{max}, except in the case of *P. nigrapinnis*. These results indicate that cichlid lenses generally do not block wavelengths of light that the fish are highly sensitive to. Among LM cichlids, lens transmittance is also positively correlated with relative *SWS1* expression and estimated single-cone PS_{max} (Hofmann, O'Quin, Marshall, and Carleton 2010), although lens T50 values are more bimodally distributed among these species (fig. 4). Interestingly, we identified four LT cichlids with lens T50 values that are intermediate to the two broad groups found among LM cichlids (fig. 4). These species are *G. christyi*, *N. cunningtoni*, *O. ventralis*, and *P. famula*. All these species are from different tribes but express either the middle- or the long-wavelength palette. Additionally, all these species' opsin expression palettes generally overlap those observed in LM cichlids, suggesting that these intermediate lens transmittance values are not associated with novel or unusual patterns of opsin expression (fig. 2; supplementary table S3, Supplementary Material online). Like the results of our analyses of opsin expression diversity and photoreceptor sensitivity, the lens T50 values we observe suggest that visual system diversity is greater among the phylogenetically and phenotypically diverse cichlids of LT. Even so, the presence of similar, positive correlations between opsin expression divergence (illustrated through average single-cone PS_{max}) and lens transmittance among cichlids from LT and LM again suggests a role for natural selection in the parallel evolution of these traits.

We find that diet and lens transmittance are both associated with the evolution of opsin expression in cichlids from LT and LM, as they are in other groups as well (Munz and McFarland 1977; Lythgoe 1979; Losey et al. 2003). However, these two factors alone cannot explain all the similarities and differences in opsin expression we observe among cichlids from these two lakes. To illustrate this point, we identified three LT cichlids that are ecologically or morphologically similar to species in LM (Kocher et al. 1993; Kassam et al. 2003). The first pair of species, *Petrotilapia famula* (LT) and *Petrochromis nigra* (LM), both graze on epilithic algae and possess parallel morphological adaptations for doing so (Kassam et al. 2003). However, our results demonstrate that *P. famula* (LT) expresses the middle-wavelength palette, whereas *P. nigra* (LM) expresses the short-wavelength palette (Hofmann et al. 2009). These taxa also exhibit lens T50 that differ by ~15 nm (Hofmann et al. 2009). Similarly, both *Lobochilotes labiatus* (LT) and *Placidochromis milomo* (LM) possess puffy, distended lips for sucking invertebrates from the surface of rocks (Kocher et al. 1993). But we find that *L. labiatus* (LT) expresses the long-wavelength palette, whereas *P. milomo* (LM) expresses the middle-wavelength palette (Hofmann et al. 2009). The lens T50 of these two species differ by >40 nm (Hofmann et al. 2010). Only the final comparison between *J. regani* (LT) and *Melanochromis auratus* (LM), which both feed on phytoplankton and algae and both express the middle-wavelength palette, supports the hypothesis of ecological as well as spectral convergence. Unfortunately, we do not have lens transmittance data for *J. regani*. We also found that LT cichlids that forage on phytoplankton exhibit levels of *SWS1* expression on par with species that forage on fish and benthic invertebrates. This pattern contrasts strongly with ecologically similar species from LM (fig. 4). This difference is likely due to the expression of the long-wavelength palette among members of the LT tribe Tropheinii, which are phytoplanktivorous. This and the other examples we detail above likely contributed to the weak conclusion of our phylogenetic ANOVA (table 3). To us, these observations suggest that other factors must also drive opsin expression evolution in African cichlids. These factors likely include additional ecological factors such as depth, as well as nonadaptive factors such as random genetic drift.

One additional ecological factor that could also explain the parallel evolution of similar opsin expression profiles among cichlids from LT and LM is the ambient light environment. Changes in spectral sensitivity due to the attenuation of light at different depths are observed among cichlids from all three East African Great Lakes (Sugawara et al. 2005; Seehausen et al. 2008). However, we were unable to test for an association between opsin expression and ambient light environment because detailed spectral measurements for LT are not available. Additionally, we had limited information regarding the sampling depth for most species. However, we note that the amount of opsin expression diversity present among cichlids from each lake seems to be correlated with the amount of spectral variation present in each lake. In other words, both LT and LM are remarkably clear and have waters with similar spectral qualities (Carleton et al. 2006). Cichlids from both these lakes exhibit a diverse range of opsin expression profiles (e.g., at least three; see fig. 2 and supplementary fig. S1, Supplementary Material online) that collectively confer sensitivity to the entire spectrum of visible light available (Hofmann et al. 2009). In contrast, LV has a spectrally narrow light environment that is red shifted relative to LT and LM (Seehausen et al. 1997; Carleton et al. 2006). Opsin expression diversity in LV is very limited (fig. 3; supplementary fig. S1, Supplementary Material online) and appears to be constrained to only those opsins sensitive to the long wavelengths of light present in the lake (Hofmann et al. 2009). These observations suggest that ambient light may also influence the evolution of opsin gene expression in African cichlids; however, future

spectral measurements of LT will be necessary to definitively test this hypothesis.

Conclusions

Repeated phenotypic evolution can provide valuable insights into which genetic mechanisms generally contribute to the evolution of phenotypic diversity. Like pelvic spine loss in sticklebacks (Chan et al. 2010) and wing pigmentation in *Drosophila* (Prud'homme et al. 2006), we infer that cichlids in LT and LM have independently evolved similar retinal sensitivities through the parallel evolution of opsin gene regulation (figs. 2 and 3). Multiple ancestral state reconstructions support the parallel evolution of two distinct opsin expression profiles among unrelated cichlids from these two lakes (fig. 3). In contrast, we identified few protein-coding mutations that were likely to shift cichlid retinal sensitivities, with the possible exception of the *SWS1* (UV) opsin (supplementary table S4, Supplementary Material online). Although opsin genes provide a classic example of how mutations within the protein-coding regions of genes can contribute to phenotypic evolution (Yokoyama 2002), the independent evolution of similar opsin expression palettes among African cichlids underscores the important contribution that regulatory mutations can also make (Britten and Davidson 1971; King and Wilson 1975; Sucena et al. 2003; Prud'homme et al. 2006).

Why changes in opsin expression are prominent among cichlids from LT and LM could be due to similar adaptations to diet and lens transmittance (fig. 4), the light environment, or all three. Alternatively, biases in the use of one mutational type versus another could be due to selection (Schluter 2000) or genetic and developmental constraints (Schluter 1996; West-Eberhard 2003). For example, regulatory mutations may have relatively higher fitness when large shifts in opsin expression are necessary for spectral adaptation. In contrast, protein-coding mutations may be better suited for fine-tuning spectral sensitivity and necessary for turning spectral sensitivity at the two ends of the visible light spectrum (Hofmann et al. 2009). Examples of convergence in cichlid opsin-coding sequences do exist, particularly in the *RH1*, or rod, opsin (Sugawara et al. 2005). However, the spectral sensitivity of the rod opsin can only evolve through protein-coding mutations in cichlids because they do not have an additional *RH1* opsin to express. But in teleosts that do possess more than one rod opsin, large shifts in dim-light spectral sensitivity are generated through changes to the regulation of these genes (Yokoyama et al. 2008).

Exactly how the parallel evolution of opsin expression has been achieved among African cichlids from LT and LM is unclear. Currently, we cannot distinguish between the hypotheses of de novo mutation, sorting of ancestral polymorphism, or parallel heterochronic shifts in opsin expression, although we favor the latter hypothesis. We have recently demonstrated that adult opsin expression has a strong genetic basis and is heritable (Carleton et al. 2010; Hofmann, O'Quin, Smith, and Carleton 2010). Further, hybrid crosses reveal that as few as two loci may underlie these important differences, including both *cis*- and *trans*-acting loci (Carleton et al. 2010). Future work will aim to use these hybrid crosses to further elucidate the molecular genetic basis for differential opsin expression in cichlids. We will also examine diversity at important *cis*- and *trans*-regulatory regions to determine what contributions these two mechanisms make to the evolution of spectral sensitivity in cichlids. These future analyses will help us distinguish between the possible scenarios that led to the parallel evolution of opsin gene expression among African cichlids.

Supplementary Material

Supplementary figure S1 and tables S1–S5 are available at Molecular Biology and Evolution online http://www.mbe.oxfordjournals.org/.

Acknowledgments

This work was supported by awards to K.E.O. from the Guy Jordan Endowment Fund of the American Cichlid Association, The Cosmos Club Foundation of Washington, DC, and a Grant-In-Aid of Research from Sigma Xi, and The Scientific Research Society. K.L.C. was supported by the University of Maryland and National Science Foundation grant IOS-0841270. H.A.H. was supported by NSF grant IOS-021795. We thank S. Renn, C. Shumway, A. Pollen, G. and W. Kazumbe, and S. Bahan for assistance in the field; Tanzania Fisheries Research Institute, the Tanzania Commission on Science and Technology, and Prof. A. Nikundiwe (University of Dar Es Salaam) for their kind support of our research; Z. Nasser for help sequencing the opsin genes; and D. Soares for providing *Astatotilapia burtoni* tissues for this study. This work benefited greatly from comments by two anonymous reviewers.

References

Albertson RC, Markert JA, Danley PD, Kocher TD. 1999. Phylogeny of a rapidly evolving clade: the cichlid fishes of Lake Malawi, East Africa. *Proc Natl Acad Sci U S A*. 96:5107–5110.

Allender CJ, Seehausen O, Knight ME, Turner GF, Maclean N. 2003. Divergent selection during speciation of Lake Malawi cichlid fishes inferred from parallel radiations in nuptial coloration. *Proc Natl Acad Sci U S A*. 100:14074–14079.

Asenjo AB, Rim J, Oprian DD. 1994. Molecular determinants of human red/green color discrimination. *Neuron* 12:1131–1138.

Becker RA, Wilks AR, Brownrigg R, Minka TP. 2010. maps: Draw geographical maps. R package, version 2.1–4. Distributed by The Comprehensive R Archive Network. Available from: http://cran.r-project.org/web/packages/maps.

Bowmaker JK. 1995. The visual pigments of fish. *Prog Retin Eye Res*. 15:1–31.

Brichard P. 1989. Cichlids of Lake Tanganyika. Neptune City (NJ): TFH Publications, Inc.

Britten RJ, Davidson EH. 1971. Repetitive and non-repetitive DNA sequences and a speculation on the origins of evolutionary novelty. *Q Rev Biol*. 46:111–138.

Browman HI, Novales-Flamarique I, Hawryshyn CW. 1994. Ultraviolet photoreception contributes to prey search behavior in two species of zooplanktivorous fishes. *J Exp Biol*. 186:187–198.

Carleton KL. 2009. Cichlid fish visual systems: mechanisms of spectral tuning. *Integr Zool*. 4:75–86.

Carleton KL, Harosi FI, Kocher TD. 2000. Visual pigments of African cichlid fishes: evidence for ultraviolet vision from microspectrophotometry and DNA sequences. *Vision Res*. 40:879–890.

Carleton KL, Hofmann CM, Klisz C, Patel Z, Chircus LM, Simenauer LH, Soodoo N, Albertson RC, Ser JR. 2010. Genetic basis of differential opsin gene expression in cichlid fishes. *J Evol Biol*. 23:840–853.

Carleton KL, Kocher TD. 2001. Cone opsin genes of African cichlid fishes: tuning spectral sensitivity by differential gene expression. *Mol Biol Evol*. 18:1540–1550.

Carleton KL, Parry JW, Bowmaker JK, Hunt DM, Seehausen O. 2005. Colour vision and speciation in Lake Victoria cichlids of the genus Pundamilia. *Mol Ecol*. 14:4341–4353.

Carleton KL, Spady TC, Kocher TD. 2006. Visual communication in East African cichlid fishes: diversity in a phylogenetic context. In: Ladich F, Collin SP, Moller P, Kapoor BG, editors. Communication in fishes. Enfield (NH): Science Publishers. p. 485–515.

Carleton KL, Spady TC, Streelman JT, Kidd MR, McFarland WN, Loew ER. 2008. Visual sensitivities tuned by heterochronic shifts in opsin gene expression. *BMC Biol*. 6:22.

Chan YF, Marks ME, Jones FC, et al. (16 co-authors). 2010. Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a Pitx1 enhancer. *Science* 327:302–305.

Chang BS, Crandall KA, Carulli JP, Hartl DL. 1995. Opsin phylogeny and evolution: a model for blue shifts in wavelength regulation. *Mol Phylogenet Evol*. 4:31–43.

Day JJ, Santini S, Garcia-Moreno J. 2007. Phylogenetic relationships of the Lake Tanganyika cichlid tribe Lamprologini: the story from mitochondrial DNA. *Mol Phylogenet Evol*. 45:629–642.

Diaz-Uriarte R, Garland T Jr. 2007. PHYLOGR: functions for phylogenetically based statistical analyses. R package, version 1.0.6. Distributed by The Comprehensive R Archive Network. Available from: http://cran.r-project.org/web/packages/PHYLOGR.

Dobberfuhl AP, Ullmann JF, Shumway CA. 2005. Visual acuity, environmental complexity, and social organization in African cichlid fishes. *Behav Neurosci*. 119:1648–1655.

Duftner N, Koblmuller S, Sturmbauer C. 2005. Evolutionary relationships of the Limnochromini, a tribe of benthic deepwater cichlid fish endemic to Lake Tanganyika, East Africa. *J Mol Evol*. 60:277–289.

Felsenstein J. 1985. Phylogenies and the comparative method. *Am Nat*. 125:1.

Fernald RD, Liebman PA. 1980. Visual receptor pigments in the African cichlid fish, *Haplochromis burtoni*. *Vision Res*. 20:857–864.

Fox J. 2008. car: Companion to applied regression. R package, version 1.2-9. Distributed by The Comprehensive R Archive Network. Available from: http://cran.r-project.org/web/packages/car.

Fuentes C, Casella G. 2009. Testing for the existence of clusters. *Stat Oper Res Trans*. 33:115–146.

Garland T Jr., Dickerman AW, Janis CM, Jones JA. 1993. Phylogenetic analysis of covariance by computer simulation. *Syst Biol*. 42:265–292.

Gompel N, Prud'homme B. 2009. The causes of repeated genetic evolution. *Dev Biol*. 332:36–47.

Gopal V, Fuentes C, Casella G. 2009. bayesclust: Tests/searches for significant clusters in genetic data. R package, version 2.1. Distributed by The Comprehensive R Archive Network. Available from: http://cran.r-project.org/web/packages/bayesclust.

Grafen A. 1989. The phylogenetic regression. *Philos Trans R Soc Lond B Biol Sci*. 326:119–157.

Halstenberg S, Lindgren KM, Samagh SP, Nadal-Vicens M, Balt S, Fernald RD. 2005. Diurnal rhythm of cone opsin expression in the teleost fish *Haplochromis burtoni*. *Vis Neurosci*. 22:135–141.

Harmon L, Weir J, Brock C, Glor R, Challenger W, Hunt G. 2009. geiger: Analysis of evolutionary diversification. R package, version 1.3–1. Distributed by The Comprehensive R Archive Network. Available from: http://cran.r-project.org/web/packages/geiger.

Hofmann CM, Carleton KL. 2009. Gene duplication and differential gene expression play an important role in the diversification of visual pigments in fish. *Integr Comp Biol*. 49:630–643.

Hoekstra HE, Hirschmann RJ, Bundey RA, Insel PA, Crossland JP. 2006. A single amino acid mutation contributes to adaptive beach mouse color pattern. *Science* 313:101–104.

Hofmann CM, O'Quin KE, Marshall NJ, Carleton KL. 2010. The relationship between lens transmission and opsin gene expression in cichlids from Lake Malawi. *Vision Res*. 50: 357–363.

Hofmann CM, O'Quin KE, Marshall NJ, Cronin TW, Seehausen O, Carleton KL. 2009. The eyes have it: regulatory and structural changes both underlie cichlid visual pigment diversity. *PLoS Biol*. 7:e1000266.

Hofmann CM, O'Quin KE, Smith AR, Carleton KL. 2010. Plasticity of opsin gene expression in cichlids from Lake Malawi. *Mol Ecol*. 19:2064–2074.

Huber R, van Staaden MJ, Kaufman LS, Liem KF. 1997. Microhabitat use, trophic patterns, and the evolution of brain structure in African cichlids. *Brain Behav Evol*. 50:167–182.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES. Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–755.

Jessen TH, Weber RE, Fermi G, Tame J, Braunitzer G. 1991. Adaptation of bird hemoglobins to high altitudes: demonstration of molecular mechanism by protein engineering. *Proc Natl Acad Sci U S A*. 88:6519–6522.

Jordan R, Howe D, Juanes F, Stauffer JJ, Loew E. 2004. Ultraviolet radiation enhances zooplanktivory rate in ultraviolet sensitive cichlids. *Afr J Ecol*. 42:228–231.

Jordan R, Kellogg K, Howe D, Juanes F, Stauffer J Jr., Loew E. 2006. Photopigment spectral absorbance of Lake Malawi cichlids. *J Fish Biol*. 68:1291–1299.

Kassam DD, Adams DC, Hori M, Yamaoka K. 2003. Morphometric analysis on ecomorphologically equivalent cichlid species from Lakes Malawi and Tanganyika. *J Zool*. 260:153–157.

Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acid Res*. 30:3059–3066.

Kidd MR, Kidd CE, Kocher TD. 2006. Axes of differentiation in the bower-building cichlids of Lake Malawi. *Mol Ecol*. 15:459–478.

King MC, Wilson AC. 1975. Evolution at two levels in humans and chimpanzees. *Science* 188:107–116.

Koblmuller S, Egger B, Sturmbauer C, Sefc KM. 2007. Evolutionary history of Lake Tanganyika's scale-eating cichlid fishes. *Mol Phylogenet Evol*. 44:1295–1305.

Koblmuller S, Schliewen UK, Duftner N, Sefc KM, Katongo C, Sturmbauer C. 2008. Age and spread of the haplochromine cichlid fishes in Africa. *Mol Phylogenet Evol*. 49:153–169.

Kocher TD. 2004. Adaptive evolution and explosive speciation: the cichlid fish model. *Nat Rev Genet*. 5:288–298.

Kocher TD, Conroy JA, McKaye KR, Stauffer JR. 1993. Similar morphologies of cichlid fish in Lakes Tanganyika and Malawi are due to convergence. *Mol Phylogenet Evol*. 2:158–165.

Kocher TD, Conroy JA, McKaye KR, Stauffer JR, Lockwood SF. 1995. Evolution of NADH dehydrogenase subunit 2 in east African cichlid fish. *Mol Phylogenet Evol*. 4:420–432.

Lee W, Conroy J, Howell WH. 1995. Structure and evolution of teleost mitochondrial control regions. *J Mol Evol*. 41:54–66.

Levine JS, MacNichol EF Jr. 1979. Visual pigments in teleost fishes: effects of habitat, microhabitat, and behavior on visual system evolution. *Sens Processes*. 3:95–131.

Losey GS, McFarland WN, Loew ER, Zamzow JP, Nelson PA, Marshall NJ, Montgomery WL. 2003. Visual biology of Hawaiian coral reef fishes I. Ocular transmission and visual pigments. *Copeia* 2003:433–454.

Lythgoe JN. 1979. The ecology of vision. Oxford: Clarendon Press.

Maddison WP, Maddison DR. 2001. Mesquite: a modular system for evolutionary analysis, version 1.12. Distributed by The Mesquite Project. Available from: http://mesquiteproject.org.

Meyer A, Kocher TD, Basasibwaki P, Wilson AC. 1990. Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature* 347:550–553.

Midford PE, Garland T Jr., Maddison WP. 2003. PDAP:PDTREE package for Mesquite. Distributed by The Mesquite Project. Available from: http://mesquiteproject.org/pdap_mesquite.

Moran P, Kornfield I. 1993. Retention of an ancestral polymorphism in the mbuna species flock (Teleostei: Cichlidae) of Lake Malawi. *Mol Biol Evol*. 10:1015–1029.

Mundy NI. 2005. A window on the genetics of evolution: MC1R and plumage colouration in birds. *Proc Biol Sci*. 272:1633–1640.

Munz FW, McFarland WN. 1977. Evolutionary adaptations of fishes to the photic environment. In: Crescitelli F, editor. Handbook of sensory physiology: the visual system in vertebrates. Berlin (Germany): Springer. p. 193–274.

Pagel M. 1999. Inferring the historical patterns of biological evolution. *Nature* 401:877–884.

Pagel M, Meade A, Barker D. 2004. Bayesian estimation of ancestral character states on phylogenies. *Syst Biol*. 53:673–684.

Pagel M, Meade A. 2007. BayesTraits: Draft Manual. Distributed by the author. Reading (UK): School of Biological Sciences, University of Reading. Available from: http://www.evolution.rdg.ac.uk.

Paradis E, Claude J, Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*. 20:289–290.

Parry JW, Carleton KL, Spady T, Carboo A, Hunt DM, Bowmaker JK. 2005. Mix and match color vision: tuning spectral sensitivity by differential opsin gene expression in Lake Malawi cichlids. *Curr Biol* 15:1734–1739.

Pollen AA, Dobberfuhl AP, Scace J, Igulu MM, Renn SC, Shumway CA, Hofmann HA. 2007. Environmental complexity and social organization sculpt the brain in Lake Tanganyikan cichlid fish. *Brain Behav Evol*. 70:21–39.

Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*. 14:817–818.

Protas ME, Hersey C, Kochanek D, Zhou Y, Wilkens H, Jeffery WR, Zon LI, Borowsky R, Tabin CJ. 2006. Genetic analysis of cavefish reveals molecular convergence in the evolution of albinism. *Nat Genet*. 38:107–111.

Prud'homme B, Gompel N, Rokas A, Kassner VA, Williams TM, Yeh SD, True JR, Carroll SB. 2006. Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. *Nature* 440:1050–1053.

Roberts RB, Ser JR, Kocher TD. 2009. Sexual conflict resolved by invasion of a novel sex determiner in Lake Malawi cichlid fishes. *Science* 326:998–1001.

Salzburger W, Meyer A, Baric S, Verheyen E, Sturmbauer C. 2002. Phylogeny of the Lake Tanganyika cichlid species flock and its relationship to the Central and East African Haplochromine cichlid fish faunas. *Syst Biol*. 51:113–135.

Schluter D. 1996. Adaptive radiation along genetic lines of least resistance. *Evolution* 50:1766–1774.

Schluter D. 2000. The ecology of adaptive radiation. New York: Oxford University Press.

Schluter D, Price T, Mooers A, Ludwig D. 1997. Likelihood of ancestor states in adaptive radiation. *Evolution* 51:1699–1711.

Seehausen O. 2004. Hybridization and adaptive radiation. *Trends Ecol Evol*. 19:198–207.

Seehausen O, Koetsier E, Schneider MV, Chapman LJ, Chapman CA, Knight ME, Turner GF, van Alphen JJ, Bills R. 2003. Nuclear markers reveal unexpected genetic variation and a Congolese-Nilotic origin of the Lake Victoria cichlid species flock. *Proc Biol Sci*. 270:129–137.

Seehausen O, Terai Y, Magalhaes IS, et al. (12 co-authors). 2008. Speciation through sensory drive in cichlid fish. *Nature* 455:620–627.

Seehausen O, van Alphen JJ, Witte F. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science* 277:1808–1811.

Shapiro MD, Marks ME, Peichel CL, Blackman BK, Nereng KS, Jonsson B, Schluter D, Kingsley DM. 2004. Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* 428:717–723.

Siebeck UE, Marshall NJ. 2001. Ocular media transmission of coral reef fish—can coral reef fish see ultraviolet light? *Vision Res*. 41:133–149.

Spady TC, Parry JW, Robinson PR, Hunt DM, Bowmaker JK, Carleton KL. 2006. Evolution of the cichlid visual palette through ontogenetic subfunctionalization of the opsin gene arrays. *Mol Biol Evol*. 23:1538–1547.

Spady TC, Seehausen O, Loew ER, Jordan RC, Kocher TD, Carleton KL. 2005. Adaptive molecular evolution in the opsin genes of rapidly speciating cichlid species. *Mol Biol Evol*. 22:1412–1422.

Sturmbauer C. 1998. Explosive speciation in cichlid fishes of the African Great Lakes: a dynamic model of adaptive radiation. *J Fish Biol*. 53:18–36.

Sucena E, Delon I, Jones I, Payre F, Stern DL. 2003. Regulatory evolution of shavenbaby/ovo underlies multiple cases of morphological parallelism. *Nature* 424:935–938.

Sugawara T, Terai Y, Imai H, Turner GF, Koblmuller S, Sturmbauer C, Shichida Y, Okada N. 2005. Parallelism of amino acid changes at the RH1 affecting spectral sensitivity among deep-water cichlids from Lakes Tanganyika and Malawi. *Proc Natl Acad Sci U S A*. 102:5448–5453.

Sugawara T, Terai Y, Okada N. 2002. Natural selection of the rhodopsin gene during the adaptive radiation of East African Great Lakes cichlid fishes. *Mol Biol Evol*. 19:1807–1811.

Swofford DL. 2003. Phylogenetic analysis using parsimony (* and other methods), version 4. Sunderland (MA): Sinauer Associates.

Taberlet P, Meyer A, Bouvet J. 1992. Unusual mitochondrial DNA polymorphism in two local populations of blue tit *Parus caeruleus*. *Mol Ecol*. 1:27–36.

Taborsky M, Hert E, Siemens MV, Stoerig P. 1986. Social behaviour of Lamprologus species: functions and mechanisms. *Ann Mus R Afr Center Sci Zool*. 251:7–11.

Takahashi T. 2003. Systematics of Tanganyikan cichlid fishes (Teleostei: Perciformes). *Ichthyiol Res*. 50:367–382.

Terai Y, Seehausen O, Sasaki T, et al. (14 co-authors). 2006. Divergent selection on opsins drives incipient speciation in Lake Victoria cichlids. *PLoS Biol*. 4:e433.

Wald G. 1935. Carotenoids and the visual cycle. *J Gen Physiol*. 19:351–371.

West-Eberhard MJ. 2003. Developmental plasticity and evolution. Oxford: Oxford University Press.

Wittkopp PJ, Williams BL, Selegue JE, Carroll SB. 2003. Drosophila pigmentation evolution: divergent genotypes underlying convergent phenotypes. *Proc Natl Acad Sci U S A*. 100:1808–1813.

Yamaoka K, Hori M, Kuratani S. 1986. Ecomorphology of feeding in "goby-like" cichlid fishes in Lake Tanganyika. *Physiol Ecol Japan*. 23:17–29.

Yokoyama S. 2002. Molecular evolution of color vision in vertebrates. *Gene* 300:69–78.

Yokoyama S. 2008. Evolution of dim-light and color vision pigments. *Annu Rev Genomics Hum Genet*. 9:259–282.

Yokoyama S, Tada T, Zhang H, Britt L. 2008. Elucidation of phenotypic adaptations: molecular analyses of dim-light vision proteins in vertebrates. *Proc Natl Acad Sci U S A*. 105:13480–13485.

Yokoyama S, Yokoyama R. 1996. Adaptive evolution of photoreceptors and visual pigments in vertebrates. *Ann Rev Ecol Syst*. 27:543–567.